

Draft Assessment Report

Evaluation of Active Substances

Plant Protection Products

Prepared according to **Regulation (EC) 1107/2009**
as it applies in Great Britain

Elemental iron

Volume 3 – B.9 (PPP) – Final Bite

Great Britain

January 2024

Version History

When	What
November 2021	Initial DAR
October 2023	Updated Post Expert Committee on Pesticides (ECP) Independent Scientific Advice (ISA) and following submission of additional information on Ecotoxicology
January 2024	Updates made after comments from the applicant

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B.9. ECOTOXICOLOGY DATA AND ASSESSMENT OF RISKS FOR NON-TARGET SPECIES

Background

The representative formulated product containing elemental iron is a ready-to use granular bait called 'Final Bite'. This formulation contains 10 g/kg iron. A [REDACTED] is also present in the formulation. The molluscicide formulated as granules is intended for use on all edible and non-edible crops in outdoor or protected situations. The intended UK GAP is presented below.

Table B.9.0-1 Critical UK GAP for Elemental Iron 10 RB 'Final Bite ®'

Crop	Situation	Application timing	Application number	Application rate (g a.s./ha)	Application interval (days)
All edible crops	Outdoor	When infestation appears (peak mainly in spring & autumn)	1-6	80	5
	Protected				
All non-edible crops	Outdoor				
	Protected				

The representative product, Final Bite, is a small solid blue granular bait (mean size: 2.5 x 2.5 mm) with a density of < 0.6 kg/L and weight of approximately 0.0133 g per granule (84000 granules/kg). The amount of iron per granule can therefore be calculated as 0.000133 g, or 0.133 mg.

B.9.1. EFFECTS ON BIRDS AND OTHER TERRESTRIAL VERTEBRATES

B.9.1.1. Effects on birds

The following data was submitted in support of this application.

Reference	Author	Species	Substance	Endpoint	Value (mg product/kg bw)
10.1.1/1	[REDACTED] 2008	<i>C. coturnix</i>	Slug and Snail Killer (1 % Iron)	14 d LD50	>2000

B.9.1.1/1 – Acute Toxicity of the formulation to birds

Title: Slug and Snail Killer Acute Oral Toxicity Test with Japanese Quail (*Coturnix coturnix japonica*)

Study code: G/53/08

Author: [REDACTED] 2008

Guideline: EPA OPPTS 850.2100 (1996)

GLP: Yes

Materials and Methods:

Test item: Slug and Snail Killer (no batch code reported)

Active substance: 0.98 % w/w Iron (analysed)

Test organism: *Coturnix coturnix japonica* (Japanese quail). Obtained from the [REDACTED]. The age of the birds at the beginning of the experiment was 5 weeks.

Acclimation: 15 days in the same conditions as the main test, with basal diet (Standard bird food produced by Contipasz Z. A., Grodnow). No mortality occurred during the acclimation period. 17 hours prior to administration of the test item, feed was withheld.

Test Conditions:

Test units: 53 x 34 x 26 cm cages made of galvanised metal net over plastic bases (360 cm² per bird). Temperature ranged between 20-24 °C, relative humidity 49-65 %. and the light regime was 16 h of light and 8 h of darkness. The birds were assigned randomly to pens, and divided by sex into two pens each containing 5 birds. After administration of the test item, basal diet (see above) and clean drinking water was available to the birds ad libitum.

Test duration: 14 days

Test concentration (s): 2000 mg/kg bw, single dose administered by gavage on the basis of individual body weight. The test item was milled and mixed with 1 % carboxymethylcellulose solution as a carrier. The dosing volume of the test item + carrier was consistent between birds and equal to 0.6 mL per 100 g body weight.

A concurrent control group was used, receiving the same test procedures sans the test item.

The test concentration and the untreated control had 2 replicate of 5 birds in each.

Observations:

Overall mortality, behaviour (closely monitored for the first 120 minutes after dosing, a further three time points on the day of dosing, then once daily for the remainder of the study). Individual body weights were checked on the day of application, and after the first and second week of the experiment. Food consumption was monitored after the first and second week of the experiment. Finally, gross pathology examination of all birds was conducted following the end of the study.

Results:

Zero mortality occurred in the test organisms at the treated dose of 2000 mg/kg bw (LD₅₀ >2000 mg/kg bw), nor were any differences in behaviour between treated and control organisms observed. Gross pathology of test organisms revealed no differences between the treated and control organisms.

Table 9.1.1./1-1: Body weight changes in birds treated with 2000 mg/kg bw Slug and Snail Killer versus untreated control group

Day	0	7	14
Dose (mg/kg bw)	Average weight (g)/dose		
0 (control)	139.9	164.1	175.9
2000	139.8	162.4	176.1

Food consumption at the end of the test (mean of all replicates) was 276.5 g/bird in the untreated control, and 271.2 g/bird in the 2000 mg/kg bw treatment group.

Validity Criteria (EPA OPPTS 850-2100):

- Control mortality was no more than 10 % (0 %)

HSE Comments:

The study was conducted to GLP. It follows EPA OPPTS guideline 850-2100 (1996) with the following deviations.

- Age of test birds was 7 weeks at the time of dosing (instead of 16 weeks)
- Space available to each bird (360 cm²) was lower than recommended for bobwhite quail (500 cm²) (similar to Japanese quail)

Neither deviation is considered to have affected the outcome of the test as the control mortality validity criterion was met.

It is noted that no batch number is provided for the formulation and it is not possible to compare the specification of iron used with the agreed specification as detailed in Volume 4 of this dossier. As such, it is not certain that the active substance in the formulation is comparable with the agreed specification, and it is not clear if this study covers the risk from the agreed specification of iron. Without this information the study cannot be considered reliable for use in the risk assessment of elemental iron.

The agreed endpoint is:

14 d LD50 >2000 mg product/kg bw (19.6 mg a.s./kg bw)

B.9.1.1/2 – Short term dietary/Reproductive studies

No studies with the formulation were submitted.

The following literature data was submitted in support of the risk assessment for birds.

B.9.1.1/2-1

Title : Study of House Sparrow (*Passer domesticus*) feeding preference to natural color [sic] and guard coat blue coated seeds (1996).

Author : Pawlina, I. and Proulx, G.

Summary:

In an avoidance trial, 36 wild sparrows (18 males and 18 females) were live-trapped and contained in an outdoor aviary for 2-4 weeks before being fed with a choice of natural coloured or blue-coloured seed. Two experiments were conducted, one using mixed seed diet and the other canola seed. All experimental cages were kept indoors with a temperature of 12 °C during the day and 8°C during the night, with a 16 : 8 light : dark cycle. As well as the experimental seed they were provided with grit and water.

The results showed that in the trial with mixed seed diet, 97 % of the seed consumed was natural coloured. In the trial with canola seed, 69 % of the seed consumed was natural coloured. However, in the canola seed trial, fewer seeds were consumed and the males displayed a statistically significant loss in weight.

The results were taken to show that the blue colouring of the seeds had a repellent effect. The increased amount of blue seeds eaten in the canola seed trial was discussed, and considered to be an effect of the natural colour of the canola being closer to the dark blue colour (being dark brown naturally); whereas in the mixed seed trial there was a very clear contrast between the natural coloured seeds and the blue seeds.

HSE Comments

This avoidance trial gave birds a choice between blue and natural-coloured mixed or canola seed. Under the conditions of the study, birds preferred eating natural-coloured seed. The study is discussed further in the risk assessment.

B.9.1.1/2-2

Title: The food and feeding behaviour of the Jackdaw, Rook and Carrion Crow (1956)

Author: Lockie, J.

Summary:

A number of Rooks, Carrion Crows and Jackdaws were shot every month between 1951 and 1953, and their gizzards were examined for diet, . The contents were listed in terms of % of gizzards containing each type of food.

Table 1. *Percentage of gizzards containing various animal foods: 1951-53*

+ = present; – = not found.

JAN.-FEB.	Rook	Jackdaw	Carrion crow
Curculionidae, imagines	6	26	—
Diptera, imagines	—	6	—
Other insects	13	12	—
Gastropoda	6	35	+
Lumbricidae	75	—	+
Number of gizzards examined	16	34	1

The weevils which could be identified were *Sitona* sp. (probably *S. lineatus* (L.)) and *Barynotus obscurus* (Fabr.). The snails were *Hellicella* sp. and *Hygromia* sp. Diptera were *Scopeuma stercorarium* (L.) and *Borborus* sp.

MAR.-APR.			
Curculionidae, imagines	11	54	17
Coleoptera, imagines (other than Curculionidae)	28	29	58
Coleoptera, larvae	22	7	17
Lepidoptera, larvae and pupae	11	11	8
Other insects	17	18	33
Gastropoda	—	21	42
Lumbricidae	83	18	83
Carrion	6	—	33
Number of gizzards examined	18	28	12

Adult Coleoptera (apart from weevils) were *Agriotes sputator* (L.), *A. obscurus* (L.), *Harpalus* sp., and *Aphodius* sp. Larval Lepidoptera were *Celaena secalis* (L.) and *Leucania* sp. Other insects were mainly *Scopeuma stercorarium* (L.) and lycosid spiders.

MAY-JUNE			
Curculionidae, imagines	17	60	—
Coleoptera, imagines (others)	33	50	+
Coleoptera, larvae	17	10	—
Lepidoptera, larvae and pupae	17	20	—
Diptera larvae	33	10	—
Other insects	67	20	+
Gastropoda	—	20	+
Lumbricidae	100	—	+
Number of gizzards examined	6	10	2

Coleoptera (other than weevils) were *Agriotes* sp., *Harpalus* sp., *Sphaeridium scarabaeoides* (L.), *Cassida* sp. and *Byrrhus pilula* L. Lepidoptera were larval *Celaena secalis* (L.) and the larvae and pupae of *Hepialus lupulinus* (L.).

AUG.-OCT.			
Curculionidae, imagines	27	48	+
Coleoptera, imagines (others)	27	35	+
Lepidoptera, larvae and pupae	36	13	—
Diptera, imagines	45	26	—
Diptera, larvae and pupae	27	—	—
Arachnida	—	9	—
Other insects	9	13	+
Gastropoda	—	9	+
Lumbricidae	55	—	+
Number of gizzards examined	11	23	3
Adult Diptera were Tipulidae, <i>Scopeuma stercorarium</i> (L.) and <i>Polietes lardaria</i> (Fabr.). The weevils were <i>Sitona</i> sp., <i>Otiorrhynchus</i> sp. and <i>B. obscurus</i> (Fabr.). Other adult Coleoptera were <i>Aphodius rufipes</i> (L.), <i>A. erraticus</i> (L.), <i>A. ater</i> (Dg.) and <i>Amara</i> sp. Lepidoptera larvae (mostly found in rooks) were likely to have been <i>Triphaena pronuba</i> (L.).			
NOV.-DEC.			
Curculionidae, imagines	18	20	—
Coleoptera, imagines (other than above)	18	—	+
Other insects	18	20	+
Lumbricidae	82	10	+
Carrion	—	—	+
Number of gizzards examined	22	10	3

HSE Comments:

The above study from the available literature was submitted in support of the risk assessment for birds consuming slugs and snails poisoned with the representative product. Gastropoda are listed for all three species examined. In general, the % of gizzards containing gastropoda ranged from 0-6 % in Rooks, 9-35 % in Jackdaws and 0-42 % in carrion crows. The implications of this data are discussed further in the risk assessment.

B.9.1.1/2-3

Title: A comparative study of the food of some British Corvidae (1968)

Author: Holyoak, D.

Summary:

The paper describes the results of gizzard analyses of 234 Carrion Crow, 264 Rooks, 22 Jackdaws, 77 Magpies and 74 Jays. The results are described in terms of % gizzards containing each type of food.

TABLE II—PERCENTAGE OF CARRION CROW GIZZARDS CONTAINING VARIOUS FOODS

	<i>Jan-Feb</i>	<i>Mar-Apr</i>	<i>May-Jun</i>	<i>Jul-Aug</i>	<i>Sep-Oct</i>	<i>Nov-Dec</i>
Apple	—	—	—	6	6	5
Wild fruits and seeds	8	7	3	17	41	35
Grain	71	65	60	78	82	93
Potato	2	—	—	—	—	10
Root crops	2	—	—	—	—	—
Animal meal	2	—	—	—	—	—
Bread	2	—	—	—	—	—
Rabbit	2	—	—	—	—	—
Carrion meat	7	16	3	—	6	15
Small Mammals	7	14	31	22	6	10
Hen and Duck eggs	—	3	6	—	—	—
Fish carrion	7	14	—	—	—	—
Earthworms	47	36	9	17	—	20
Snails	7	3	6	—	18	5
Coleoptera imagines	31	29	26	—	12	15
Other insects	3	29	71	50	—	—
Number of Gizzards	86	58	35	18	17	20

TABLE III—PERCENTAGE OF ROOK GIZZARDS CONTAINING VARIOUS FOODS
Includes data from Lockie (1956)

	<i>Jan-Feb</i>	<i>Mar-Apr</i>	<i>May-Jun</i>	<i>Jul-Oct</i>	<i>Nov-Dec</i>
Acorns	—	—	—	27	13
Grain	93	98	67	100	100
Other farm produce	33	8	5	—	4
Weed seeds	7	7	—	—	9
Elder, Plum and Apple	—	—	—	20	—
Carrion	2	5	14	7	—
Birds' eggs	—	2	5	—	—
Slugs and Snails	5	25	14	13	4
Earthworms and eggs	78	91	76	53	83
Spiders	—	—	5	—	—
Curculionidae imagines	6	6	14	20	4
Other Coleoptera imagines	6*	36	24	27	17
Coleoptera larvae	1*	11	10	7*	—*
Diptera imagines	—	2*	5*	40	—*
Diptera larvae and pupae	—	—*	43	20	4*
Lepidoptera larvae and pupae	2*	6	24	33	4*
Other insects	23	11	38	13	17
Number of Gizzards	82	123	21	15	23

Note: Some of the figures for the proportion of gizzards containing insect foods from several groups are too low (indicated by asterisks) as Lockie lumped these with 'other insects' in some months but not in others.

TABLE IV—PERCENTAGE OF JACKDAW GIZZARDS CONTAINING VARIOUS FOODS
Includes data from Lockie (1956)

	<i>Jan-Feb</i>	<i>Mar-Apr</i>	<i>May-Jun</i>	<i>Jul-Oct</i>	<i>Nov-Dec</i>
Acorns	—	—	—	9	3
Grain	90	51	44	97	100
Other farm produce	42	18	—	—	3
Weed seeds	56	40	7	37	28
Elder, Plum and Apple	2	—	—	14	3
Carriion	4	—	—	—	3
Slugs and Snails	27	5	7	9	6
Earthworms and eggs	—	7	—	3	3
Spiders	—	—	—	9	—
Curculionidae imagines	15	54	43	54	28
Other Coleoptera imagines	21*	18	52	26	13
Coleoptera larvae	2*	7	11	6*	3*
Diptera imagines	4	1*	4*	43	—*
Diptera larvae and pupae	2	4*	15	11	3*
Lepidoptera larvae and pupae	—	9	19	9	—*
Other insects	10	16	11	9	16
Number of Gizzards	52	76	27	35	32

Note: Use of asterisks as in Table III.

TABLE V—PERCENTAGE OF MAGPIE GIZZARDS CONTAINING VARIOUS FOODS

	<i>Jan-Feb</i>	<i>Mar-Apr</i>	<i>May-Jun</i>	<i>Jul-Aug</i>	<i>Sep-Oct</i>	<i>Nov-Dec</i>
Acorns	—	—	—	—		(50)
Chestnuts	—	—	—	—	(50)	
Wild fruits and seeds	15	5	4	17		
Blackberry (<i>Rubus fruticosus</i>)	—	—	—	8		
Peas	—	5	—	—		
Grain	62	32	12	75		(100)
Rabbit	—	14	8	—		
Mammal carrion	—	5	4	8		
Small Mammals	31	5	16	25		
Passerine birds' eggs	—	5	—	—		
Game bird eggs	—	5	23	8		
Hen and Turkey eggs	—	—	—	17		
Woodlice (<i>Isopoda</i>)	—	—	23	—		
Snails	8	32	31	17	(50)	
Earthworm eggs	8	—	—	—		
Coleoptera imagines	46	64	77	83	(50)	(50)
Other insects	23	27	20	8	(50)	
Number of Gizzards	13	22	26	12	2	2

TABLE VI—PERCENTAGE OF JAY GIZZARDS CONTAINING VARIOUS FOODS

	<i>Jan-Feb</i>	<i>Mar-Apr</i>	<i>May-Jun</i>	<i>Jul-Aug</i>	<i>Sep-Oct</i>	<i>Nov-Dec</i>
Acorns	100	47	28		(100)	(86)
Chestnuts	6	—	8			
Hazel-nuts	—	—	—			(14)
Wild fruits and seeds	—	21	16	(50)		
Strawberry	—	—	28			
Grain	6	16	8	(75)		(29)
Small Mammals	—	5	4			
Passerine birds' eggs	—	11	8			
Passerine pulli	—	—	8			
Snakes (<i>Natrix helvetica</i>)	—	5	—			
Snails	—	5	—			
Coleoptera imagines	50	95	96	(100)	(67)	(14)
Lepidoptera larvae	—	21	16			
Other insects	—	11	8		(33)	
Number of Gizzards	16	19	25	4	3	7

HSE Comments:

The above study from the available literature was submitted in support of the risk assessment for birds consuming slugs and snails poisoned with the representative product. Slugs and/or snails are listed for all five species examined. In general, the % of gizzards containing gastropoda ranged from 4-25 % in Rooks, 5-27 % in Jackdaws, 8-32 % in Magpies, 0-5 % in Jays and 3-18 % in carrion crows. The implications of this data are discussed further in the risk assessment.

B.9.1.2. Effects on terrestrial vertebrates other than birds

The following endpoints are from studies evaluated by UK Toxicology specialists and were found to be acceptable for use in the risk assessment. Please refer to B6 of this dossier for more details. Also included are concentrations of iron in commercial diets as submitted by the applicant in support of the ecotoxicology dossier (see Vol 3CA B9 for more details).

Table 9.1.2 -1 Summary of endpoints in mammals

Test species	Test substance	Time scale (test type)	Endpoint	Data point Author, year
Rat	Feo compared with FeII (carbonyl iron, ferrous sulphate)	Acute oral toxicity	LD₅₀ > 50 g Fe/kg bw	CA 5.2.1/01: Whittaker, P, <i>et al.</i> (2002)
Rat	Carbonyl iron	Long-term (90 days)	NOAEL = 35 mg/kg of diet, equivalent to 3.2 mg Fe/kg bw/day	CA5.3.2/01 Whittaker, P, <i>et al.</i> (1996)
Rat	Carbonyl iron	Long-term (90 days)	NOAEL >200 mg Fe /kg bw/day	CA5.3.2/02 Zhu <i>et al.</i> (2016)
n/a	Iron in commercial rodent diet	n/a	240 mg Fe/kg feed	Vol 3CA B9.1.2.2/1 LabDiet 5001
n/a	Iron in commercial rodent diet	n/a	45 mg Fe/kg feed	Vol 3CA B.9.1.2.2/2 Reeves et al. 1993

B.9.2.1/2 – Short term dietary/Reproductive studies

No studies with the formulation were submitted.

B.9.2. RISK ASSESSMENT FOR BIRDS AND OTHER TERRESTRIAL VERTEBRATES

The following table summarises the data available for use in the risk assessment for terrestrial vertebrates:

Test species	Test substance	Time scale (test type)	Endpoint
Japanese Quail	Slug and Snail Killer (1 % iron)	Acute oral toxicity	LD50 = >2000 mg product/kg bw (equivalent to >19.6 mg a.s./kg bw)
		Acute oral toxicity, extrapolated**	LD50 = 3776 mg product/kg bw (equivalent to 37 mg a.s./kg bw)
n/a	n/a	Reproductive/long term toxicity	NOAEL = 5 mg a.s./kg bw/d*
Rat	Fe ₀ compared with Fe _{II} (carbonyl iron, ferrous sulphate)	Acute oral toxicity	LD₅₀ > 50 g Fe/kg bw
Rat	Carbonyl iron	Long-term (90 days)	NOAEL = 35 mg/kg of diet, equivalent to 3.2 mg Fe/kg bw/day
Rat	Carbonyl iron	Long-term (90 days)	NOAEL = 200 mg Fe /kg bw/day
n/a	n/a	Reproductive/long term toxicity	NOAEL = 24 mg Fe/kg bw/d*
n/a	n/a	Reproductive/long term toxicity	NOAEL = 4.5 mg Fe /kg bw/d*

*calculated based on lowest concentration of iron in a commercial diet expressed in mg a.s./kg diet x 0.1 in line with EFSA guidance (2009).

**In accordance with EFSA guidance (2009), Table 1, an extrapolation factor of 1.888 can be applied to this endpoint (10 animals, zero mortalities)

Issues with the Data provided – literature sources, and the Agreed Specification

A number of literature sources are referred to in the risk assessment which are evaluated in Vol 3CA B9 as well as this document. It is noted that the literature sources are not derived from the literature review submitted in support of this application. The period covered by the Elemental Iron literature review is 2006-2017, although one article submitted (again, not derived from the literature review) is from 2013 (Dieumou *et al.*, 2013). Therefore it is not certain that the additional data submitted are the only ecotoxicologically relevant data for iron and non -target organisms outside of the period covered by the literature review.

The standard literature review submitted in support of conventional active substance registration is designed to catch any useful data potentially missed by the standard dataset required under Regulation EU 283/2011. It is not designed to replace the standard dataset, being limited to the ten years prior to submission. It is therefore unknown if the data derived from outside of this ten year window has been subject to the same level of scrutiny as that derived from the literature review. This raises uncertainty with the overall reliability of the dataset submitted.

Furthermore, it has not been possible to compare the active substance used in the formulation studies with the agreed specification (see Volume 4). This also applies to the data drawn from the available literature.. As such, it is not certain that the active substance in the formulation is comparable with the agreed specification, and it is not clear if the data provided covers the risk from the agreed specification of iron. This information must be provided before drawing a clear conclusion on the risk from this active substance.

Risk assessment using the data provided

The long-term/reproductive endpoint for birds is based on the lowest concentration of iron in commercially available poultry diets (50 mg a.s./kg food) as described in Vol 3CA B9. Although no data is available describing long-term/reproductive effects of iron in birds, it is assumed that this level of iron in commercial feed does not have a detrimental effect on breeding (at least from a commercial perspective) and so is considered appropriate as a surrogate long-term/reproductive endpoint, although there is inherent uncertainty in extrapolating from broiler chickens to natural populations of birds in the field. In line with EFSA guidance (2009), a default conversion factor of 0.1 is applied to this concentration, in order to generate a long term 'NOEL' of 5 mg Fe/kg bw/d.

For mammals, the endpoints are drawn from Vol 3CA B6. As can be seen in the results table above, the results for long-term/reproductive toxicity vary considerably, with the worst-case result showing iron deposition in the liver from doses above and including 35 mg Fe/kg bw/d. The NOAEL from this study is therefore based on the control concentration of iron (which caused no adverse effects) resulting in an endpoint of 3.2 mg Fe/kg bw/d. This worst-case endpoint is considered protective of natural populations of mammals in the field.

The risk assessment for granular formulations is usually performed in a quantitative way (e.g. toxicity/exposure-ratios), bearing in mind the difficulty of assessing the exposure of birds and mammals to granules as noted in the EFSA Guidance Document on birds and mammals (EFSA, 2009)¹.

It is possible that birds and mammals may be exposed to granules in different ways

- a) Ingesting granules as a source of food
- b) Ingesting granules as grit (birds only)
- c) Birds may mistake granules for small seed
- d) Birds and mammals may ingest granules when they eat food contaminated with soil
- e) Birds and mammals may consume food contaminated with residues resulting from granular applications.

The representative product, Final Bite, is a small solid blue granular bait (mean size: 2.5 x 2.5 mm) with a density of < 0.6 kg/L and weight of approximately 0.0133 g per granule (84000 granules/kg). The amount of iron per granule can therefore be calculated as 0.000133 g, or 0.133 mg.

A) Ingesting granules as a source of food

No data is available regarding the calorific value of the representative product, so a first tier risk assessment for ingesting granules as a source of food based on cereal seeds being an approximate equivalent, is described below.

EFSA guidance (2009) suggests that species of concern appropriate for the first tier risk assessment are an omnivorous bird (house sparrow, 27.7 g) and an omnivorous mammal (wood mouse, 21.7 g). Given the relatively large size of 'Final Bite' granules, which may make them difficult for smaller birds to consume, a medium-sized bird species is also included (partridge, 390 g). As a worst-case assumption all species have been considered to consume 100 % cereal seeds (granule equivalent).

Calculation of Daily dietary Dose (DDD)

The basic acute daily dietary dose (DDD) for bird and mammal risk assessment is given by the following equation:

$$\text{Daily Dietary Dose} = \text{FIR/bw} \times \text{RUD} \times \text{AR} \times \text{TWA} \times \text{MAF}$$

Where:

RUD = Residue per unit dose

AR = Application rate

FIR/bw = food intake rate relative to bodyweight

TWA = Time-weighted average factor (acute risk assessment only)

MAF = Multiple application factor

In the case of products applied as granules/pellets, no default RUD values are available or are required. Instead the concentration of active substance in the food item for birds/mammals ingesting granules as a source of food is the concentration of iron in the granule (i.e. 10000 mg Fe/kg). This concentration is equivalent to the RUD x AR in the above calculation.

To calculate a FIR/bw for the first tier assessment, a diet of 100% granules is conservatively assumed. First, the daily energy requirement has been determined using the equations presented in Appendix G of the guidance document (EFSA, 2009) and the appropriate bodyweight for the relevant species. In the absence of specific on moisture content and assimilation efficiency for 'Final Bite' granules, default cereal seed values have been assumed from Appendix G of EFSA (2009). The amount of granules/cereal seeds required to achieve the daily energy expenditure for the relevant species, adjusted for bodyweight, is calculated in the table B.9.2.1 using the following equation:

¹ EFSA Journal 2009; 7(12):1438 page 43

$$FIR = \frac{DEE}{FE \times \left(1 - \frac{MC}{100}\right) \times \left(\frac{AE}{100}\right)} \text{ (g fresh weight/day)}$$

Where:

DEE	Daily Energy Expenditure of the indicator species (kJ/day)
FE	Food energy (kJ/dry g)
MC	Moisture content (%)
AE	Assimilation efficiency (%)

Table B.9.2-1: Tier 1 - Estimates of food intake rate relative to bodyweight

Indicator species	Bodyweight (g)	Daily energy expenditure (kJ/d)	Food energy (kJ/dry g)	Moisture content (%)	Assimilation efficiency (%)	Food intake rate (fresh) (g/d)	FIR/bw
House sparrow	27.7	101.7	18.4	14.7	80	8.1	0.292
Partridge	390	608	18.4	14.7	80	48.4	0.124
Wood mouse	21.7	58.8	18.4	14.7	84.3	4.4	0.21

In the absence of specific data on how rapidly iron degrades/dissipates in granules, a TWA of 1 is assumed in the first tier risk assessment as a conservative measure.

It is noted that 'Final Bite' granules can be applied multiple times. This would suggest inclusion of a MAF > 1 is appropriate. However, in the absence of data on the decline/dissipation of the active substance from granules or data on the degradation of granules, it is not possible to derive a precise MAF. It is considered likely that 'Final Bite' granules will be reapplied only where there are few granules remaining from the previous application, with granules likely to have been consumed by molluscs, broken down or been removed. Therefore, a MAF of 1 will be assumed in the first tier risk assessment, though it is acknowledged that this is not necessarily a worst-case assumption.

The first tier DDD values for birds and mammals consuming granules as food are calculated in the following table and compared to the relevant toxicity endpoints, to derive TERs for the acute and long-term/reproductive risk assessments in the following tables.

Table B.9.2-2: Tier 1 - Estimates of exposure and acute risk to birds and mammals following ingestion of granules as a source of food

Generic focal species	Food	FIR/bw (kg dw/kg bw/d)	Content of iron in the product (mg a.s./kg)	MAF	DDD (mg a.s./kg bw)	Toxicity (mg a.s./kg bw)	TER	Acceptability trigger
House sparrow	Granules based on cereal seeds	0.292	10000	1	2920	37	0.013	≥10
Partridge		0.124	10000	1	1240	37	0.03	≥10
Wood mouse		0.21	10000	1	2100	50000	23.8	≥10

Table B.9.2-3: Tier 1 - Estimates of exposure and long-term/reproductive risk to birds and mammals following ingestion of granules as a source of food

Generic focal species	Food	FIR/bw (kg dw/kg bw/d)	Content of iron in the product (mg a.s./kg)	TWA	MAF	DDD (mg a.s./kg bw/day)	Toxicity (mg a.s./kg bw/day)	TER	Acceptability trigger
House sparrow	Granules based on cereal seeds	0.292	10000	1	1	2920	5	0.0017	≥5
Partridge		0.124	10000	1	1	1240	5	0.004	≥5
Wood mouse		0.21	10000	1	1	2100	3.2	0.0015	≥5

The acute risk to mammals from consuming the seeds as food, substituting cereal seeds for granules in the absence of specific data, indicates an acceptable risk at first tier with a small margin of safety. Considering the conservative nature of this assessment (assuming diet of 100 % granules, 100 % of time spent foraging in treated area), any uncertainty inherent in extrapolating from seeds to granules is covered. The acute risk to birds and reproductive risk to both groups is not acceptable at this first tier and requires further consideration; this is discussed in the weight of evidence/refined risk assessment below.

B) Ingesting granules as grit (birds only).

EFSA Guidance (2009) provides a quantitative acute and reproductive risk assessment scheme for the consumption of granules as grit (page 44). As the size of the granule is 2.5 mm, the equation for large granules is used :

$$\begin{aligned}\text{Daily grit dose (Acute)} &= 2453 \times (G_{\text{density}} / (71 + G_{\text{density}})) \times G_{\text{loading}} \\ \text{Daily grit dose (reproductive)} &= 1306 \times (G_{\text{density}} / (71 + G_{\text{density}})) \times G_{\text{loading}}\end{aligned}$$

G_{density} = number of granules on soil surface)

G_{loading} = amount of active substance in one granule.

With a weight of 0.0133 g/granule, and a concentration of 1 % iron w/w, the G_{loading} value is 0.000133 g or 0.133 mg iron/granule.

G_{density} can be calculated based on the application rate of 8 kg product/ha, or 0.8 g product/m² and the average granule weight of 13.3 mg (see Document J section 1.4.3). The G_{density} is therefore equal to 0.8/0.0133 = 60 granules/m²

Therefore,

$$\begin{aligned}\text{DGritDacute} &= 2453 \times 60 / (71 + 60) \times 0.133 = 149.4 \text{ mg a.s./bird} \\ \text{DGritDrepro} &= 1306 \times 60 / (71 + 60) \times 0.133 = 79.55 \text{ mg a.s./bird/d}\end{aligned}$$

The above calculations assume a 300 g bodyweight for a large bird. Therefore, DGritD values need to be converted to a per kg equivalent for comparison with toxicity endpoints.

$$\begin{aligned}\text{DGritDacute} &= 149.4 \text{ mg a.s./bird} \div 0.3 = 498 \text{ mg a.s./kg bw} \\ \text{DGritDrepro} &= 79.55 \text{ mg a.s./bird/d} \div 0.3 = 265 \text{ mg a.s./kg bw/d}\end{aligned}$$

The corresponding TER ratios are :

$$\begin{aligned}\text{TERacute} &= 37/498 = 0.074 \\ \text{TERrepro} &= 5/265 = 0.019\end{aligned}$$

These values are lower than the acute and reproductive trigger values of 10 and 5, respectively, indicating an unacceptable risk at the first tier. A refined risk assessment is required.

C) Birds mistaking granules for seeds

The risk assessment scheme for this scenario is based on a small granivorous bird ; as the granule size is 2.5 mm this scenario does not apply (only large birds will be capable of consuming the granule).

D) Birds and mammals ingesting granules when eating soil-contaminated food

Firstly the daily dry soil dose for both acute and long term exposure is calculated using a default value and the dosage in kg a.s./ha, before being used with the toxicity endpoints to determine a TER. The dosage of Iron according to the GAP is 0.08 kg a.s./ha.

Birds

$$\text{DDSD acute} = 0.283 \times \text{dosage}$$

$$\text{DDSD repro} = 0.025 \times \text{dosage}$$

$$\text{TERacute} = 37/0.022 = 1608$$

$$\text{TERrepro} = 5/0.002 = 2500$$

An acceptable risk can be concluded for birds, as the TER values are greater than the acute and long term trigger values of 10 and 5 (respectively) with a large margin of safety.

Mammals

$$\text{DDSDacute} = 0.097 \times \text{dosage}$$

$$\text{DDSDrepro} = 0.005 \times \text{dosage}$$

$$\text{TERacute} = 50000/0.0078 = 6443299$$

$$\text{TERrepro} = 3.2/0.0004 = 8000$$

An acceptable risk can be concluded for mammals, as the TER values are greater than the acute and long term trigger values of 10 and 5 (respectively) with a large margin of safety.

Weight of Evidence/Refined risk assessment

An unacceptable risk was identified at first tier for birds ingesting seeds as grit and an unacceptable risk is concluded for the acute risk to birds, and long term risk to birds and mammals ingesting granules as a source of food. A refined risk assessment using a weight of evidence approach is presented.

The applicant presented the following lines of argument which are discussed in turn.

1) Iron is an essential component of bird and mammal diets

The applicant has made the case that iron is an important nutrient in homeostasis and is a supplement in many rodent and bird diets used in the laboratory as well as commercial rearing (see Vol 3CA B9). Commercial diets contain iron at concentrations ranging from 50-130 mg Fe/kg feed in the case of birds (chickens, turkeys), and up to 240 mg Fe/kg feed in the case of mammals (mice, rats) On this basis, it is posited that natural populations of birds and mammals exposed to elemental iron from the proposed uses of Final Bite will be capable of metabolising any excess iron consumed by accident (i.e. from consuming granules as food and/or grit). However, animals that have not evolved with a diet relatively rich in iron have been found to lack the regulatory processes required to metabolise this excess intake, leading to conditions such as iron storage disease (Haemochromatosis). Indeed, the study by Whitaker *et al.* (1996) showed that in laboratory rats fed with 35 mg Fe/kg bw/d, excess iron accumulated in the liver. The argument that iron is a natural material important for homeostasis and therefore can be considered low-risk is negated by the high concentration of iron in the representative product (10000 mg/kg) and the evidence that high iron intake can cause iron storage disease in both birds and mammals that are not evolved to consume relatively high levels of iron in their diets. There is also inherent uncertainty in extrapolating surrogate NOEL values from commercial diets to natural populations of birds and mammals which may not be accustomed to as high a dietary intake of iron. Overall, this argument should be used with caution as it is reliant on there being low exposure and should not be the basis for the assumption of low risk.

2) The blue colour of the granules will repel birds and mammals from eating the granules

The applicant has posited that the blue colour of the granules will deter birds and mammals from selectively consuming them while foraging, citing an avoidance trial with coloured seed and sparrows (Pawlina and Proulx, 1996²). This trial investigated the amount of coloured seed consumed versus the amount of natural coloured seed consumed by captive wild sparrows (canola and mixed seed, in two separate experiments). The study indicated that the birds preferred natural coloured seed over blue-coloured seed, with 97 % of the mixed seed consumed being of natural colour, and 69 % of canola seed consumed being of natural colour. However, avoidance trials do not necessarily represent field conditions where effects of exposure to poor weather conditions, feeding pressure (both contributing to nutritional need, forcing birds to eat things they would normally not preferentially eat if given the choice) and predation (whereby a small dose does not cause mortality in a trial but might cause a bird to be more vulnerable to predators in field conditions) come into effect. This one study is not sufficient to demonstrate that birds will instinctively avoid eating granules of the representative formulation as a source of food when faced with no other source of nutrition. No data is available for mammals.

Further data is required to prove that birds and mammals will avoid eating blue-coloured granules in the field.

3) Granules will not be available to birds and mammals in sufficient numbers to cause adverse effects or for long periods of time.

Being a molluscicide bait, the Applicant has proposed that it is likely that most granules of the representative product will be consumed by the target organism or otherwise degrade into the soil and as such will not be available in the long term to terrestrial vertebrates. As such the duration of exposure to the test item is likely to be minimal. Exposure is likely to be limited to accidental consumption of small quantities of the product shortly after application. Whilst on the one hand this argument has merits, this is considered further below. In addition, this argument does not however exclude long term effects from short term exposure.

The representative product, Final Bite, is intended to be applied by mechanical spreading or by hand when the infestation appears, meaning that the likelihood of a bird or mammal spending its entire day foraging in the treated area is variable. Up to six applications of 8 kg product/ha (equivalent to 58 granules/m² per application) are requested. It can be considered that application made in greenhouses (i.e. permanent protection with full enclosure) will result in negligible exposure to terrestrial vertebrates. Outdoor exposure will vary depending on the level of infestation, and therefore the frequency of application. An earthworm field study (Axmann, 2019) submitted in support of this application made six applications of the representative product, each equivalent to 8 kg product/ha, with a five day application interval. This could be considered to be a worst-case application with an annual allowance of product applied in the space of a single month. The worst-case number of granules/m² was 156 over the course of the treatment period. This however was based on just two 1m² plots which were photographed for the purpose of application verification. The study did not investigate how long it took for the granules to be consumed by target organisms or otherwise degrade into the soil. However, the evidence available from the study is indicative of the worst-case number of pellets available per m² available immediately after application.

Overall, there is limited actual evidence to rely on regarding the availability of granules to birds and mammals, although for indoor applications (greenhouse/permanent protection with full enclosure) it can be assumed that exposure will be negligible. In the short term at least, it can be assumed that immediately after a single application at least 58 pellets/m² will be present in any given outdoor scenario. There is evidence to suggest that after applying a full year's worth of granules within a single month (Axmann, 2019) results in approximately 156 granules/m² over the course of treatment. This is from one study and there is no information regarding the length of time that mammals and birds could be exposed to the granules under differing environmental conditions and what the potential impact could be from one or more applications.

4) Consuming slugs and snails that have been poisoned by the representative product is very unlikely

Birds and mammals might also be exposed by consuming gastropods that have been poisoned by the representative product. The applicant has provided data on gastropod consumption in Corvids (Holyoake, 1968; Lockie, 1956) which showed that gastropods make up a small amount of the diet of Rooks, Jackdaws, Magpies, Jays and Crows. The % of gizzards examined containing slugs and snails were highest in Magpies (up to 32 %, and relatively high for most of the year) and lowest in Jays (no more than 5 %, generally 0). However, there is no data to back up the assertion that corvids are the key focal species at risk, and the data itself certainly does not discount the potential for the consumption of gastropods. Furthermore, as the target organism is soft bodied, it is unclear whether the

² See Vol3CA Section 9.

assessment process used would have been sufficiently sensitive to detect the presence of gastropods accurately. In addition, there is no information regarding the immediate habitat and hence whether slugs would have been present in significant numbers.

No data have been provided for mammals, though data is publically available³ that shows that Common Shrews can consume as much as 78 % of their diet as gastropods.

There is also no data comparing residues of iron and [REDACTED] such as [REDACTED] in slugs that have consumed the product with slugs that have not.

Overall, there is insufficient data to conclude an acceptable risk to birds and mammals from this route of exposure.

Overall conclusion – dietary risk

The UK does not consider the risk to birds and mammals from outdoor use of the representative product to have been adequately addressed. Firstly, using data sourced from the available literature instead of a standard toxicity dataset means that it is not certain that the data submitted are the only ecotoxicologically relevant data for iron and non-target organisms - outside of the period covered by the standard literature review, which only covers the period 2006-2017. Furthermore, it has not been possible to compare the active substance used in the formulation toxicity studies and the iron referred to in the data from the available literature with the agreed specification (see volume 4 of this dossier). This would need to be addressed before an acceptable risk could be concluded.

Even assuming the above issues could be resolved, the data provided is insufficient to show an acceptable risk from the proposed use of elemental iron.

The lack of reproductive toxicity information for birds means that the risk assessment is reliant on information about iron content in commercial diets for poultry, for which there is inherent uncertainty in extrapolating to natural populations of birds. For mammals there is at least information on the acute and long-term toxicity of iron, but the worst-case data indicates that doses as low as 35 mg Fe/kg bw/d cause iron accumulation in rats. Using the relatively high concentrations of iron in commercial diets as a surrogate NOAEL suffers from the same reliability and applicability issues as using iron content from poultry commercial diets.

Even assuming the toxicity data available represents a reasonable worst-case dataset, the risk assessment has failed to address the principle cause for concern for the representative product, which is consuming granules as food. The argument that blue colouring will deter birds and mammals from consuming the granules is backed up by only a single study on birds, using coloured seeds, which does not address the effects of conditions in the field, and does not compare the effects of choice versus no choice. There is insufficient evidence to suggest that terrestrial vertebrates will not use the product as a food source, and no first tier risk assessment can be conducted in the absence of data regarding the calorific value of the granules.

There is also no evidence to back up the assertion that the representative product will not be available to birds and mammals for long periods of time, in fact data from Axmann (2019) indicates that it is possible that granules will be available for a long time. Assuming that the granules provide a food source to terrestrial vertebrates, the maximum single application rate of 60 granules/m² is sufficient to provide enough granules to fail the first tier risk assessment, as illustrated by the calculations below.

Acute Toxicity

Birds – LD50 (extrapolated) = 37 mg Fe/kg bw

Trigger value for acute assessment = 10

Maximum acceptable dose = 37/10 = 3.7 mg Fe/kg bw

Amount of iron in a single granule = 0.133 mg Fe/granule

Number of pellets to exceed maximum acceptable dose in a 1 kg bird = 3.7/0.133 = 27.8 pellets

Number of pellets to exceed maximum acceptable dose for a small bird (27.7 g) = 27.8 * 0.0277 = 0.77 pellets

³ 1998 Update CONTRACT PN0910/PN0919 MILESTONE REPORT Mammals and Farming: information for risk assessment J.E. Gurney, J. Perrett D.R. Crocker & J.A. Pascual

Number of pellets to exceed maximum acceptable dose for a medium-sized bird (390 g) = $27.8 * 0.39 = 10.8$ pellets

Area required to forage to exceed maximum acceptable dose for small bird (27.7 g) = $0.77/60 = 0.013 \text{ m}^2$

Area required to forage to exceed maximum acceptable dose for medium-sized bird (390 g) = $10.8/60 = 0.18 \text{ m}^2$

Long-term/reproductive Toxicity

Birds – Toxicity endpoint = 5 mg Fe/kg bw

Trigger value for assessment = 5

Maximum acceptable dose = $5/5 = 1 \text{ mg Fe/kg bw}$

Amount of iron in a single granule = 0.133 mg Fe/granule

Number of pellets to exceed maximum acceptable dose in a 1 kg bird = $1/0.133 = 7.52$ pellets

Number of pellets to exceed maximum acceptable dose for a small bird (27.7 g) = $7.52 * 0.0277 = 0.21$ pellets/d

Number of pellets to exceed maximum acceptable dose for a medium-sized bird (390 g) = $7.52 * 0.39 = 2.93$ pellets/d

Area required to forage to exceed maximum acceptable dose for a small bird (27.7 g) = $0.21/60 = 0.0035 \text{ m}^2/\text{d}$

Area required to forage to exceed maximum acceptable dose for a medium-sized bird (390 g) = $2.93/60 = 0.049 \text{ m}^2/\text{d}$

Mammals – Toxicity endpoint = 3.2 mg a.s./kg

Trigger value for assessment = 5

Maximum acceptable dose = $3.2/5 = 0.64 \text{ mg a.s./kg bw}$

Amount of iron in a single granule = 0.133 mg Fe/granule

Number of pellets to exceed maximum acceptable dose in a 1 kg mammal = $0.64/0.133 = 4.81$ pellets/d

Number of pellets to exceed maximum acceptable dose in a small mammal (21.7 g) = $4.81 * 0.0217 = 0.1$ pellets/d

Area required to forage to exceed maximum acceptable dose = $0.1/60 = 0.0017 \text{ m}^2/\text{d}$

These calculations arguably present an overly conservative worst-case scenario, but illustrate the potential risk to terrestrial vertebrates from the requested application rate of the representative product. No information is available to show conclusively that birds and mammals will not use the granules as a food source, there is no data to show that exposure to granules will be limited, and no meaningful information has been provided for resolving the potential risk of consuming target organisms poisoned by the granules. As a result, no acceptable risk can be concluded for outdoor use of the representative product.

Further consideration of risks to birds and mammals

In light of the HSE assessment above, further consideration of the risks from Final Bite to birds and mammals was requested. Following discussions with HSE, the Applicant provided further detail regarding the literature searches performed (Anon, 2022), the representativeness of the test material used in the ecotoxicology studies, and an updated weight of evidence consideration of the risks to birds and mammals from use of Final Bite (Schabacker and von Blanckenhagen, 2023a). The updated submissions are considered by HSE in the sections below.

Representativeness of the test material

Additional consideration of the representativeness of the material tested in the non-target organism toxicity studies compared to the technical specification for elemental iron has been provided. This is reviewed by HSE in volume 4. It is concluded that the toxicity data for non-target organisms, including birds and mammals, is representative of the technical specification for elemental iron.

Literature search

In their initial dossier the Applicant provided a single concept literature search covering the 10 year period prior to the submission of the dossier. This is reviewed in section B.9.11 (a.s.) and covers all non-target organism groups. In the bird and mammal risk assessment sections of the Applicant's submission, as part of a weight-of-evidence approach, published literature were utilised which were published prior to the literature search period.

HSE raised concerns over the transparency of using published data from a time period not covered by the literature review and sought advice from the UK Expert Committee on Pesticides. The following advice was received:

'The ECP advised that there was uncertainty with the approach taken by the applicant and that a more transparent literature review would increase confidence in the data set, as there was a danger that favourable literature sources could otherwise be used. They recognised that for a common material, such as iron, this has the potential to be a very time-consuming piece of work. Therefore, a very focused ecotoxicology-specific approach would be most suitable. The committee also noted that the most reliable data drawn from the available literature would be in the form of scientific peer reviewed articles/studies.'

'The guidelines for conducting 10-year systematic literature reviews are limiting and result in a highly constrained output. They may fail to capture all relevant knowledge and, in the case of iron, the ECP would also expect the majority of published information to lie outside the prescribed 10-year period. Therefore, it would be acceptable to use data not taken from a 10-year systematic literature search. Such data are likely to be found in the peer-reviewed literature but this, of itself, does not guarantee the quality of the research. However, each item used in this way needs to be assessed by HSE and scientific judgement applied. There is a need for the methods used to select data from outside the systematic review to be described, justified and transparent.'

As part of their updated submission, the Applicant has provided additional detail regarding the literature search performed to identify relevant information for the bird and mammal risk assessment (Anon, 2022). This information has been used to support updated risk assessments for birds and mammals (Schabacker and von Blanckenhagen, 2023a).

The literature search performed for the bird and mammal risk assessment adopted a targeted approach, rather than a more conventional systematic method, as described in EFSA guidance on literature reviews (EFSA, 2011). This is discussed below.

Article 8(5) of Regulation (EC) 1107/2009 specifies that: 'Scientific peer-reviewed open literature, as determined by the Authority, on the active substance and its relevant metabolites dealing with side-effects on health, the environment and nontarget species and published within the last ten years before the date of submission of the dossier shall be added by the applicant to the dossier'.

The Applicant has proposed that given iron is a well-known, essential element, rather than a novel active substance, focusing the literature search on just the last 10 years would not be appropriate. HSE agrees that there is a clear need to extend the literature search further back, in order to capture basic physiological data for elemental iron, which the Applicant is proposing to use as part of a weight of evidence consideration.

The following search engines/libraries were utilised.

Table B.9.2-4: Bibliographic databases searched

Source	Applicant's comments
PubChem	PubChem is the world's largest collection of freely available chemical information. It provides information about chemicals by name, molecular formula, structure, and other designations.
PubMed	PubMed is a free search engine accessing primarily the MEDLINE database of references and abstracts on life sciences and biomedical topics.
Google Scholar	Google Scholar is a freely accessible web search engine that indexes the full text of scholarly literature across an array of publishing formats and disciplines. Google Scholar index includes most peer-reviewed online journals of Europe and America's largest scholarly publishers.
RIFCON library	Contains more than 11,000 references with contexts on toxicology, pesticides, and relevant organisms.

The Applicant has searched multiple databases and has provided explanation to justify their selection. While the first three databases searched are not directly focused on environmental risk assessment, they are very extensive and include scientific publications in a range of relevant disciplines. While the RIFCON library is less

transparent, it provides a useful additional source of information, with a clear ecotoxicological/ecological focus. Therefore, the databases searched are considered sufficient.

Given the approach adopted, specific dates on which these databases were searched have not been provided. This limits the transparency of the literature search and is discussed further below.

In order to inform the risk assessment, specific terms were searched for, which were considered relevant for the bird and/or mammal risk assessments. The search terms used are summarised in the following table. The primary search term used was iron, with various combinations of other terms.

Table B.9.2-5: Search terms used for the targeted literature search

Substance	Physiology	Exposure	Toxicity	Species
Iron	Metabolism	Exposure	Toxicity	Birds
	Physiology	Concentration	Effect	Avian
	Homeostasis	Diet	Mode of action	Poultry
	Regulation	Food	Detoxification	Mammals
	Transport	Environment		Mammalian
Pellets	Supplementation	Soil		Rat
Granules	Deficiency	Overload		Mice
Molluscicide	Absorption	Bioavailability		Vertebrates
Metals	Uptake			Invertebrates
	Excretion			Slugs
	Requirements			Snails
	Trace element			Plants
	Micronutrient			
	Nutrition			

The search terms used are considered suitable for identifying potentially relevant information from published literature to inform the risk assessments for birds and mammals.

The search initially focused on the most recent time period to find the latest publications, before being extended further back in time, as required. The Applicant's report states that '*the search was terminated when a manageable number of current articles were found*'. Given the volume of information that would be expected to be produced using the above search terms, this approach is understandable but it is noted that it is not fully transparent, i.e. the length of the search time period is not provided for specific combinations of search terms and '*a manageable number*' is a subjective term.

The above approach was intended to primarily identify review articles and textbook chapters that consider specific subjects relating to iron, which may be relevant for the bird and mammal risk assessments.

Results of all searches of bibliographical databases performed are not included in Anon (2022). An example is provided (see table below). This illustrates the large number of search hits found using the search terms and highlights the need to further sift the results.

Table B.9.2-6: Example of search hits found using particular combinations of search terms

	PubChem	PubMed	Google scholar
Iron	244,201	247,740	4,710,000
Iron metabolism	100,410	139,051	2,840,000
Iron metabolism bird	85	1,346	62,400
Iron metabolism mammal	36	92,327	43,100

Search hits were filtered based on titles. Only literature types aimed at a biological, agricultural, or medical audience were considered further, such as textbooks, review articles, substance-related studies, and peer-reviewed scientific literature.

Remaining search hits were assessed using rapid relevance criteria to determine if they contain relevant information for the bird and/or mammal risk assessments. The criteria used by the Applicant are shown in the following table.

Table B.9.2-7: Rapid relevance criteria used in literature search

Criteria	Questions	Rating
Accuracy	Is the information reliable? Is the information free of errors? Is the information based on proven facts? Can the information be verified against other reliable sources?	If one of the questions was answered with "no", the citation was excluded (Score: include, exclude)
Relevance	Is the information relevant? Does the information covered meet the information need? Is the information contained valuable and pertinent? Is it basic or in-depth coverage?	The relevance is rated based on a scale (Score: 1: relevant (The text contains relevant information), 2: partially relevant (Parts of the publication contain relevant information), 3: irrelevant) (The information is not relevant to the topic)
Authority	Are the authors qualified to write on this topic? Are they associated with a reputable organization (university, research institutes) in this field?	Authors publishing in the field of agriculture, animal physiology, medicine, and veterinary medicine (Score: yes, no)
Objectivity	What is the purpose of the information? Is the information biased?	Publications aimed at a specialist agricultural, biological, toxicological, or medical audience were included (Score: yes, no)
Timeliness	When was the information published? Is the information current or outdated?	In the case of similar focal points of the information compilation (e.g., general iron physiology), more recent publications were preferred (Score: current c, outdated o)

It is clear that the above criteria do not solely relate to relevance. In fact, only one of the criteria used relates to relevance, with the other four relating to reliability. For the relevance sub-category, literature were defined as relevant, partially relevant or irrelevant. HSE considers that the use of these broad categories is reasonable.

It is noted that the questions used to establish relevance in Table B.9.2-7 are rather vague and non-specific in nature, i.e. they don't establish clear criteria that are particular to the risk assessment area. It would be preferable and more transparent to establish clear relevance criteria for the specific risk assessment question before the literature search was performed, as recommended in the EFSA guidance on literature reviews (EFSA, 2011). However, in this case the search terms used do include specific subjects, such as metabolism and homeostasis, which when used in combination with the relevance questions, provide a clearer basis for assessing relevance. It is also noted that since the intended focus of the literature search is on review articles and textbooks, setting more precise relevance criteria would be difficult.

It is not specified whether the assessments performed against the rapid relevance criteria were conducted by looking at the title, abstract or full text of the reference. This is not transparent, though it is assumed that in Anon (2022) literature were considered at an appropriate level of detail to determine whether the rapid relevance criteria were satisfied or not.

In Anon (2022) there is no explicit discussion of reliability assessment as part of the literature search. However, it is apparent that some of the criteria used for the rapid relevance assessment inform on the reliability of the reference. Reliability refers to the extent to which a study is free from bias and its findings reflect true facts

(EFSA, 2011). Anon (2022) explains that Klimisch scores were not used to categorise reliability as they were not deemed appropriate for assessing review articles. As part of the rapid relevance assessment, accuracy, authority, objectivity and timeliness have been assessed. These broad criteria are selected as being more appropriate for assessing review publications, compared to standard criteria used to assess the methodological quality of primary studies (e.g. replication, statistical power etc.). While HSE agree that different reliability criteria are needed for a review publication compared to a study that generates primary data, several of the questions specified in Table B.9.2-7 lack a clear definition and are open to interpretation. For example, ‘*is the information reliable?*’ and ‘*is the information biased?*’ are very broad questions that are not transparent.

Literature that were not excluded based on the above rapid relevance criteria were taken forward for further consideration as part of the weight-of-evidence assessment. It is not reported how many references were excluded based on the rapid relevance criteria. As a result of this process a total of approximately 50 references were identified for further evaluation. These references are listed in Appendix 1.

HSE conclusions regarding the literature search for birds and mammals

HSE has considered the literature search performed by the Applicant (as described in Anon, 2022) in the context of EFSA guidance on this subject (EFSA, 2011). It is accepted that some deviation from the standard approaches discussed in EFSA (2011) was necessary, due to nature of this elemental substance, the extent to which some of the search topics have been investigated and discussed historically, the high volume of potential references to sift, and the changing scope/focus of research on this substance over time.

However, there are several areas where the searches performed and decision criteria applied could have been more transparently documented. This means that it would be very difficult for anyone reading Anon (2022) to replicate the process followed by the authors.

The approach followed by Anon (2022) appears reasonable for finding the most up-to-date thinking on specific subjects relating to iron and birds/mammals (e.g. iron homeostasis in mammals), to be used as part of a weight-of-evidence consideration. However, it is less clear that the search strategy adopted would identify all relevant primary data generated during a specific time period, including any novel data on iron toxicity to birds/mammals. Therefore HSE has performed additional searches for relevant literature for each of the key areas discussed below.

Weight of evidence consideration of risks to birds and mammals

In the following sections the information obtained during the literature search phase is discussed. This is in the context of how the Applicant has proposed that the references can be used to demonstrate that the representative uses of ‘Final Bite’ will not result in unacceptable risks to birds and mammals (see Appendix 2 for the Applicant’s full consideration). HSE has considered the literature referenced and divided this into the following main discussion areas:

- Effects of elemental iron on birds and mammals
- Uptake and regulation of iron
- Potential for exposure of wild birds and mammals to iron via ‘Final Bite’ granules
- Background exposure of birds and mammals to iron via normal diet

Key supporting studies have been reviewed by HSE in Appendix 3.

Effects of elemental iron on birds and mammals

Elemental iron acts against molluscs through disrupting oxygen uptake by hemocyanin. This mode of action is specific to molluscs, which have hemocyanin as a blood pigment. Vertebrates do not have hemocyanin as a blood pigment, so they will not be impacted by the same mode of action. However, exposure of vertebrates to iron could impact their health by other modes of action, so further consideration of the potential for iron exposure to cause toxic effects in vertebrates is needed.

Iron is an essential nutrient for vertebrates, playing a crucial role in cellular respiration processes. Inadequate intake of iron can cause decreased cellular function in many organs, leading to serious health consequences.

However, intake of excess iron can cause build-up of iron in organs, impacting their functioning, and can be potentially life threatening. Due to the lack of an active excretory mechanism, iron overload develops when iron intake/uptake exceeds needs (Mete et al., 2003).

The literature review conducted found many papers and books which provide insight into iron toxicity in general and specific mechanisms of toxicity. These include Ponka et al. (2007), Schümann et al. (2014), Coffey and Ganz (2017), Brue (1994), Papanikolaou and Pantopoulos (2005), Sherry et al. (2006), and Aggett (2012). These references have been checked by HSE. While these references contain relevant background information regarding iron overload and modes of action, they do not seek to determine a threshold for iron exposure, above which toxic effects would be expected to be experienced by birds or other terrestrial vertebrates.

The literature search has not identified any additional studies that provide quantitative data on the effects of oral iron exposure on bird and/or mammal health. A detailed consideration of two long-term studies investigating iron toxicity in rats has been provided (in Schabacker & von Blanckenhagen, 2023b) and this is reviewed by HSE below. Other lines of evidence that inform on the potential for iron to have toxic effects on birds and/or mammals are also discussed below.

Acute avian toxicity

As discussed in the initial HSE assessment, a GLP study investigating acute toxicity to Japanese quail is available with 'Slug and Snail Killer', a granule containing 1% iron (██████ 2008). The study largely conformed to the guideline EPA OPPTS 850.2100 (1996). There were no mortalities in the treatment group, no behavioural effects and no differences found in gross pathology. The LD₅₀ was determined to be greater than the maximum concentration tested (2000 mg formulation/kg bw). However, given the low iron concentration in the formulation, this is only equivalent to a dose of 19.6 mg a.s./kg bw. Using a set extrapolation factor of 1.888 from EFSA (2009) to approximate the 'true' LD₅₀, this was determined to be 37 mg a.s./kg bw.

Regarding the limit dose study design, the guideline OECD 223 (2016) states that: *'The limit dose must be adequate for assessment purposes, and it is usually 2000 mg/kg bw'*. Similarly, the guideline 850.2100 (2012) specifies: *'For test substances expected to have relatively low toxicity, a limit test may be conducted with a single dose level at 2,000 mg/kg bw or the maximum expected environmental residue concentration, whichever is higher'*. Therefore testing a maximum dose of 2000 mg test item/kg bw is in line with standard practices for pesticide active substances. However, 2000 mg/kg bw is not a maximum limit and both guidelines indicate that a higher dose can be tested, where required for risk assessment purposes. It is not clear what the maximum dose is that could be reasonably tested in an acute avian toxicity study or whether it would be sufficiently high that it could allow an acceptable risk from iron to be demonstrated in a first tier assessment. It is however acknowledged that the ████████ (2008) was conducted a long time prior to the current application for use of iron as a molluscicide, so the potential exposure level in the environment would not have been an aspect of study design.

It is noted that for mammals a considerably higher dose was tested in the available acute toxicity study, with a LD₅₀ > 50000 mg a.s./kg bw determined for rats. While it is acknowledged that there is a lack of specific information submitted to support this point, it is expected that birds and mammals would have broadly similar acute sensitivity to iron. Therefore, it is likely in this case that applying the default extrapolation factor to the unbound endpoint from the acute bird study still significantly underestimates the 'true' LD₅₀ for birds.

The worst-case first tier exposure scenario for birds is the direct consumption of granules as a source of food. First tier potential exposure levels (DDD_s) of 2920 mg a.s./kg bw for the house sparrow and 1240 mg a.s./kg bw were calculated for the partridge generic focal species. Of these, the partridge is considered the more relevant scenario for the 'Final Bite' risk assessment, given the relatively large size of the granules. At first tier an acceptability trigger of ≥ 10 is used to determine whether unacceptable impacts on birds can be reasonably excluded. In order for the TER for the partridge to be ≥ 10 for the consumption of granules as food scenario, the acute avian LD₅₀ would need to be ≥ 12400 . This is significantly above the maximum dose tested in ████████ (2008) but is below that tested for mammals (Whittaker et al., 2002). This comparison assumes that the bird consumes sufficient granules to satisfy its daily energy requirement.

Acute toxicity of different forms of iron

Acute avian toxicity data are available for a range of forms of iron. In table B.9.2-8 acute avian toxicity endpoints for different forms of iron are reported. These values are taken from the active substance reviews of ferric phosphate, ferric sulphate and ferric pyrophosphate, in addition to ████████ (2008). In all cases the LD₅₀ was

determined to be above the maximum dose tested, though the maximum doses tested differed by two orders of magnitude. Therefore, it cannot be concluded from these endpoints whether there are clear differences in acute toxicity to birds between the different forms of iron tested.

Table B.9.2-8: Acute avian toxicity of different forms of iron

Substance	Acute LD ₅₀ (mg a.s./kg bw)	Source
Iron (formulated as 'Slug and Snail Killer')	>19.6	█ (2008)
Ferric phosphate (formulated as 'NEU 1165 M')	>20	Van Dreumel & Reijnders ⁴ (1996)
Ferric phosphate (formulated as 'Ferric Orthophosphate RB 1.62 W')	>27	Barfknecht (2006) ¹
Ferric pyrophosphate	>2000	Mhaske (2013) ⁵
Ferrous sulphate heptahydrate	>2250*	Grimes & Jaber (1986) ⁶

*Equivalent to >1230.75 mg FeSO₄/kg bw

Similarly, acute mammalian toxicity endpoints for the different forms of iron are presented in table B.9.2-9. For carbonyl iron, ferric phosphate and ferric pyrophosphate the endpoints are all above the highest dose tested and were at least 2000 mg/kg bw. A range was defined for ferrous sulphate heptahydrate, which indicated that this form of iron is more acutely toxic to mammals.

Table B.9.2-9: Acute mammalian toxicity of different forms of iron

Substance	Acute LD ₅₀ (mg a.s./kg bw)	Source
Carbonyl iron	>50000	Whittaker et al. (2002)
Ferrous sulphate	~1100	Whittaker et al. (2002)
Ferric phosphate	>5000	Van Huygevoort (2000) ¹
Ferric pyrophosphate	>2000	Mrzyk (2013) ²
Ferrous sulphate heptahydrate	1185-1750	Yeary et al. (1966), Jacobs et al. (1979) ³

It is noted that the acute mammalian study (Whittaker et al., 2002) tested █ which differs from █ iron in the manufacturing process. These are both forms of elemental iron and are of high purity. In section B.6 it was considered that data for the █ form could be used in assessing the oral toxicity of the █ form.

Schabacker and von Blanckenhagen (2023a) postulate that elemental iron is less toxic to vertebrates than iron salts. This is on the basis that before the iron is absorbed it must first be converted from elemental iron into ferrous ions by gastric acid, and that this is the rate-limiting step. This is supported through reference to a technical data sheet for carbonyl iron powder⁷. This data sheet states that as a result of slow gastrointestinal oxidation, toxic effects resulting from overdose of carbonyl iron take longer to materialise compared to ferrous salts. It is noted that section B.6.1.1 (table 6.1-1) indicates the bioavailability of elemental iron powder (H-reduced) in rats is 13-54% of the bioavailability of ferrous sulphate. This supports the notion that elemental iron will be more slowly and less completely taken up following ingestion compared to ferrous salts.

⁴ Renewal assessment report: Ferric Phosphate. Volume 3, Annex B.9 Ecotoxicology. 12 June 2014.

⁵ EFSA (European Food Safety Authority), 2020. Conclusion on the peer review of the pesticide risk assessment of the active substance ferric pyrophosphate – Appendix A. EFSA Journal 2020;18(1):5986, 37 pp. doi:10.2903/j.efsa.2020.5986

⁶ European Food Safety Authority; Conclusion on the peer review of the pesticide risk assessment of the active substance iron sulfate. EFSA Journal 2012;10(1):2521. [48 pp.] doi:10.2903/j.efsa.2012.2521.

⁷ <https://www.yumpu.com/en/document/read/37735226/technical-data-sheet>

The carbonyl iron data sheet includes a table comparing endpoints from mammalian acute toxicity studies conducted with different forms of iron. This is reproduced below.

Table B.9.2-10: Comparison of acute toxicity of different iron forms from data sheet for [REDACTED] carbonyl iron powder

Species	FeSO ₄ LD ₅₀ (mg/kg)	Fe ²⁺ LD ₅₀ (mg/kg)	Carbonyl iron powder LD ₅₀ (mg/kg)	Study
Rat	1490-5000	298-1000	30000	Shelanski, 1950; Boyd and Shanas, 1963
Guinea Pig	1500-1750	300-350	20000	Shelanski, 1950; Boyd and Shanas, 1963
Dog	800	160	>25000	GAF, 1990
Young Rat	950	190	19000	ISP, 1997a

This comparison table also indicates that elemental iron is less acutely toxic to mammals, compared to dosing with ferrous sulphate or ferrous ions. However, the underlying studies are not published, so HSE has not been able to confirm the reliability of this comparison.

Overall, the available data do indicate that carbonyl and elemental iron are less acutely toxic to mammals than ferrous salts. This would suggest that it is likely that birds would also be less acutely sensitive to elemental iron than ferrous salts. As the LD₅₀ for elemental iron from the [REDACTED] (2008) acute avian toxicity study is an unbound value there is uncertainty as to the 'true' avian LD₅₀ for elemental iron. Given that ferrous sulphate heptahydrate is the more acutely toxic form of iron to mammals, it is unlikely that elemental iron would be more toxic to birds than ferrous sulphate heptahydrate. Therefore, if a higher concentration had been tested in [REDACTED] (2008), it would be expected to result in a LD₅₀ ≥ 1230.75 mg Fe/kg bw. However, the level of mortality in birds when exposed to elemental iron at this dose has not been experimentally confirmed.

Iron accumulation in organs and health impacts for birds and mammals

In vertebrate species ingestion of large quantities of absorbable iron in the diet can lead to the storage of iron in body tissues, especially the liver. Iron overload occurs when iron intake exceeds needs and iron accumulates in tissues. Accumulation of iron deposits in tissues (especially the liver) is known as haemosiderosis and where this results in health impacts, the terms haemochromatosis or iron storage disease are often applied. Accumulation of iron in the liver of vertebrates, hepatic haemosiderosis, may or may not result in damage to the functioning of the liver. The literature review provided by the Applicant has identified two publications which contain experimental information on iron accumulation in the livers of birds: Dierenfeld et al. (1994) and Mete et al. (2003). These publications are briefly summarised in Volume CA section B.9.1.1 and are discussed below.

Dierenfeld et al. (1994) summarises data on iron concentrations in livers of dead birds, including those housed in the Wildlife Conservation Park (Bronx Zoo, US). Two families of the order Passeriformes, Paradisaedae (birds of paradise) and Sturnidae (mynahs, starlings), were found to have high liver iron concentrations, and these species are the focus of the publication. The authors state that these families are also known to be associated with incidents of hepatic iron overload.

In Paradisaedae the average liver iron concentration was 4245.8 mg/g (wet weight) for birds kept in the zoo and fed a diet containing 65 ppm iron. In contrast the average residue in free-ranging birds was 469 mg/g (wet weight). However, while the average iron concentration in livers from birds fed the iron fortified diet was about an order of magnitude higher than the average liver concentration for free-ranging birds, it is noted that the sample size for the free-ranging population was very low (n = 2) and that the standard deviation for the birds fed the 65 ppm diet was high (±2958.5 mg/g).

For Sturnidae the average liver concentration for birds fed a diet containing 156 ppm iron was 1984.6 ± 1413.9 mg/g (n = 20), and for free-ranging birds was 700.3 ± 125.9 mg/g (n = 24). Therefore, there is also an apparent

difference in liver concentrations between free-ranging birds and birds fed an artificial diet, though again the high variability complicates interpreting the significance of this apparent difference.

Dierenfeld et al. (1994) state that for other Passeriformes liver iron concentrations were in the range 500-1000 mg/g wet weight, i.e. lower than the average values for Paradisaedae and Sturnidae.

The results reported in Dierenfeld et al. (1994) indicate that iron can build-up in the livers of captive birds fed an artificial diet containing iron, though the differences from the free-ranging birds sampled should be treated with caution, given the high variability. The accumulation of iron in the liver has the potential to impact the fitness/survival of birds, though it is not presented in the study whether the liver iron concentrations in Dierenfeld et al. (1994) led to health effects.

The authors consider whether the excess iron concentrations in livers of Paradisaedae and Sturnidae could be due to genetic factors or diet. They consider that had predisposition to iron storage disease been exclusively genetic in origin, then differences in liver concentrations between zoo and free-ranging birds would not have been observed. They propose that diet is a prime factor influencing development of iron storage disease, with species that predominantly eats fruits or insects in the wild more susceptible to the disease. This is postulated on the basis of fruits and insects containing lower iron concentrations than other foods, though the data considered by HSE below do not consistently support there being a significantly lower iron concentration in fruits and insects, particularly insects.

The relevance of the data reported in Dierenfeld et al. (1994) for the 'Final Bite' risk assessment can be considered further. Clearly, Paradisaedae is not a bird family that would encounter 'Final Bite' granules or molluscs that have consumed such granules in GB. Sturnidae is the starling family, which contains species that could be relevant for the GB risk assessment. However, given Sturnidae includes more exotic species such as Mynah birds, and given bird livers were sampled from zoo animals, it is considered likely that the majority of the dataset comes from exotic species that are not native to GB. Therefore, the data from Dierenfeld et al. (1994) is of limited relevance for the GB assessment for elemental iron.

Mete et al. (2003) investigated the causes of observed high variability in incidences of iron storage disease between bird species, selecting Hill mynah birds as a species that frequently experiences this disease and selecting domestic chicken as a species which does not. Birds were fed diets containing 54 mg Fe/kg as Fe(II) sulphate and iron uptake was investigated at the intestinal absorptive cell level. Liver iron concentrations were found to be at least 10-fold higher in mynahs and this was determined to be due to higher intestinal iron uptake of Fe (II). The higher intestinal uptake in mynah birds was the case despite the elevated iron concentration in the liver, suggesting that this species is less able to regulate its iron levels. While this study provides useful information on the mechanism behind differences in susceptibility to iron storage disease between bird species, toxic symptoms in birds were not investigated and it does not contain information directly relevant to the GB risk assessment for elemental iron, given the species tested.

HSE has performed additional literature searches and has found multiple other publications which discuss iron accumulation/iron storage disease in birds/mammals. Key studies are evaluated in Appendix 3 and are discussed below.

Crissey et al. (1993) – This study is summarised below. It is poorly reported but contains relevant information. The species tested (European starling) is potentially relevant for the risk assessment, since this species could consume granules or poisoned slugs. Birds were fed diets containing low (148 ppm) and high (3035 ppm) iron concentrations. Unfortunately data from the study are not well-reported, so analysis is reliant on the authors' verbal description of results. Accumulation of iron in livers did occur for both dietary groups but it is notable that a difference in accumulation between groups was not evident after 10 weeks exposure, but was observed after 18 weeks. This indicates the importance of the time of exposure in determining the extent of iron accumulation. While hepatic haemosiderosis was observed in starlings in this study, all birds appeared visually healthy during the study and did not lose bodyweight (though it is noted that birds in the high iron diet group had enlarged livers).

Wadsworth et al. (1983) – In this study hepatic haemosiderosis was investigated in captive birds during post-mortem examinations. The study does not inform on the levels of dietary iron intake required for hepatic haemosiderosis, and it cannot be determined whether accumulation of iron in bird livers resulted in the death of the bird or other health effects. However, the study does contain information on which bird species more

frequently experienced hepatic haemosiderosis, with these being birds from the orders Ciconiiformes, Passeriformes, Cuculiformes and Coraciiformes. The order Ciconiiformes includes wading birds, storks and herons. The order Cuculiformes includes the cuckoo and hoatzin families. The order Coraciiformes includes kingfishers and hornbills. It is considered that birds from these orders would be highly unlikely to be exposed to elemental iron from 'Final Bite' granules via diet. The order Passeriformes includes sparrows, thrushes and finches, amongst other species. While 'Final Bite' granules may be too large for some passerine species to consume, this is not necessarily the case for all species from this order and it is also noted that some passerine species consume molluscs. Therefore passerine species could be exposed to elemental iron from 'Final Bite' granules via ingestion of granules/molluscs.

Cork et al. (1995) – Presence of administered iron in tissues was investigated in white leghorn chickens in this study. Iron accumulation was observed in livers of treated birds but not controls. No mortality occurred in treated or control birds. Retrospective data found accumulation of iron in bird livers was fairly common in native and non-native New Zealand species (28% of cases), though the extent varied between species and individuals. The retrospective part of the study did not reliably inform on whether iron accumulation in the liver contributed to bird death, though the authors considered, based on this dataset and other reports, that *'although hepatic haemosiderosis is frequently a histological finding not associated with overt liver disease, it is often associated with concurrent malignant and infectious diseases'*.

Ward et al. (1988) and Ward et al. (1991) – These studies investigated uptake and accumulation of iron in birds and mammals. Accumulation of iron in livers was found to be relatively common in a wide range of bird species, especially Passeriformes, though it did not occur at all in some individuals. Data with starlings and mice showed that where hepatic iron concentrations were already high, absorption of iron was reduced, indicating that animals were able to regulate their iron levels, to some extent. Seasonal differences in iron accumulation were also evident for starlings. While accumulation of iron in bird livers was frequently observed in these datasets, there were no indications of this impacting liver functioning. There is generally a lack of information on iron exposure levels/concentrations in diet for these studies, which limits consideration of the relevance of the data for field exposure situations.

In summary, there are a number of studies where hepatic haemosiderosis has been found following intake of iron by birds. This effect is not solely limited to exotic species or captive birds, with hepatic iron accumulation found in some free ranging GB bird species. It is not possible to determine a dietary dose of iron that would be expected to result in hepatic haemosiderosis for a particular GB species, but the data raise the potential for exposure to elemental iron via granules or molluscs to result in iron accumulation in bird livers for relevant species, particularly Passerines and starlings. However, the majority of the data from the available studies found that the iron accumulation observed did not impact the functioning of the liver or the overall health of the bird.

The reported incidents where there were health implications relate to species not native to GB and/or under captive conditions (e.g. mynah birds). There are no studies that found hepatic haemosiderosis leading to disease in GB bird species under field conditions, though it would be fair to say that the studies available were not designed for this specific purpose. In a review paper on this subject, **Cork (2000)** states that *'there has not been any conclusive evidence that the presence of stainable iron in the liver of birds has any clinical significance although it has been linked to the presence of concurrent infectious and neoplastic diseases'*. Therefore the incidents seen with mynah birds etc. may relate to an indirect rather than direct effect of iron exposure. Less information is available for mammals, though the situation appears similar, with iron storage disease reported in non-native species only (e.g. New World monkeys and black rhinoceros).

Multiple reasons for the difference in susceptibility to hepatic haemosiderosis and associated health impacts (direct or indirect) between species have been postulated in the literature. These include genetic factors, evolutionary responses to local iron availability or the presence of fruit in the diet (ascorbic acid can enhance iron uptake). The rate of absorption of dietary iron is clearly a key determinant but it is unlikely that this is influenced by a single factor that would explain the differences seen between species. It is also noted that differences in susceptibility between captive and free ranging species could be impacted by the availability of multiple food sources for free ranging species. Where a bird or mammal is able to select between different food items containing different iron concentrations, they may be able to regulate their iron levels better than where there is no choice of food. This point is further discussed below.

Overall, it is apparent from the literature that dietary iron can accumulate in the livers of birds and mammals and in some cases lead to health effects, though this appears less likely (and may not occur at all) in free ranging GB bird and mammal species.

Iron toxicity incident data

Dierenfeld et al. (1995) discusses incidents of hepatic iron overload associated with disease reported for bird species in zoos, including mynah birds, birds of paradise, tanagers, hornbills, quetzals and toucans. While these incidents suggest that exposure to excess iron can result in negative health effects for birds, the species these incidents relate to are not native to GB and are not considered relevant to the GB risk assessment for birds consuming 'Final Bite' granules or molluscs that have fed on such granules. This point is discussed further in the section on iron accumulation in liver and health impacts for birds and mammals.

Pavone et al. (2014) report on a mortality outbreak in captive birds belonging to the family Turdidae in Italy. Other species held were not affected. The species involved, blackbird, fieldfare, song thrush and redwing, can be found in GB agricultural areas and have the potential to come into contact with elemental iron from Final Bite granules, particularly via consumption of molluscs. Therefore, the study contains information on species that are of relevance to the risk assessment. The authors conclude that exposure to excess iron in artificial diets was the cause of the mortality seen. Given that only these species were impacted, it suggests differences in susceptibility to iron storage disease for different bird species. It is notable that the birds involved were captive and it is unknown whether this was a co-factor in the mortality observed (e.g. increased stress or parasitism). The analysed concentration of iron found in the food granules was 111 mg/kg granules.

In **Haldane and Davis (2009)**, information is available from poisoning incidents with dogs in Australia. The ingested doses of iron ranged from 728–4400 mg/kg bw. These doses are well below the acute LD₅₀ and no mortality was observed, noting that dogs underwent chelation therapy. Therefore, these findings don't inform the acute risk assessment. The doses consumed are above those tested in Zhu et al. (2016) and at the upper end of those tested in Whittaker et al. (1996). The fact that iron levels in blood were elevated and dogs experienced sublethal effects at these doses is in line with results from the other studies used in the long-term/reproductive risk assessment. It is noted that the Haldane and Davis (2009) findings relate to a granules containing iron(Fe³⁺) EDTA complex and are not Final Bite.

The above incidents of iron accumulation impacting bird or mammal health do not relate to free ranging animals. The absence of reports of negative impacts of exposure to iron on bird or mammal health under field conditions may indicate that such animals are less susceptible to iron storage disease than captive animals. This is a significant finding. However, it must also be considered that the likelihood of reporting of such incidents would be expected to be lower for free ranging animals compared to captive animals.

The incidents of iron storage disease in birds reported in the literature primarily relate to exotic species, which are not directly relevant for the elemental iron risk assessment. However, this is not exclusively the case, with the mortality reported in Turdidae birds in Pavone et al. (2014) being particularly notable. The incident reported in Pavone et al. (2014) indicates that in species that are potentially relevant for the GB elemental iron risk assessment, accumulation of iron can occur to a sufficient extent to result in mortality (with captive birds at least). These birds were exposed to an iron concentration of 111 mg/kg in their diets. This is within the range of 50–141 mg/kg reported for commercial poultry diets (see section on iron in commercial diets of captive birds), which would further suggest there is high variability in susceptibility to iron storage disease between bird species. The 111 mg iron/kg in bird diets in Pavone et al. (2014) is considerably lower than the 10000 mg iron/kg in Final Bite granules, noting such a comparison does not account for the amount of food/granules consumed. Given Pavone et al. (2014) do not report the calorific content of the food that was given to birds, it is not possible to accurately determine the daily dose of iron consumed by captive birds.

Long-term studies with mammals

The Applicant has provided further consideration of two critical studies investigating toxicity in mammals from exposure to iron via an expert statement (Schabacker & von Blanckenhagen, 2023b). These studies are considered in the context of long-term/reproductive risks to birds and mammals from the proposed uses of the representative formulation 'Final Bite'.

Table B.9.2-11: Selected short-term toxicity data for elemental iron (reproduced from section B.6)

Reference	Route of exposure and duration	Test item, doses	LOAEL mg/kg bw/d	NOAEL mg/kg bw/d	Species	Main adverse effects
Whittaker P. <i>et al.</i> , (1996) Publication	Oral 84 d	Fe ⁰ (carbonyl) 35 (control), 350, 3500, 20,000 mg iron/kg diet Approximately equivalent to 3.2, 35, 350, 1850 mg/kg bw/d	35	3.2	Rat	3.2 mg/kg bw/d No observed effects 35 mg/kg bw/d Liver iron deposition (haemosiderosis) and lipid peroxidation 350 mg/kg bw/d Mortality 2/10, liver iron deposition (haemosiderosis) and lipid peroxidation, cardiomyopathy, splenic and pancreatic atrophy 1850 mg/kg bw/d Mortality 5/18, iron deposition (haemosiderosis), cardiomyopathy, splenic and pancreatic atrophy
Zhu Q. <i>et al.</i> , (2016) Publication	Oral 90 d	Fe ⁰ (carbonyl) 0, 100 or 200 mg/kg bw/d	> 200	200	Rat	No adverse effects observed on a variety of parameters

In the sub-sections below, the two studies and the analysis of these in Schabacker and von Blanckenhagen (2023b) are discussed.

Whittaker *et al.* (1996)

This study was conducted in the Centre for Food Safety and Applied Nutrition (FDA, USA). The aim was to induce an iron-overloaded rat model via oral exposure – as a comparison to the human condition, ‘genetic haemochromatosis’. Groups of ‘weanling’ male Sprague Dawley rats were fed diets supplemented with carbonyl iron (purity unknown) at 35, 350, 3500 or 20000 mg iron/kg of diet for 12 weeks. There were 11, 10, 10 and 18 rats per group, respectively. The concentration level of 35 mg/kg diet (~3.2 mg Fe/kg bw/day) represented a ‘control’ exposure and the data from the three ‘treatment’ groups (approximately 35, 350 and 1850 mg/kg bw/d) were compared to the control group. At termination, all animals were subjected to the following investigations: necropsy with a focus on the liver, heart, pancreas and spleen; non-heme iron content and lipid peroxidation in the liver, apoptosis in the pancreas and histopathology (Prussian blue staining for Fe stored as haemosiderin, and general hematoxylin & eosin staining).

The Whittaker *et al.* (1996) study is a non-GLP publication. The HSE toxicology evaluation of this study concluded it was not wholly reliable for risk assessment, due to deficiencies in reporting (e.g. lack of data on bodyweight or food consumption).

EFSA guidance on bird and mammal risk assessment (EFSA, 2009), specifies in section 2.3 that repeated dose 28-day oral toxicity studies with rodents (OECD 407, 1998a) and subchronic oral toxicity 90 day rodent studies (OECD 408, 1998b) may be considered in assessing the reproductive risk to mammals. There are key similarities in the methodology of the Whittaker *et al.* (1996) to these study designs, i.e. there is prolonged oral exposure of rodents via diet and investigation of toxic effects. Therefore, results from the Whittaker *et al.* (1996) study are considered potentially relevant for the reproductive/long-term risk assessments for mammals.

Key results from Whittaker *et al.* (1996) are briefly summarised below.

- Reduced survival at 350 and 1850 mg/kg bw/d (20% and 28% reduction compared to 'control').
- 5/7 rats which died prematurely had heart damage attributed to iron overload.
- Statistically significant increase in non-heme iron in liver at 350 and 1850 mg/kg bw/d (813% and 3126% increase compared to 'control').
- Statistically significant increase in non-heme iron in heart at 1850 mg/kg bw/d (29% increase compared to 'control').
- Increased incidence of spleen and pancreas atrophy at 350 and 1850 mg/kg bw/d.
- Increased incidence of cardiomyopathy at 350 and 1850 mg/kg bw/d.
- Significant increase of conjugated dienes in liver at 35, 350 and 1850 mg/kg bw/d, indicating lipid peroxidation (statistically significant at 350 mg/kg bw/d and above).

An overall NOAEL of 3.2 mg/kg bw/d was identified from this study.

In the expert statement (Schabacker and von Blanckenhagen, 2023b), the authors note that Whittaker *et al.* (1996) was not conducted to GLP and does not follow an OECD guideline. HSE notes that while the study was not conducted to GLP, this is typical for published literature and there is very limited information investigating the toxicity of iron to birds or mammals from GLP studies. While it is not ideal that the study did not follow a recognised OECD (or similar) guideline, the conduct and results generated by the study are considered relevant for the regulatory risk assessment question (i.e. determination of the risk to mammals from oral intake of iron in diet).

Additionally, Schabacker and von Blanckenhagen (2023b) point out that the aim of the study by Whittaker *et al.* (1996) was not to test exposure of wild rodents to agricultural iron use, but to establish a model of iron overload in rats as a comparison to genetic haemochromatosis in humans. The study was therefore designed for this purpose and not for a realistic exposure of wildlife. While this is correct, it does not mean that relevant results from the study cannot also inform the risk assessments for wild mammals (and birds).

There are limitations regarding reporting in this study, which are discussed in Schabacker and von Blanckenhagen (2023b) and by HSE in section B.6. There are also uncertainties relating to the relevance of the study in addressing the risk assessment situation for Final Bite. These are discussed below and it is agreed that these do limit the robustness of the study and its relevance for the risk assessment.

- There is a lack of information on the bodyweight of study animals and how much food they consumed. This means that the daily doses reported above are extrapolated values. Therefore, the dose values stated are not precise and there is a degree of uncertainty with them.
- Iron would be expected to be present as a micronutrient in the husbandry diet given to test animals. It is unclear from the publication whether this has been taken into account when expressing concentrations of iron in test diets (i.e. whether these reflect added carbonyl Fe plus Fe present in normal diet). If iron in the diet was not accounted for, then the true level of exposure in the study could be underestimated. A datasheet for AIN-76A Semipurified Diet⁸ indicates that it contains 3.5% AIN mineral mix, which contains 6 g/kg Ferric Citrate (16-17% Fe). Therefore, AIN mineral mix contains approximately 1 g/kg Fe, meaning AIN-76A contains 35 mg Fe/kg. This is equivalent to the control concentration, so it is apparent that the concentrations presented in Whittaker *et al.* (1996) do account for iron in the normal diet.
- Iron was added to test diets in the [redacted] Fe form, rather than as elemental [redacted]-Fe has a small particle size, which could result in higher bioavailability than other forms of iron (Whittaker *et al.*, 2002 – see section B.6.2.1).
- Final Bite also includes a [redacted] which rats were not dosed with in Whittaker *et al.* (1996). Schabacker and von Blanckenhagen (2023b) postulate that the [redacted] 'is highly likely to limit the effects of iron if ingested by wildlife'. This point is discussed in a subsequent section.

Zhu et al. (2016)

This study was conducted at the Department of Food Science and Technology, East China University of Science and Technology, Shanghai. Groups of 10 male Wistar rats received daily oral gavage doses of 0 (control diet), 100 or 200 mg/kg bw carbonyl Fe (unknown purity), for 13 weeks. Blood samples were taken at termination for haematology and clinical chemistry investigations. Following complete necropsy, the weights of liver, kidneys, spleen and testes were recorded and subsequent histopathology of these organs, stomach and ileum was performed.

The Zhu *et al.* (2016) study is a non-GLP publication. The HSE toxicology evaluation of this study concluded it was acceptable, with limitations (the laboratory did not deploy specific histopathological staining against iron deposits).

Since the study involved prolonged oral exposure of rodents and investigated toxic effects, it is considered potentially relevant for the reproductive/long-term risk assessments for mammals, though it is noted that exposure was via gavage, rather than diet. EFSA guidance (EFSA, 2009) indicates that the use of gavage dosing can result in high systemic levels that induce adverse findings that cannot be produced when equivalent doses (in mg/kg bw/d) are given via the diet.

There were no adverse effects on rats at up to the maximum dose administered in the Zhu *et al.* (2016) study (i.e. 200 mg/kg bw/d).

The Zhu *et al.* (2016) study is discussed in the expert statement (Schabacker and von Blanckenhagen, 2023b). It is noted that this study was not designed to investigate the exposure of wild rodents to iron in agricultural scenarios but despite this, relevant results from the study can potentially inform the risk assessment. The same shortcomings regarding the study not being to GLP or following an OECD guideline are noted, as discussed above for Whittaker *et al.* (1996).

Comparison of Whittaker et al. (1996) and Zhu et al. (2016) studies

In both the Whittaker *et al.* (1996) and Zhu *et al.* (2016) studies rats were orally exposed to carbonyl Fe over a period of 84-90 days. Despite this similarity, the lowest reported no effect doses are quite different, with a NOAEL of 3.2 mg/kg bw/d in Whittaker *et al.* (1996) and a NOAEL of ≥ 200 mg/kg bw/d in Zhu *et al.* (2016). While there are issues due to the level of reporting in the Whittaker *et al.* (1996) study, many of the uncertainties associated with this study are also applicable for the Zhu *et al.* (2016) study. Therefore, there isn't a convincing case to demonstrate that one of these studies is relevant for the Final Bite risk assessment, while the other is not.

One difference in design between the studies that could explain the difference in results observed was the method of dosing, with rats dosed via diet in Whittaker *et al.* (1996) and via gavage in Zhu *et al.* (2016). It is considered plausible that the difference in dosing method could impact the likelihood of iron being excreted or transported to organs, where it can accumulate. Dosing via diet may result in exposure over a longer time period than dosing via gavage, hence there will be a longer period during which elemental iron is in contact with gastric acid, thus forming ferrous ions, with subsequent transport through the mucosal cell. However, the data available are insufficient to reach a definitive conclusion whether the dosing method is responsible for the difference in results seen between the studies. As noted by Schabacker and von Blanckenhagen (2023b), '*it is currently unclear what is responsible for these differences*'.

Neither the Whittaker *et al.* (1996) or Zhu *et al.* (2016) study results allow ED_x endpoints to be determined. While use of NOAEL endpoints is standard in reproductive/long-term risk assessment for birds and mammals, the precision of the endpoints is limited by dose spacing. It is noted that in the Whittaker *et al.* (1996) study dose spacing was relatively wide, with test concentrations equivalent to 3.5, 35, 350 and 1850 mg/kg bw/d. At the LOAEL, 35 mg/kg bw/d, the effects observed were iron deposition in the liver, along with lipid peroxidation. There was no mortality, limited cardiomyopathy and no atrophy of the spleen or pancreas at 35 mg/kg bw/d, whereas such clear symptoms of toxicity were observed at higher doses. As such, clear adverse impacts on the health of test organisms that would influence survival or reproductive success were only observed at 350 mg/kg bw/d and above in Whittaker *et al.* (1996). In the Zhu *et al.* (2016) study there were no adverse effects at 100 or 200 mg/kg bw/d, i.e. doses between the 35 and 350 mg/kg bw/d levels tested in Whittaker *et al.* (1996). It is also noted that in the Zhu *et al.* (2016) study iron deposition in organs was not directly measured, so it is possible that there was build-up of iron in organs at 100 or 200 mg/kg bw/d in the Zhu *et al.* (2016) study, with no associated

adverse effects. Therefore, it is also possible that the difference in NOAEL values between the studies is an artefact of the doses selected and the parameters investigated in these studies.

Long-term/reproductive risk to mammals using NOAEL from Zhu et al. (2016)

In order to consider the potential impact of the selection of NOAEL on the long-term/reproductive risk assessment for mammals, the first tier TER calculation is presented below using a NOAEL of 200 mg a.s./kg bw/d.

Table B.9.2-12: Tier 1 - Estimates of exposure and long-term/reproductive risk to mammals following ingestion of granules as a source of food

Generic focal species	Food	FIR/bw (kg dw/kg bw/d)	Content of Metaldehyde in the product (mg a.s./kg)	TWA	MAF	DDD (mg a.s./kg bw/day)	Toxicity (mg a.s./kg bw/day)	TER	Acceptability trigger
Wood mouse	Granules based on cereal seeds	0.21	10000	1	1	2100	200	0.095	≥5

The resulting TER is below the trigger value of 5, indicating that at tier 1 it has not been demonstrated that there will be no unacceptable impact to mammals.

Uptake and regulation of iron

Iron absorption

The ferric (Fe^{3+}) and the ferrous (Fe^{2+}) forms are the only oxidative states of iron stable in the aqueous environment of the animal body and in food. Absorption of iron is more efficient in the ferrous state because ferric iron is less soluble at the alkaline pH of intestinal fluid (Lewis et al., 2006). Therefore, in order for poorly soluble ingested elemental iron (Fe^0) to enter the circulatory system of the animal, it must first be converted to ferrous iron (Fe^{2+}).

The two major forms of dietary iron are heme iron, bound within a protoporphyrin ring and abundant in animal hemoproteins such as hemoglobin or myoglobin, and non-heme iron, bound to other molecules (Coffey & Ganz, 2017). Elemental iron in 'Final Bite' granules is present in the non-heme form.

As most non-haem iron in the diet is in the ferric form (Fe^{3+}), it first needs to be reduced to the ferrous form (Fe^{2+}) before it can be absorbed (Wallace, 2016). Non-heme iron is primarily absorbed in the duodenum, where acidic gastric secretions enhance iron solubility (Coffey & Ganz, 2017). Iron is absorbed by proteins in the mucosal epithelium of the luminal surface of the duodenum. It can then either be sequestered within the iron-storage protein ferritin or exported to plasma. To enter the systemic circulation, ferrous iron must cross the basolateral membrane of intestinal enterocytes. This is achieved via the iron exporting transmembrane protein ferroportin, which is currently the only known iron exporter.

Iron from animals (heme iron) exists in the ferrous form. While the uptake mechanisms for heme iron differ from those for non-heme iron, they appear less well understood (based on the literature reviewed). Coffey and Ganz (2017) suggest uptake occurs via receptor-mediated endocytosis rather than passive diffusion. It is noted that heme iron is only soluble at neutral or alkaline pHs such as those of the jejunum and ileum (Aggett, 2012). Therefore, heme and non-heme iron are most efficiently absorbed in different sections of the small intestine. Heme solubility is also increased significantly by the presence of protein.

Given how efficiently iron is recycled within the body, only a relatively small fraction of iron from the diet needs to be absorbed. Normally, heme iron (from animal sources) is considered to be approximately 20-25% available to the animal, while non-heme, vegetative sources are usually less than 5% available (Brue, 1994). Unabsorbed iron will be excreted in faeces by the animal.

The efficiency with which iron is absorbed is dependent on the plasma iron concentration. The production of hepcidin inhibits the functioning of ferroportin, as discussed in the section below on homeostasis. It is noted that the efficiency of iron absorption increases during pregnancy and is sustained during lactation (Aggett, 2012; Ramsay and Campbell, 1954). This is due to a higher iron requirement during these periods.

There are also other factors which can influence the absorption of iron from the diet. Absorption of iron can be enhanced by organic acids such as ascorbic acid, malic acid, tartaric acid, lactic acid, and citric acid, which occur naturally in fruit and vegetables (Aggett, 2012). Chelators or ligands can bind with non-heme iron to either inhibit or enhance its absorption (Lewis et al., 2006).

Other inhibitors of non-heme iron's availability include phytates found in wholegrain cereals, legumes, nuts, and seeds.

On the basis of the available literature, it is apparent that the extent of uptake of iron from the diet is dependent on the form of iron, the presence of other chemicals, and the current iron levels within the animal. The fact that iron is present in 'Final Bite' granules as Fe (not Fe²⁺) and is in the non-heme form are both factors that would inhibit the bioavailability of ingested iron.

The absorption, distribution, metabolism and excretion of iron by the oral route are discussed in detail in section B.6.1.1.

Homeostasis

As discussed above in the section on toxicity, both deficiency and overabundance of iron in the body can lead to negative health impacts to birds and mammals. The literature search identified a number of publications which discuss how birds and/or mammals are able to regulate their iron levels. Iron absorption and distribution is homeostatically regulated to reduce the risks of deficiency and overload (Schumann et al., 2007). This homeostatic capacity is discussed briefly here.

Dietary iron is the sole source of iron in the bodies of birds and mammals. The primary method of iron loss is bleeding. Within the body iron is recycled efficiently, with negligible excretion. Any iron found in the faeces is usually a result of unabsorbed iron from the diet (Brue, 1994). As a result of the limited capacity for excretion to reduce excess iron levels within the body, the capacity to manage intestinal uptake is key to iron regulation. Aggett (2012) describes the process by which gastrointestinal absorption of iron can be up-regulated or down-regulated by mammals, in response to the requirements of the organism. This is controlled at systemic and cellular levels. Each cell controls its iron uptake and storage to meet its individual iron requirement. Within cells iron is bound to the protein ferritin.

The mechanisms regulating systemic iron homeostasis are largely centred on the liver and involve two molecules, hepcidin and ferroportin, that work together to regulate the flow of iron from cells into the systemic circulation (Wallace, 2016). Ferroportin is a transmembrane protein that transports iron from the inside of a cell to the outside of the cell. The peptide hormone hepcidin is synthesized by hepatocytes. It is produced and secreted by the liver in response to the availability of iron. By inhibiting ferroportin, hepcidin regulates both the acquisition of new iron from the diet and the release of iron from stores in hepatocytes and macrophages. Hepatocytes sense circulating iron levels directly and also respond to iron-induced bone morphogenetic proteins (Coffey & Ganz, 2017). An increase in plasma iron concentration (amount of iron bound to transferrin) stimulates hepcidin production, which reduces intestinal iron uptake and release from storage. Hepcidin is down-regulated by increased iron needs and by hypoxia, and is up-regulated by inflammation (Aggett, 2012).

Iron released into plasma is rapidly bound by the protein transferrin. This plasma protein is responsible for the distribution of iron to tissues. However, the binding of absorbed iron to transferrin is saturable (Coffey & Ganz, 2017). Iron overload can occur where transferrin is already saturated with iron. In cases of iron overload, plasma iron concentrations chronically exceed the binding capacity of transferrin, resulting in non-transferrin bound iron in plasma. This is then transported via the plasma and can accumulate in tissues, including the liver, pancreas, and heart. Excess iron can be stored in the protein ferritin or as a pigment called hemosiderin, a deposit of protein and iron.

While it is clear from the literature available that birds and mammals are able to tightly regulate the uptake of iron, in response to deficiency or excess iron in their diet, there clearly are limits to this buffering capacity, at least under certain circumstances. As noted in Schumann et al. (2017): *'repleted iron stores and preceding high iron intakes reduce intestinal iron absorption which, however, offers no reliable protection against oral iron*

overload'. This is further evidenced by the toxicity data discussed above, where in some situations iron accumulates in organs, such as the liver, to a sufficient extent that it impacts the functioning of these organs and the survival of the organism. Therefore, it cannot simply be concluded that the homeostatic regulation of iron within the body precludes the possibility that consumption of granules containing elemental iron (or molluscs that have consumed such granules) can result in negative health impacts to birds and mammals. The critical aspect is whether the amount of iron consumed in the diet can be successfully buffered by controlling gastrointestinal uptake, or whether a critical threshold is exceeded.

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

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[REDACTED]

[REDACTED]

Potential for exposure of wild birds and mammals to iron via 'Final Bite' granules

Avoidance due to colour of granules

As part of the weight-of-evidence assessment (Schabacker and von Blanckenhagen, 2023a), the following has been stated: '*Blue is a rather unattractive colour for birds and mammals and used as a visual cue for minimising interactions with toxic baits (Cowan and Crowell 2017)*'. The potential for the colour of granules to reduce consumption has been briefly considered in the initial HSE evaluation. It was concluded that the one available study (Pawlinka and Proulx, 1996) was not sufficient to demonstrate that birds will avoid eating Final Bite granules as a food source, under conditions where alternative food items may be limited and pressure to feed may be high. The reference to the publication Cowan and Crowell 2017 is new and so will be considered further here.

Cowan and Crowell (2017) is a review publication from New Zealand. It does not contain primary data and is aimed at addressing issues related to whether bait used for possum control that is dyed green and contains cinnamon oil will be of reduced attractiveness to birds. This publication does include some additional discussion of whether blue dye should be used as an alternative to green dye, referencing studies by Greig-Smith & Rowney (1987) and Nicholls et al. (2000). These studies have not been submitted. The study title indicates that Greig-Smith & Rowney (1987) involves starlings and house sparrows, i.e. species that are relevant for GB. The Nicholls et al. (2000) study appears to be with American kestrels, which are not a relevant species for this GB

assessment. Additionally, Cowan and Crowell (2017) also refer to some other research with blue baits and native New Zealand bird species (Hartley et al. 1999, 2000, Weser & Ross, 2013) but again these are considered of limited relevance for this GB assessment. Cowan and Crowell (2017) does not contain any information on avoidance by mammals, though the authors note that avoidance behaviour in mammals is more likely a consequence of taste or odour, rather than a visual cue. Regarding Greig-Smith & Rowney (1987), the abstract for this study indicates that there was avoidance of blue-coloured food by both sparrows and starlings. It is noted that dyed turkey crumbs were used as the test item and that birds were kept in captivity.

Overall there are some indications from the literature that blue coloured food items can be avoided by birds. However, there is a lack of data on avoidance by relevant GB species when exposed to blue granules under field conditions. Whether such avoidance behaviour would occur with Final Bite granules in the field is unknown. Therefore, while the colour could reduce exposure to birds via consumption of Final Bite granules, this line of evidence has a high degree of uncertainty. Additionally, this line of evidence is not applicable for mammals.

Avoidance due to taste

In Schabacker J. & von Blanckenhagen F. (2023a) the authors state that iron has a well-known, distinctive, and noticeable taste. On this basis rodents would be expected to react to the adverse taste and inhibit consumption. This line of argumentation is supported by a study by Noorjahan et al. (2014), which has been evaluated by HSE. In this study consumption of water by rats was reduced with increasing concentration of an [REDACTED]. Therefore, under the conditions of the study the metallic taste of the test item did inhibit consumption. This is potentially a relevant finding for the risk assessment, though it is noted that because an animal is able to detect iron in water does not necessarily mean they would be able to detect iron when ingesting a more complex, solid mixture, such as a Final Bite granule. Additionally, the extent to which such an effect would reduce exposure of wild mammals from 'Final Bite' granules under field conditions is unknown. It is also noted that no equivalent information is available for birds, which may have a less developed sense of taste than rats.

Unfamiliarity of birds and mammals with Final Bite granules

The fact that Final Bite granules will be an unfamiliar sight to birds and mammals has been suggested by Schabacker and von Blanckenhagen (2023c) to be another factor which would decrease the likelihood of granules being consumed. The authors propose that it would take time for animals to get used to the granules and try out what may be perceived as a new food source, noting that granules disappear after a relatively short time (i.e. before animals have adjusted to their presence). However the proposed uses of Final Bite include all edible and non-edible crops and up to 6 applications could be made per crop field. Given the potential widespread application of granules over an extended time period, and potential year-on-year use, it is not clear that granules would remain an unfamiliar potential food item for birds and mammals. The potential for granules to degrade over time is discussed in a separate section.

Unattractiveness due to low calorific content of granules

In Schabacker and von Blanckenhagen (2023c) the calorific content of Final Bite granules is compared to the energy content of other food items that may be consumed by birds or mammals in treated fields. These are summarised in the following table. The energy contents for the other food items are defaults taken from the EFSA bird and mammal guidance (EFSA, 2009). Taking into account the proportion of [REDACTED] in Final Bite granules (see Volume 4) and using a [REDACTED] the energy content of granules has been estimated by HSE. While this calculation does not take into account the energy from other co-formulants that are present in Final Bite granules, given that apart from [REDACTED] these constitute a low proportion of the overall formulation, their omission would be expected to make only a minor difference to the calculated energy content of Final Bite granules.

Table B.9.2-13: Food energy contents of Final Bite granules relative to other potential food items

¹¹ McCance and Widdowson's 'composition of foods integrated dataset' on the nutrient content of the UK food supply ([Composition of foods integrated dataset \(CoFID\)](https://www.gov.uk/government/datasets/composition-of-foods-integrated-dataset-cofid) - GOV.UK (www.gov.uk)).

Food item	Grass, cereal shoots	Non-grass herbs	Cereal seeds	Weed seeds	Arthropods	Soil invertebrates	Final Bite slug granules
kJ/dry g	17.6	17.8	18.4	21.7	22.7	19.4	10.9-11.95

It is apparent that Final Bite granules have a considerably lower calorific content per unit weight compared to other food items that may be present in treated fields and that may be consumed by birds or mammals. Therefore, assuming the energy expended to find food items is equivalent, other available food items may be expected to be consumed in preference to Final Bite granules, since such a foraging strategy would optimise energy intake/usage. This information would indicate that assuming a diet of 100% granules across multiple days is unrealistic, though it does not exclude the possibility of birds or mammals consuming Final Bite granules completely. The likelihood of granules being intentionally consumed for their energy content is expected to vary according to the availability of other food items, which may differ throughout the year (e.g. suitable alternative food items may be more scarce in winter for some species).

Availability of alternative food items

When bird or mammal species which have the potential to consume Final Bite granules forage within treated fields, there are likely to be other food items available to them which they could consume. For example, a granivorous species would consume weed or crop seeds. As other food items will be available, these are likely to be consumed, in addition to any Final Bite granules that are eaten. This has not been accounted for in the first tier risk assessment, which assumes only granules are consumed when foraging in the treated field.

No information has been provided to inform on the different food items that may be available in treated fields and their quantities. HSE has therefore considered any information in the bird and mammal risk assessment guidance document that may be relevant. EFSA (2009) assumes 100 small seeds/m² are visible to a small bird (i.e. to a depth of 1 mm) in a first tier scenario for granule applications (used in the default calculation for the birds mistaking granules for small seeds scenario). Assuming an application rate for Final Bite of 60 granules/m², this means granules constitute 37.5% of the total small seeds or granules available on the soil surface.

If it is conservatively assumed that granules are of similar attractiveness as a food source compared to small seeds, it could be estimated that in these circumstances that the diet of a small granivorous bird when foraging in a treated field could include 37.5% granules (noting that in reality there would be variability in both the number of granules available per m² and the number of seeds per m²). However, while this could be taken into account in the quantitative risk assessment, it is evidence from the margin of failure in the first tier risk assessment that such a refinement would not be sufficient to be able to demonstrate there is no unacceptable risk to birds or mammals.

This line of evidence could be further developed with information on the number of seeds of a relevant size found per m² in a suitable range of crop fields, and/or robust information on the relative preference of relevant bird/mammal species for Final Bite granules (or blank granules) as a food item compared to weed/crop seeds.

Schabacker and von Blanckenhagen (2023a) suggest it is possible that birds or mammals consuming Final Bite granules would switch their foraging behaviour in relation to excess iron intake, i.e. when experiencing iron overload they could respond by preferentially consuming other food items and not granules, food items that contain lower iron concentrations. No publications have been provided to demonstrate that this behaviour occurs in birds or mammals but it is a line of evidence that could be developed further. Supporting evidence demonstrating this behaviour would need to be available for relevant species under field conditions. There is a study by Noorjahan et al. (2014) in which rodents reduced their consumption of spiked water in response to increasing concentrations of an [REDACTED]. However, this is more likely an avoidance response to an adverse taste, rather than rats adjusting their consumption in response to their physiological needs. Currently it is unclear whether birds and mammals might be capable of tasting and actively regulating their iron uptake from diet items via food selection in response to their physiological needs.

Proportion of time that a bird or mammal would spend foraging in a treated field (PT)

The first tier risk assessment assumes that the bird or mammal consumes 100% of its food from fields to which Final Bite granules have been applied. This is potentially a conservative assumption and one that can be refined in higher tier assessments of the long-term/reproductive risk. However, in order to refine this factor, appropriate supporting information would need to be supplied. This is usually in the form of studies investigating the proportion of their foraging time that relevant bird or mammal species spend in fields of the relevant crop, e.g. via radio-tracking their behaviour. In this case no such information has been provided. It is noted that the proposed uses of Final Bite include all edible and non-edible crops. Therefore, it would be difficult in practice to refine the PT factor, given the product could, in theory, be applied to any crop fields within a particular area. As such this point cannot be considered further.

Duration of granule availability

The representative product, Final Bite, can be applied up to six times at a rate of 8 kg product/ha (equivalent to 60 granules/m² per application). The quantity of granules applied in a given time period will depend on the level of infestation. The time period over which granules are available on the soil surface as a potential food item for birds or mammals will depend on the number of applications and how quickly the granules are removed/degraded. Schabacker and von Blanckenhagen (2023a) state that '*pellets are preferably spread in humid conditions where slugs become active. Therefore, the pellets are expected to swell and soften quickly and soon disintegrate*'. Additionally consumption of granules by slugs will reduce granule availability.

As discussed above and in section B.9.7, an earthworm field study (Axmann, 2019) submitted in support of this application included six applications of the representative product, each equivalent to 8 kg product/ha, with a five day application interval. This could be considered to be a worst-case application for the proposed GAP. The worst-case number of granules/m² was 156 over the course of the treatment period. This study did not directly investigate how long it took for the granules to be consumed by target organisms or otherwise degrade in the soil but the results do indicate that in some circumstances, build-up of granules following multiple applications can occur (noting that approximately 60 granules/m² are applied per application). However, it is noted that the purpose of this study was to investigate effects of iron granules on earthworms, therefore the timing of application and number of applications was not driven by slug pressures, as would be expected for actual use of Final Bite. Therefore, the maximum number of granules found per unit area in Axmann (2019) may be an overestimate of that expected following the realistic application of elemental iron granules, where slug pressures are high and hence removal of granules by slugs may also be high.

In Schabacker and von Blanckenhagen (2023b) the authors calculate that in the earthworm field study by Axmann (2019) the granule dissipation was equivalent to a mean reduction of 59.4% in 5 days. The authors note that as there was no mollusc infestation in the Axmann (2019) study fields, the granules would likely disappear much faster in the field under real conditions, when slugs feed on the granules.

In section B.3 granule degradation is considered. Two rainproof testing trials were provided from the UK in 2017 and 2018. Trials were conducted in a greenhouse with simulated rainfall (either 0mm, 4mm or 10mm per day with rainfall not exceeding 0.5mm per minute). Granule integrity was recorded over a 14 day period on a scale of 1-10 with 1 = granule intact and 10 = complete breakdown and disintegration of granule. With either 4 mm or 10 mm rain all granules were intact and of normal size and shape for at least 5 days after application. By 14 days after application there was moderate swelling and slight cracking of granules. These studies therefore indicate that granule integrity was generally well maintained for a 2-week period when exposed to artificial rainfall in a controlled environment.

The extent to which granules breakdown over time under field conditions has been investigated in trials intended to determine the efficacy of Final Bite. These field trials are considered in section B.3 and the relevant results for granule degradation are summarised in table B.9.2-14. Only data from UK field trials are shown, since these are considered most representative of GB conditions. Granule integrity/breakdown was assessed using a simple percentage estimate, with results from the trials highly variable. In one trial complete breakdown of the granules occurred with 12 days, whereas in another study trial granules integrity had only declined by 20% over a 4 week period. This variability in results could be due to differences in weather or local environmental conditions between sites. It is noted that the default averaging period assumed in long-term/reproductive risk assessments for birds and mammals under EFSA guidance (EFSA, 2009) is 21 days. In the majority of the field trials in table

B.9.2-14 granules had not completely deteriorated over this timeframe. This would indicate that degradation of granules over time alone would not be sufficient basis to exclude the potential for long-term exposure to occur.

Table B.9.2-14: Information on granule integrity and dead slug presence following application of 8 kg Final Bite/ha from UK efficacy field trials

Trial ID	Crop	Presence of dead slugs at surface	Granule integrity data
UK16MEBRSOL620A	Brassicas	Negligible	Approx. 40% breakdown 14 DAA
UK16MEBRSOL620B	Brassicas	Negligible	Approx. 45% breakdown 14 DAA
UK17MEBRSNW636A	Oilseed rape	Negligible	Approx. 40% breakdown 14 DAA; complete deterioration by 30 DAA
UK17MEBRSNW636B	Oilseed rape	Negligible	Approx. 25% breakdown 13 DAA; complete deterioration by 28 DAA
UK17MEBRSNW636C	Oilseed rape	Negligible	Approx. 50% breakdown 21 DAA; complete deterioration by 28 DAA
UK17MEBRSNW636D	Oilseed rape	Negligible	Approx. 50% breakdown 15 DAA; complete deterioration by 28 DAA
UK17MEBRSOL630A	Brassicas	Negligible	Approx. 10% breakdown 14 DAA; complete deterioration by 21 DAA
UK17MEBRSOL630B	Brassicas	Negligible	Approx. 40% breakdown 21 DAA
UK17MESOLTU629F	Potato	Negligible	Complete deterioration by 28 DAA
UK17METRZAS628A	Winter wheat	Negligible	Approx. 70% breakdown 14 DAA
UK17METRZAW637A	Winter wheat	Negligible	Near complete deterioration by 21 DAA
UK17METRZAW637B	Winter wheat	Negligible	Approx 20% breakdown by 14 DAA; 90% breakdown by 28 DAA
UK17METRZAW637C	Winter wheat	Negligible	Approx. 70% breakdown 14 DAA
UK17METRZAW637D	Winter wheat	Negligible	Approx. 90% breakdown 14 DAA
UK18MEBRSNS232A	Oilseed rape	Negligible	Approx. 40% breakdown 14 DAA
UK18MEBRSNS232B	Oilseed rape	Negligible	Approx. 20% breakdown 28 DAA
UK18MEBRSOL236A	Brassicas	Negligible	Approx. 30% breakdown 21 DAA
UK18MESOLTU235A	Potato	-	Approx. 20% breakdown 27 DAA
UK18MESOLTU235B	Potato	-	Approx. 20% breakdown 27 DAA
UK18MESOLTU235C	Potato	-	Approx. 45% breakdown 21 DAA
UK18MESOLTU235D	Potato	-	Complete deterioration by 12 DAA
UK18MESOLTU235E	Potato	-	Complete deterioration by 14 DAA
UK18MEYCERS234A	Winter barley	Negligible	Approx. 40% breakdown 28 DAA

DAA = Days after application

No studies have been submitted which directly investigate the rate of removal of Final Bite granules by molluscs under field conditions. However, in a number of caged arena trials the recovery of a known, added quantity of granules was assessed at various time points. The data from the UK arena trials are summarised in table B.9.2-15. In these trials slugs or snails were kept inside the study areas. Therefore, it is clear that there is the potential for slugs/snails to remove granules via ingestion under these trial conditions, though whether the number of confined slugs/snails is representative of realistic, best-case or worst-case feeding under field conditions is uncertain.

In 4/23 of the caged arena trials the number of granules found on the soil surface was reduced by 50% or more within 2-3 days. Therefore, rapid removal of granules occurred in a minority of studies. In the majority of studies it took up 14 days before granule numbers had reduced by 50% or more (13/23 trials). There were 4/23 trials in which the granule density was not reduced by 50% within 21 days. These results indicate high variability in the rate of removal of granules by slugs/snails, under trial conditions. Based on these results it cannot be assumed that removal of granules by molluscs will be sufficient to exclude the potential for long-term exposure of birds or mammals.

Table B.9.2-15: Information on granule counts over time from UK efficacy outdoor caged arena trials following application of 8 kg Final Bite/ha

Trial ID	Crop	Pellet recovery
UK16MENNNNN617A	Oilseed rape	Pellets per 0.25 m ² 0 DAA = 26 2 DAA = 13 6 DAA = 9.25 12 DAA = 9.25
UK16MENNNNN617B	Lettuce	Pellets per 0.25 m ² 0 DAA = 21.8 3 DAA = 8.8 7 DAA = 6.5 14 DAA = 4.3
UK17MEBRSNS627A	Oilseed rape	Pellets per 0.25 m ² 0 DAA = 17.5 2 DAA = 9.3 7 DAA = 6.3 14 DAA = 1.8 21 DAA = 0.3 28 DAA = 0
UK17MEFRASS634A	Strawberry	Pellets per 0.25 m ² 0 DAA = 13 2 DAA = 12.5 4 DAA = 11.5 6 DAA = 8.5 8 DAA = 10.5 10 DAA = 6.8 12 DAA = 4 14 DAA = 3
UK17MELACSA632B	Chinese cabbage	Pellets per 0.25 m ² 0 DAA = 15.3 2 DAA = 13.5 7 DAA = 7.5 13 DAA = 2.3 21 DAA = 0
UK17MEYORNA633A	Chinese cabbage	Pellets per 0.25 m ² 0 DAA = 18.8 3 DAA = 17.3 7 DAA = 13.5 12 DAA = 9.5 21 DAA = 2.5
UK17MEYORNA633A	Ornamentals	Pellets per 0.25 m ² 0 DAA = 18.5 3 DAA = 13.8

		7 DAA = 13 12 DAA = 10.8 21 DAA = 9.8
UK18MEBRSOL237A	Brassicas	Pellets per 0.25 m2 0 DAA = 9 2 DAA = 6 7 DAA = 5.8 12 DAA = 2.8 21 DAA = 5.5
UK18MEBRSOL237B	Brassicas	Pellets per 0.25 m2 0 DAA = 9.8 2 DAA = 11 8 DAA = 7.8 14 DAA = 4.3 21 DAA = 2.8
UK18MEBRSOL237C	Brassicas	Pellets per 0.25 m2 0 DAA = 14 2 DAA = 16 7 DAA = 11.3 14 DAA = 8 21 DAA = 7.8
UK18MEBRSOL249A	Brassicas	Pellets per 0.25 m2 0 DAA = 15.8 3 DAA = 17.8 10 DAA = 11.8 14 DAA = 9 21 DAA = 6.3
UK18MEBRSOL249B	Brassicas	Pellets per 0.25 m2 0 DAA = 10 2 DAA = 11 7 DAA = 7.8 14 DAA = 5.8 21 DAA = 3
UK18MEBRSOL249C	Brassicas	Pellets per 0.25 m2 0 DAA = 18.8 2 DAA = 16 7 DAA = 13.3 14 DAA = 11 21 DAA = 11.5
UK18MEFRASS239A	Strawberry	Pellets per 0.25 m2 0 DAA = 14.8 2 DAA = 18.3 4 DAA = 14.5 6 DAA = 12 8 DAA = 13.8 10 DAA = 13 12 DAA = 12.8 14 DAA = 11
UK18MEFRASS239B	Strawberry	Pellets per 0.25 m2 0 DAA = 8 2 DAA = 10.5 4 DAA = 8.3

		6 DAA = 10.5 8 DAA = 4.8 10 DAA = 6.5 12 DAA = 4.5 14 DAA = 2.5
UK18MEFRASS239F	Strawberry	Pellets per 0.25 m2 0 DAA = 14.3 2 DAA = 16.3 4 DAA = 12.5 6 DAA = 11.5 8 DAA = 12.8 10 DAA = 14.3 12 DAA = 12.8 14 DAA = 13.3
UK18MEFRASS248A	Strawberry	Pellets per 0.25 m2 0 DAA = 11.5 2 DAA = 13.8 4 DAA = 16.5 6 DAA = 11.5 8 DAA = 18.5 10 DAA = 16 12 DAA = 7.5
UK18MEFRASS248B	Strawberry	Pellets per 0.25 m2 0 DAA = 9.3 2 DAA = 13.8 4 DAA = 12 6 DAA = 10.8 8 DAA = 10 10 DAA = 6.3 12 DAA = 10.5 14 DAA = 6.5
UK18MELACSA238B	Chinese cabbage	Pellets per 0.25 m2 0 DAA = 12.3 3 DAA = 12.5 7 DAA = 11.8 10 DAA = 11.5 21 DAA = 5.3
UK18MEYORNA240A	Ornamentals	Pellets per 0.25 m2 0 DAA = 16.3 2 DAA = 23 7 DAA = 16 14 DAA = 5.3 21 DAA = 1.3
UK18MEYORNA240B	Ornamentals	Pellets per 1 m2 2 DAA = 49.8 9 DAA = 32.3 14 DAA = 6.8 21 DAA = 0
UK18MEYORNA247A	Ornamentals	Pellets per 0.25 m2 0 DAA = 13.3 2-3 DAA = 12 7-10 DAA = 9

		12-14 DAA = 4 21 DAA = 0.8
UK18MEYORNA247B	Ornamentals	Pellets per 0.25 m ² 0 DAA = 11.3 3 DAA = 5.8 7 DAA = 5.8 13 DAA = 3.5 21 DAA = 2.3

Extent to which birds or mammals may consume molluscs

In the EU review of the active substance metaldehyde, data from published literature were investigated to determine the extent to which molluscs are consumed by a range of bird species associated with agricultural land. The following table is reproduced from section B.9 of the additional report to the draft assessment report for metaldehyde¹².

Table B.9.2-16: Summary of dietary data for potentially slug-eating species from the metaldehyde DAR additional report

Species	Habitat	Diet	Slugs in diet
<i>Alauda arvensis</i> (skylark)	Open landscape with low vegetation ³	During winter only seeds and green plants; during summer also insects; rarely spiders, small gastropods and earthworms ³	7% molluscs annual average composition by volume ¹
<i>Corvus corone</i> (carrion crow)	Woods and large hedges for nesting, sleeping; open landscape for feeding ³	Principally invertebrates and cereal grain; also small vertebrates, bird eggs, carrion and scraps. ² Quite variable, depending on available food; among others also slugs and snails, among others Arionidae; in April 8 of 37 stomachs contained up to 19 slugs ³	9% snails annual average, composition by number ¹ . Up to 42% occurrence ²
<i>Corvus frugilegus</i> (rook)	Agricultural landscape ³	Variable and diverse; animal and vegetable; earthworms and insects mainly, sometimes large amount of slugs ³	7.3% gastropods annual average, up to 25% occurrence ²
<i>Corvus monedula</i> (jackdaw)	Feeding in open landscape; grassland, meadows and pasture, harvested and ploughed fields ³	Quite variable, depending on available food; food supplemented by gastropods, but always only small amounts ³	12.6% gastropods wet weight (Jan-Dec), 8.7% occurrence ²
<i>Emberiza citrinella</i> (yellowhammer)	Edge along agricultural land (hedges, small woods, ditches, ways, clearings, etc.) ³	Mainly seeds, also insects. ³	2% slugs annual average, composition by volume ¹

¹² [Public consultation on the active substance metaldehyde | EFSA \(europa.eu\)](https://www.efsa.europa.eu/en/public-consultation/public-consultation-on-the-active-substance-metaldehyde)

Species	Habitat	Diet	Slugs in diet
<i>Erithacus rubecula</i> (robin)	Hedges and woods, open area with short vegetation important as feeding habitat ¹	Mainly insects, also fruits, spiders, isopods, Myriapoda, Annelida, small gastropods (important as calcium source) ³	1% composition by number (Nov - Feb), up to 45% by occurrence (whole year) ¹
<i>Garrulus glandarius</i> (jay)	Woods or scrub, farmland as feeding visitor from adjacent woodland ²	Invertebrates, fruits and seeds. Small vertebrates occasionally taken, also carrion and domestic scraps ²	3.5% composition by volume ²
<i>Parus major</i> (great tit)	Hedges and woods ¹	Predominantly insectivore	11% other food, mainly pine seed and grit/snail shell (May-Jun) ¹
<i>Phasianus colchicus</i> (pheasant)	Woods or scrub ¹	Generally omnivorous, eating cereal grain, seeds, berries and other fruits, green shoots, roots and tubers, small arthropods, molluscs and occasionally small vertebrates ²	1.6% composition by number ²
<i>Pica pica</i> (magpie)	Predominantly a lowland bird of open or lightly wooded country ²	Invertebrates, especially beetles, fruits and seeds; occasionally small vertebrates and all kinds of carrion, refuse and domestic scraps. ²	1.1% by number (whole year in Russia), 50% occurrence (Sep. – Oct.), 32% occurrence (Mar – Apr.) in UK ²
<i>Prunella modularis</i> (dunnock)	Dark thickets, e.g. in spruce and mixed forests ³	During summer mainly animal food, rest of the year vegetables; insects, spiders, harvestmen; additionally small gastropods ³	up to 4.7% composition by volume, up to 33% occurrence in faeces samples ²
<i>Sturnus vulgaris</i> (starling)	Generally cultural landscape, also old forests and open grassland ³	Quite divers and highly flexible; mainly insects, earthworms and gastropods (not further specified) ³	7% molluscs composition by number annual average; up to 14% occurrence ¹
<i>Turdus iliacus</i> (redwing)	Forests ³	Mainly earthworms, also insects and gastropods ³	Quantitative proportion of diet similar to <i>Turdus philomelos</i> , 30.4% molluscs occurrence ³
<i>Turdus merula</i> (blackbird)	Forests, also small woods and near villages ³	Omnivore, mainly earthworms, beetles and ants, regularly also slugs and snails. ³	3% molluscs annual average composition by volume ¹ , up to 23% gastropods composition by number ³
<i>Turdus philomelos</i> (song thrush)	Coniferous forests ³	Variable, mainly earthworms, insects, but also snails and slugs (e.g. <i>Deroceras sp.</i>). ³	5% slugs and snails composition by number annual average ¹ , up to 44.3% molluscs composition by number ³

Species	Habitat	Diet	Slugs in diet
<i>Turdus pilaris</i> (fieldfare)	Structured open landscape, meadows, fields. ³	Mainly earthworms and insects, also gastropods (among others <i>Arion</i> and <i>Deroceras</i>). ³	Up to 40.2% gastropods by number (winter diet) ³
<i>Turdus viscivorus</i> (mistle thrush)	Forests, mainly forest edges and clearings ³	Insects, spiders, centipedes, also gastropods ³	3.5% slugs and snails composition by number ⁴

¹ Buxton et al. (1998)

² Riffel (2002)

³ Glutz von Blotzheim (2001)

⁴ BWP (2006)

The data in table B.9.2-16 indicate that the proportion of slugs in bird diets varies between species and can vary over time. The worst-case proportion of molluscs in bird diets is 0.443 from the song thrush data. Therefore it is apparent that for some bird species at some times of year, molluscs make up a significant part of their diet. Similarly for mammals, species of shrews, badgers and in particular, hedgehogs are known to include molluscs in their diets (Gurney et al., 1998). It should also be noted that dietary data based on analysis of stomach contents or faeces may underestimate the proportion of molluscs consumed, given they are soft-bodied organisms which may be broken up relatively quickly within the animal.

Overall, it is clear that based on dietary data, it cannot be excluded that some bird and mammal species could consume significant quantities of molluscs containing elemental iron.

Availability of poisoned molluscs to birds and mammals

An expert statement has been provided by Dr Catherine Whaley, head of crop research at i2L Research Ltd (Whaley, 2022). This statement discusses the behavioural responses of slugs and snails exposed to granular iron based molluscicides. The following key points are noted from this statement:

- Elemental iron has a very similar mode of action to ferric phosphate. The iron binds to a protein in the stomach and an insoluble complex is formed. This complex then leads to anoxia within the cells and ultimately results in death.
- i2L Research have performed over 90 trials (laboratory, semi-field and field) using elemental iron, and published literature and efficacy generated data show that both ferric phosphate and elemental iron are similarly toxic to slugs.
- Iron works as a contact poison, interfering with calcium metabolism in molluscs. When ingested it can cause irreparable damage to digestive tissue which prevents slugs and snails from feeding and eventually leading to death.
- Consumption of less than one granule containing 10 g/kg elemental iron is sufficient to induce mortality in an adult field slug (*Deroceras reticulatum*).
- There is a lag phase of 3-4 days between ingestion of a granule by a slug/snail containing iron and resultant mortality.
- Ferric phosphate and elemental iron do not target mucosal cells and therefore do not impair movement. Slugs and snails are therefore able to move away before dying.
- Slugs/snails poisoned by pellets often move below the soil. Work conducted at Harper Adams reported that following exposure to ferric phosphate, 50-60% of slugs moved below the soil surface within the first 24 hours. All slugs moved either to refuges or into the upper soil profile before dying.
- The visibility of slugs/snails that have died below the surface will be reduced to scavenging birds and mammals.

These lines of evidence are supported by a review of the potential for slug control with ferric phosphate (Horgan, 2006). This review explains that slugs/snails stop feeding immediately after ingesting ferric phosphate

granules. The iron phosphate then interferes with calcium metabolism, causing cellular pathological changes. The process from feeding to dying takes 3-6 days. Similarly Kozłowski et al. (2014) investigated slug mortality when exposed to varying concentrations of ferric phosphate in granules, with mortality occurring between days 5-9 at the highest dose. In this study plant damage was reduced within 24 hours, indicating that slugs stopped feeding days before mortality occurred.

Based on the Whaley (2022) statement, Schabacker and von Blanckenhagen (2023a) conclude that due to the nature and mode of action of the active ingredient of the granules, almost all poisoned snails would be below the soil surface and not readily accessible to birds and foraging animals. The available literature supports this conclusion. Additionally, HSE have checked the UK field studies that have been submitted to support the efficacy of Final Bite. In all UK field trials the number of dead slugs found on the soil surface following application of Final Bite granules was negligible (see Table B.9.2-14).

Following ingestion of a Final Bite granule, the slug/snail is expected to rapidly seek refuge (e.g. underground) and will then die over the course of the following days/week. This reduces the likelihood that slugs/snails which have consumed granules (and thus which contain iron residues), will be visible to birds or mammals and hence will be consumed by such animals. While some exposure via this pathway cannot be completely ruled out, since birds or mammals could consume slug/snails after granule ingestion but before they have retreated to a refuge, this behavioural response will greatly reduce the potential for birds or mammals to be exposed to elemental iron via consumption of poisoned molluscs.

Incomplete oxidation of elemental iron

As discussed above, iron is predominantly absorbed in the ferrous (Fe^{2+}) state in the gastrointestinal tract. It cannot be readily absorbed in its elemental form (Fe^0). To be absorbed elemental iron from Final Bite granules must first be oxidised to Fe^{2+} , which occurs in the presence of gastric acid. Schabacker and von Blanckenhagen (2023c) propose that a complete reaction with 100% conversion cannot be expected to occur within a few hours, i.e. before gastric emptying. HSE agrees that it is likely that where a substantial dose of elemental iron is ingested, there will only be enough time for a proportion of the iron to be oxidised to the ferrous form and subsequently absorbed. However, as noted by Schabacker and von Blanckenhagen (2023c), *'it is impossible to estimate the reaction rate for the rodent stomach as there are too many factors involved (residence time, movement, temperature, surface/volume ratio)'*

Following discussion with HSE, calculations of the potential amount of Fe^{2+} absorbed from consumption of a single granule have been provided in Schabacker and von Blanckenhagen (2023c). These are reproduced in the table below. It is noted by the authors and agreed by HSE that there is a high degree of uncertainty associated with these estimates and as a result 3 scenarios have been included for the proportion of Fe^0 converted to Fe^{2+} (33.3%, 50% and 66.6% conversion).

Table B.9.2-17: Approximate calculation of Fe^{2+} absorption from Final Bite granules

Conversion of Fe^0 to Fe^{2+}	Percentage uptake of released Fe^{2+} #	Percentage of iron absorbed	Estimated absorption of iron per granule*
33.30%	5%	1.67%	0.00222 mg a.s./granule
50.00%	5%	2.5%	0.00333 mg a.s./granule
66.60%	5%	3.33%	0.00443 mg a.s./granule

Absorption from [REDACTED] (1984)

* Assuming loading of 0.133 mg a.s./granule

In Schabacker and von Blanckenhagen (2023c) the authors go on to compare the estimated amount of absorbed iron per granule against toxicity endpoints from the Whittaker et al. (1996) and Zhu et al. (2016) studies, in order to estimate the number of granules that would need to be consumed to exceed the NOAEL (accounting for both incomplete conversion and incomplete absorption). However, it is noted that in the toxicity studies animals were dosed with carbonyl iron, so presumably the amount of iron absorbed would also be reduced by incomplete conversion from Fe^0 to Fe^{2+} and iron absorption in these animals would be expected to be substantially lower than 100%. Therefore the NOAEL values overestimate the absorbed dose. As a result, this is not a like-with-like

comparison. Measurements or calculations of the actual amount of iron absorbed by animals in the Whittaker et al. (1996) and Zhu et al. (2016) studies are not available and cannot be determined from the available data. In light of this, on reflection, HSE does not consider this to be a productive line of argumentation.

Background exposure of birds and mammals to iron via normal diet

Iron in commercial diets of captive birds

As summarised in table 9.1.1.3-1, example data were provided for iron concentrations in commercial avian feed. Iron concentrations in poultry feed ranged from 50-130 mg/kg feed. Similarly data on commercial rodent diets found iron concentrations of 45-240 mg/kg feed. From the updated literature search, Brue (1994) specifies a recommended minimum iron requirement of 60-80 mg/kg for companion bird diets, specifically for most psittacines (parrots) and commonly kept passerines. These data provide reassurance that exposure to iron via diet at the specified range of concentrations does not negatively impact the health of birds and mammals. It is assumed that iron levels in commercial diets do not negatively impact bird or mammal health, though it is noted that long-term effects have not been assessed. However, given that Final Bite granules contain 1% elemental iron (i.e. 10000 mg/kg), comparison with concentrations that birds or mammals can be exposed to in commercial diets is not meaningful, given the concentration in Final Bite is over 40 times higher.

Two of the publications containing information on iron concentrations in commercial poultry diets include sufficient information to also calculate the daily intake of iron via consumption of diet. These are Firman (1991) and Eissler and Firman (1996). The other publications, i.e. Diemou et al. (2013), Moore et al. (2003) and Boling & Firman (1997) do not include necessary information on bird absolute bodyweight (only data on bodyweight gain is included). The amount of iron consumed relative to the bodyweight for birds in the two publications has been determined by HSE and is summarised in the table below.

Table B.9.2-18: Iron intake by poultry from artificial diets

Study	Bird	Age	Feed iron content (mg/kg)	Daily iron intake (mg/kg bw/d)
Firman (1991)	Male broilers	1-6 weeks	80	8.05
	Female broilers	1-6 weeks	80	7.96
	Leghorn-type	1-6 weeks	80	7.28
Eissler & Firman (1996)	Turkeys	0-4 weeks	141	7.41

In the first tier risk assessment the potential dietary exposure was assessed from ingestion of Final Bite granules as a source of food. At first tier a diet of 100% granules was assumed. The resulting Daily Dietary Dose (DDD) for the long-term/reproductive risk assessment was determined for the generic focal species small omnivorous bird “house sparrow” and large bird “partridge”, as summarised below:

- DDD for small omnivorous bird “house sparrow” from 100% p granule consumption = 2920 mg/kg bw/d
- DDD for large bird “partridge” from 100% granule consumption = 1200 mg/kg bw/d

Therefore, the first tier DDD calculations for ingestion of granules as food are all well above the range of dietary intakes for captive poultry calculated in Table B.9.2-18. Therefore, under tier 1 assumptions (e.g. all diet is granules and all diet is obtained from the treated field), predicted exposure levels of iron via ingestion of granules are well above dietary iron intake levels that may be expected for captive poultry.

Given that Final Bite granules contain 0.133 mg Fe/granule, a daily iron intake of 8.05 mg/kg bw/d for a bird from commercial diet (the maximum calculated from Firman, 1991 and Eissler & Firman, 1996), is equivalent to consumption of 60.5 Final Bite granules/day for a 1 kg bird, or 23.6 granules/day for a medium-sized bird (bodyweight of 390 g, no assessment factor included).

Iron contents of natural food items for wild birds and mammals

In Schabacker and von Blanckenhagen (2023a) information on iron concentrations in potential food items for birds and mammals has been considered. For insects publications by Mwangi et al. (2018) and McDonald (2006) have been used. These publications are considered here.

Mwangi et al. (2018) review the potential role for insects as sources of iron and zinc in human nutrition. Information on iron contents is included for 17 insect species. This includes eleven edible species that are mass-produced and six species that are collected from nature. The relevant data are summarised in the following table.

Table B.9.2-19: Iron concentrations in insect species consumed by humans (from Mwangi et al., 2018)

Species	Scientific name	Mass-reared or wild-caught	Fe (mg/100g dry weight)	
			Mean	SD
House cricket	<i>Acheta domesticus</i>	Mass-reared	9.1	4.47
Jamaican field cricket	<i>Gryllus assimilis</i>	Mass-reared	17.93	
Two-spotted cricket	<i>Gryllus bimaculatus</i>	Mass-reared	9.66	
Tropical house cricket	<i>Gryllobates sigillatus</i>	Mass-reared	4.23	
Migratory locust	<i>Locusta migratoria</i>	Mass-reared	13.7	4.35
Desert locust	<i>Schistocerca gregaria</i>	Mass-reared	8.38	
Lesser mealworm	<i>Alphitobius diaperinus</i>	Mass-reared	21.80	
Yellow mealworm	<i>Tenebrio molitor</i>	Mass-reared	5.3	2.16
Superworm	<i>Zophobas morio</i>	Mass-reared	3.8	1.00
Silkworm	<i>Bombyx mori</i>	Mass-reared	15.9	7.23
Waxworm	<i>Galleria mellonella</i>	Mass-reared	5.0	1.75
Termite	<i>Macrotermes subhyalinus</i>	Wild-caught	61.9	8.61
Arboreal termite	<i>Nasutitermes spp.</i>	Wild-caught	24.6	
Palm worm	<i>Rhynchophorus palmarum</i>	Wild-caught	5.1	1.25
Sago grub	<i>Rhynchophorus ferrugineus</i>	Wild-caught	9.0	0.55
Longhorn grasshopper	<i>Ruspolia differens</i>	Wild-caught	14.8	1.80
Cornfield grasshopper	<i>Sphenarium purpurascens</i>	Wild-caught	18.0	

The primary sources for the data used in Mwangi et al. (2018) have not been provided. Therefore, the reliability/relevance of the above values has not been confirmed. It is noted that in wild-caught species iron concentrations ranged from 5.1–61.9 mg/100 g. The wild-caught situation is considered more relevant for the potential iron concentrations in insects foraged by insectivorous birds and mammals in fields treated with Final Bite granules.

McDonald (2006) also includes information on iron concentrations in different species of invertebrates. This is summarised in the following table.

Table B.9.2-20: Iron concentrations in insect species consumed by humans (from McDonald, 2006)

Species	Diet	Iron (mg/100 g)
Honeypot ant, <i>Melophorus</i> spp	Wild	3.5
Lerp scale, <i>Psylla eucalypti</i>	Wild	7.8
Witchety grub, <i>Cossidae</i> spp	Wild	10.2
Bogong moth, <i>Argrotis infusa</i>	Wild	15.9
Green tree ant, <i>Oecophylla smaragdina</i>	Wild	40
Mealworm	Wheat, grain, carrots	4

Mighty Mealy	Wheat, brain, supplements	2.6
Super Mealworm	Wheat, grain, carrots	5
Cricket, adult	Cornmeal, wheat, soybean hulls, meat meal molasses, fish meal	11
Cricket, juvenile	Shipped with raw potato	20
Wax Worm	None	8
Fruit Fly	Commercial feed	45
Earth Worm	Wild	1110
Earth Worm	Commercial, peat humus soil	580

The source of this information in McDonald (2006) is unclear. Therefore the reliability/relevance of the data cannot be assessed in detail. It is apparent that iron concentrations in earthworms were particularly high. For other species the iron concentrations ranged from 2.6-45 mg/kg, which is similar to the range seen in Mwangi et al. (2018).

HSE notes that other references are available which contain information on iron concentrations, particularly with a view to insect species that can form part of human diets. Ojha (2021) is a review publication including information on iron concentrations in various insect species. While HSE has not been able to confirm all of the referenced data, two of the underlying studies have been checked. Kohler et al. (2019) reports iron concentrations from samples of Bombay locusts, scarab beetles, house crickets and mulberry silkworms taken from street vendors and supermarkets in Thailand. Zielinska et al. (2015) measured nutritional information in commercially supplied crickets, mealworms and locusts. The results from Kohler et al. (2019) and Zielinska et al. (2015) are summarised in the following table.

Table B.9.2-21: Iron concentrations in insect species consumed by humans (from Ojha, 2021)

Species	Fe (mg/100 g dry weight)	Source
Bombay locust	3.45	Kohler et al. (2019)
Scarab beetle	9.1	
House cricket	3.11-5.81	
Mulberry silkworm	2.83-3.18	
Tropical house cricket (<i>Gryllodes sigillatus</i>)	4.23	Zielinska et al. (2015)
Mealworm (<i>Tenebrio molitor</i>)	3.29	
Desert locust (<i>Schistocerca gregaria</i>)	8.38	

The iron concentrations measured were within the range from the McDonald (2006) and Mwangi et al. (2018) data sets.

Data on iron concentrations in seeds and fruits from various plant species are available from a USDA database (<https://fdc.nal.usda.gov/>). The iron contents for seeds and fruits presented in Schabacker and von Blanckenhagen (2023a) have been checked and are reported in the following table.

Table B.9.2-22: Iron concentrations in seeds and fruits (from USDA)

Food item	Average measured iron content (mg/100g dry weight)
Pumpkin seeds	8.36
Flaxseeds	5.78
Sunflower seeds	4.37
Raspberries	0.45
Blueberries	0.34
Strawberries	0.26

Cherries	0.11
Peaches	0.34
Grapes	0.2

The McCance and Widdowson's 'Composition of foods integrated dataset' (CoFID) on the nutrient content of the UK food supply has also been checked for information on iron concentrations in various seeds and fruits.

Table B.9.2-23: Iron concentrations in seeds and fruits (from CoFID)

Food item	Iron content (mg/100g dry weight)
Caraway seeds	32.3
Celery seeds	44.9
Coriander seeds	16.32
Cumin seeds	66.36
Dill seeds	16.3
Fennel seeds	12.3
Poppy seeds	11.1
Pumpkin seeds	10
Sunflower seeds	6.4
Raspberries	0.41
Blueberries	0.55
Strawberries	0.25
Cherries	0.25
Peaches	0.4
Grapes	0.23

Where data are available from both datasets the reported iron concentrations are similar. Concentrations of iron in seeds ranged from 4.37-66.36 mg/100 g dw, which is similar to the range seen in the insect data.

Concentrations of iron in fruit ranged from 0.11-0.55 mg /100 g dw and were consistently lower than in seed or insect samples.

For iron concentrations in green plant material Schabacker and von Blanckenhagen (2023a) use two references: Früh (2009), Stephan (2010) and Ancuceanu et al. (2015). English translations of Früh (2009) and Stephan (2010) have not been provided by the applicant, hence they have not been considered by HSE.

Ancuceanu et al. (2015) is a review publication which includes information on iron concentrations in plants from 1228 species. It is summarised in Appendix 3 by HSE. The authors adopted a semi-systematic approach, based on a long list of plant genera randomly sampled in a stratified manner. Searches for information on plant iron concentrations were performed in Pubmed, Proquest Central, Google Scholar and the "Plants for the future" database. The authors also searched for data on the uptake of non-heme iron by humans. Data on iron concentrations are available for different taxa and in different plant parts, with key information for the current risk assessment presented in the table below.

Table B.9.2-24: Iron concentrations in plants (from Ancuceanu et al., 2015)

Plant part	Iron concentration (mg/kg dw)		Number of species sampled
	Range	Median	
Dicot leaves	152-193	163	438
Monocot leaves	141-240	188	82
Dicot seeds	60-99.8	80.5	83
Monocot seeds	4-70	59	11
Dicot fruit	58-87.7	69.9	178
Monocot fruit	37.6-186.2	67.8	13

In order to be able to estimate how much iron relevant bird or mammal species for the risk assessment may consume incidentally via their natural diets, the datasets on iron concentrations in potential food items, as discussed above, can be considered.

For invertebrates the datasets presented in Mwangi et al. (2018), McDonald (2006) and Ojha (2021) are available. The average iron concentration across insect species in Ojha (2021) was 51.3 mg/kg dw. In the data summarised in McDonald (2006) there were particularly high concentrations of iron in earthworms, far outside the range of the other species. The average iron concentration across species other than earthworms was 144 mg/kg dw in McDonald (2006). In Mwangi et al. (2018) data were available from both mass-reared and wild-caught species. The average iron concentration in mass-reared invertebrates was 104 mg/kg dw and in wild-caught invertebrates was 222 mg/kg dw. Therefore, the difference between the mass-reared and wild-caught species was relatively small (a factor of approximately 2) and the overall average iron concentration was 146 mg/kg dw. Taking the average values from the Mwangi et al. (2018), McDonald (2006) and Ojha (2021) datasets (i.e. 146, 144 and 51.3 mg/kg dw), the overall average iron concentration in invertebrates is 113.8 mg/kg dw.

The available data for green plant material come from the Ancuceanu et al. (2015) review. The data on iron concentrations in dicot and monocot leaves are considered the most relevant for this food item. Taking an average of the median iron concentrations for dicot and monocot leaves results in a value of 175.5 mg/kg dw.

For seeds iron concentration data are available from the USDA and CoFID databases and Ancuceanu et al. (2015). The average iron concentration across seeds in USDA was 61.7 mg/kg dw. Similarly, if the average value is taken of the median dicot and monocot seed concentrations in Ancuceanu et al. (2015), the result is a value of 69.75 mg/kg dw. In contrast, the average iron concentration from the seed values taken from CoFID is 240 mg/kg dw. It is unclear why the CoFID iron concentration in seeds is significantly higher than the other datasets. It is noted that the CoFID and USDA datasets are focused on a limited number of plant species relevant for human diets. Given the Ancuceanu et al. (2015) is more comprehensive and systematically selected, the concentration of iron in seeds from this source will be used in preference.

Fruit data are also available from the same datasets, i.e. the USDA and CoFID databases and Ancuceanu et al. (2015). Iron concentrations in fruit were low in the USDA and CoFID databases, with average values of 2.83 and 3.48 mg/kg dw respectively. The average iron concentration in fruit between dicots and monocots in Ancuceanu et al. (2015) was considerably higher at 68.85 mg/kg dw. It is unclear why there is the clear different between the datasets. While the Ancuceanu et al. (2015) is more comprehensive and systematically selected, given the results are at odds with both the other datasets, it is considered appropriate to use both a low iron concentration estimate of 3.16 mg/kg dw (average of USDA and CoFID datasets) and a high iron concentration of 68.85 mg/kg dw in further calculations.

Table B.9.2-25: Summary of background iron concentrations in food items for birds/mammals

Food item	Iron residue used (mg/kg dw)	Source
Invertebrates	113.8	Mean from the Mwangi et al. (2018), McDonald (2006) and Ojha (2021) datasets
Green plant material	175.5	Mean of the median concentrations in dicot and monocot leaves from Ancuceanu et al. (2015)
Seeds	69.75	Mean of the median concentrations in dicot and monocot seeds from Ancuceanu et al. (2015)
Fruits (low iron)	3.16	Mean of the mean values from USDA and CoFID datasets.
Fruits (high iron)	68.85	Mean of the median concentrations in dicot and

		monocot fruits from Ancuceanu et al. (2015)
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Background exposure levels via natural diet for wild birds and mammals

In order to help put into context potential iron exposure levels from birds or mammals consuming Final Bite granules or poisoned slugs, background exposure levels to iron via the normal diets of birds and mammals can be considered.

No data have been provided by the Applicant that would enable this consideration to focus on particular bird species that may be present in fields treated with 'Final Bite' granules. It is also noted that 'Final Bite' granules can be applied to fields of a wide range of crops. In order to understand potential background exposure levels of birds and mammals foraging in such fields, HSE has identified a suitable range of generic focal species for consideration, as defined in the first tier risk assessment scheme under EFSA bird and mammal guidance (see Annex 2 in EFSA, 2009). These generic focal species are not necessarily likely to be exposed to iron via Final Bite granules but they cover a range of species and different dietary guilds, providing a fuller consideration of the range of background exposures to iron via diet. These generic focal species are as follows:

- Small granivorous bird "finch"
- Small omnivorous bird "lark"
- Small insectivorous bird "wagtail"
- Small insectivorous/worm feeding species "thrush"
- Frugivorous bird "crow"
- Medium herbivorous/granivorous bird "pigeon"
- Large herbivorous bird "goose"
- Small insectivorous mammal "shrew"
- Small omnivorous mammal "mouse"
- Small herbivorous mammal "vole"
- Frugivorous mammal "dormouse"
- Large herbivorous mammal "lagomorph"

To determine background levels of iron in diets, the specific dietary makeup and corresponding food intake rates relative to bodyweight have been identified from Appendix A of EFSA (2009) and are summarised in the following table.

Table B.9.2-26: Dietary information and food intake rates relative to body weight for generic focal species from EFSA (2009)

Generic focal species	Dietary item	Proportion in diet	Bodyweight (g)	Daily food intake rate relative to bodyweight
Small granivorous bird "finch"	Weed seed	1	15.3	0.28
Small omnivorous bird "lark"	Green plant material	0.25	28.5	0.13
	Weed seeds	0.25		0.13
	Arthropods	0.5		0.26
Small insectivorous bird "wagtail"	Arthropods	1	17.6	0.79
Small insectivorous/worm feeding species "thrush"	Arthropods	1	19.7	0.76
Frugivorous bird "crow"	Fruit	1	448	0.93
Medium herbivorous/granivorous bird "pigeon"	Green plant material	1	490	1.29
Large herbivorous bird "goose"	Green plant material	1	2645	0.3

Small insectivorous mammal "shrew"	Arthropods	1	9.7	0.55
Small omnivorous mammal "mouse"	Green plant material	0.25	21.7	0.0675
	Weed seeds	0.5		0.135
	Arthropods	0.25		0.0675
Small herbivorous mammal "vole"	Green plant material	1	25	1.33
Frugivorous mammal "dormouse"	Fruit	1	57.5	1.16
Large herbivorous mammal "lagomorph"	Green plant material	1	1543	0.41

The potential background exposure level to iron via diet has been calculated for each generic focal species in the following table. This was done by multiplying the FIR/bw for each food item (from table B.9.2-26) by the iron concentration for that food item. It is acknowledged that background exposure levels of iron as consumed in bird or mammal diets will be variable. The values calculated below are intended to give an estimation of the general magnitude of the iron dose that can be consumed via normal diet in the wild and these estimates are subject to various uncertainties.

Table B.9.2-27: Estimation of background iron intake for generic focal species via diet

Generic focal species	Dietary item	Daily food intake rate relative to bodyweight	Iron concentration in food item (mg/kg)	Iron intake relative to bodyweight (mg/kg bw)
Small granivorous bird "finch"	Weed seed	0.28	69.75	19.53
Small omnivorous bird "lark"	Green plant material	0.13	175.5	-
	Weed seeds	0.13	69.75	-
	Arthropods	0.26	113.8	-
	SUM	-	-	61.48
Small insectivorous bird "wagtail"	Arthropods	0.79	113.8	89.90
Small insectivorous/worm feeding species "thrush"	Arthropods	0.76	113.8	86.49
Frugivorous bird "crow"	Fruit (low iron)	0.93	3.16	2.94
	Fruit (high iron)	0.93	68.85	64.03
Medium herbivorous/granivorous bird "pigeon"	Green plant material	1.29	175.5	226.40
Large herbivorous bird "goose"	Green plant material	0.3	175.5	52.65
Small insectivorous mammal "shrew"	Arthropods	0.55	113.8	62.59
Small omnivorous mammal "mouse"	Green plant material	0.0675	175.5	-
	Weed seeds	0.135	69.75	-
	Arthropods	0.0675	113.8	-
	SUM	-	-	28.95

Small herbivorous mammal "vole"	Green plant material	1.33	175.5	233.42
Frugivorous mammal "dormouse"	Fruit (low iron)	1.16	3.16	3.67
	Fruit (high iron)	1.16	68.85	79.87
Large herbivorous mammal "lagomorph"	Green plant material	0.41	175.5	71.96

The estimated iron intake values via normal diet for the generic focal species ranged from 2.94-226.4 mg iron/kg bw, with the lowest value for a 100% fruit diet and the highest value for a 100% green plant material diet. If only the fruit residue data from Ancuceanu et al. (2015) is used, then the range becomes 19.53-226.4 mg iron/kg bw.

In the first tier risk assessment the potential dietary exposure was assessed from ingestion of Final Bite granules as a source of food. At first tier a diet of 100% granules was assumed. The resulting Daily Dietary Dose (DDD) for the long-term/reproductive risk assessment was determined for the generic focal species small omnivorous bird "house sparrow", large bird "partridge", and small omnivorous mammal "wood mouse", as summarised below:

- DDD for small omnivorous bird "house sparrow" from 100% granule consumption = 2920 mg/kg bw
- DDD for large bird "partridge" from 100% granule consumption = 1200 mg/kg bw
- DDD for small omnivorous mammal "wood mouse" from 100% granule consumption = 2100 mg/kg bw

Therefore, the first tier DDD calculations for ingestion of granules as food are all above the range of background dietary intakes for bird and mammals calculated in Table B.9.2-27. Therefore, under tier 1 assumptions (e.g. all diet is granules and all diet is obtained from the treated field), predicted exposure levels of iron via ingestion of granules are well-above the potential background iron intakes via normal diet in the wild.

For the consumption of granules as food scenario, a medium-sized bird species that consumes seeds/grain is considered a relevant scenario for consideration. Assuming a 100% seed diet, 390 g bodyweight and iron intake relative to bodyweight via diet of 19.53 mg/kg bw (table B.9.2-27), a background dose level via normal diet of 7.6 mg iron is determined. With a granule iron content of 0.133 mg Fe/granule, this background dose level via diet would be equivalent to consumption of 57 'Final Bite' granules.

Similarly, a small mammal species that consumes seeds/grain is also considered a relevant scenario for consideration. Assuming a 100% seed diet, 21.7 g bodyweight and iron intake relative to bodyweight via diet of 28.95 mg/kg bw (table B.9.2-27), a background dose level via normal diet of 0.63 mg iron is determined. With a granule iron content of 0.133 mg Fe/granule, this background dose level via diet would be equivalent to consumption of 4.7 'Final Bite' granules.

Higher tier risk assessment – Weight of evidence consideration

Problem formulation

Final Bite granules can be applied outdoors to fields planted with a range of different crops, at a range of different growth stages and at different times of year. Therefore, birds or mammals may come into contact with the granules when foraging in such fields. Granules of Final Bite have calorific value and therefore could be directly consumed by birds and/or mammals as a source of food. Granules could also be consumed by birds as a source of grit. Additionally, molluscs which have fed on granules may be available after treatment and may contain residues of elemental iron. Therefore, birds or mammals which consume molluscs could also be exposed to iron indirectly via application of Final Bite granules. These routes of potential exposure via ingestion require further consideration.

An acceptable acute risk to mammals was demonstrated in the first tier assessment, therefore only the following risks require additional evaluation:

- Acute risks to birds from consumption of granules and/or molluscs
- Long-term/reproductive risks to birds from consumption of granules and/or molluscs

- Long-term/reproductive risks to mammals from consumption of granules and/or molluscs

While dermal exposure is also possible for any birds or mammals coming into contact with granules or contaminated molluscs, it is considered a less significant route of exposure than oral exposure and, in line with EFSA (2009) guidance, no assessment of the risk via dermal exposure is considered necessary. Given the low vapour pressure of the active substance, inhalation is not a relevant exposure route.

Elemental iron could move from granules into soil, with exposure of soil invertebrates via this route and subsequent exposure of organisms higher up the food chain. However, as discussed in section B.9.8, it is considered that the additional amount of iron that this product would contribute to soil would be negligible in comparison to natural concentrations of iron in soil. The potential for exposure of fish and other organisms via the aquatic food chain is also considered negligible given the low solubility of iron in water. Risks via secondary poisoning therefore do not require further consideration.

The active substance elemental iron is an essential micronutrient in animals. Due to its function as an essential micronutrient, organisms have developed mechanisms to cope with reduced or elevated iron concentrations, to a certain extent (homeostasis). The critical question therefore becomes whether intake of iron via ingestion of granules or poisoned slugs can be sufficient to exceed the range which the organism can tolerate/regulate, thus resulting in damage to the organism. Should such damage occur, the next critical questions are whether this impacts the survival and/or reproductive performance of the individual organism, and in turn whether this can lead to effects at the population level. Therefore, these questions are the focus of the HSE assessment.

Decision criteria under Regulation 1107/2009

Regulation 1107/2009 requires that for authorisation of a plant protection product to occur it must be clearly established that use of this product will have no unacceptable impact on birds and mammals under field conditions. To this end the EFSA guidance document on bird and mammal risk assessment (EFSA, 2009) specifies two protection goals:

- An “actual” protection goal of:
 - *“clearly establishing that there will be no visible mortality and no long-term repercussions for abundance and diversity.”*
- A “surrogate” protection goal of:
 - *“making any mortality or reproductive effects unlikely”*

The first-tier risk assessment is designed to satisfy the surrogate protection goal but at higher tiers either protection goal can be considered. In the case of elemental iron the data and risk assessment provided do not allow for a full consideration of the actual protection goal (since no data are available on population level effects) and therefore both the first and higher tier risk assessments will focus on the surrogate protection goal, i.e. demonstrating that any mortality or reproductive effects are unlikely following the use of the representative formulation.

Acute risk to birds from consumption of granules

When considering the intentional ingestion of granules as a food source scenario, the first tier acute TERs were determined to be 0.013 for the house sparrow and 0.03 for the partridge. For the ingestion of granules as grit scenario, the first tier acute TER for a large bird was 0.074. These TER values are below the acceptability trigger of ≥ 10 by wide margins.

There is convincing evidence to indicate that the avian LD₅₀ of 37 mg a.s./kg bw (2008) used in the first tier TER calculations overestimates the ‘true’ toxicity of elemental iron and Final Bite granules to birds. There were no mortalities in the (2008) study and the LD₅₀ used is extrapolated above the dose range tested. Studies with other forms of iron (e.g. ferric sulphate heptahydrate) indicate significantly lower toxicity than the (2008) extrapolated endpoint. Based on how elemental iron is converted and absorbed in the gastrointestinal tract, elemental iron would not be expected to be more acutely toxic to birds than other forms of iron, such as ferrous sulphate heptahydrate. In fact in the acute mammalian studies carbonyl iron (used as a proxy for elemental iron) was less toxic to rats than ferrous sulphate heptahydrate.

While the available acute bird toxicity endpoint is considered to overestimate the 'true' toxicity of elemental iron, conducting an additional acute bird toxicity study using higher doses of elemental iron is not recommended. Further vertebrate studies should only be conducted where essential.

Therefore, to provide insight into the level of acute risk posed by potential consumption of Final Bite granules, available acute toxicity data from a range of sources have been considered. In the following table acute toxicity endpoints that are relevant for birds are summarised and these are compared to equivalent exposure level calculations for small and medium-sized birds ingesting granules.

Table B.9.2-28: Contextualising the acute risk to birds from consumption of granules

Dose (mg Fe/kg bw)	Description	Equivalent exposure level for a medium-sized bird (390 g)	Equivalent exposure level for a small bird (27.7 g)
>2.5	LD ₅₀ /10 using Japanese quail value from [REDACTED] (2008)	Consumption of 7.33 granules per bird	Consumption of 0.52 granules per bird
3.7*	LD ₅₀ /10 using extrapolated Japanese quail value from [REDACTED] (2008)	Consumption of 10.8 granules per bird	Consumption of 0.77 granules per bird
>123	Extrapolating acute LD ₅₀ /10 from ferrous sulphate heptahydrate (Grimes & Jaber, 1986)	Consumption of 361 per bird	Consumption of 25.6 per bird
>5000	Extrapolating acute LD ₅₀ /10 from mammals (Whittaker et al., 2002)	Consumption of 14662 per bird	Consumption of 1041 per bird

*Bound value extrapolated from a limit test study conducted at a low dose, so 'true' LD₅₀/10 is likely to be higher

The EFSA guidance document on bird and mammal risk assessment contains information on the number of small and large seeds that birds have been observed to consume in a single feeding bout. These data comes from UK research (Prosser, 1999¹³) and are summarised in the following table. The guidance document notes that the methodology used by Prosser to derive these numbers was conservative in some aspects (e.g. it was a spill scenario) but not in others (the same bird may have returned to the feeding site several times a day, and one bout may not equate to a 'meal').

Table B.9.2-29: Mean and maximum number of large and small seeds taken by birds in a single feeding bout in field studies (reproduced from EFSA, 2009)

	Number of large seeds			Number of small seeds		
	Mean*	90 th percentile**	Maximum	Mean*	90 th percentile**	Maximum
Large granivorous bird	12	116	266	75	1744	4487
Small granivorous bird	3	11	11	12	85	240

* Geometric mean of mean values for different species and seed types.

** 90th percentile of maximum values for relevant species and seed types.

Given the size of Final Bite granules, they are analogous to large seeds and the Prosser (1999) data provide an indication of potential consumption levels. The 90th percentile consumption value for a large granivorous bird consuming large seeds is 116 seeds per feeding bout. This value can be considered a realistic worst-case estimate of the number of large seeds that a relevant bird species could consume during a feeding event. It is noted that

¹³ https://www.hse.gov.uk/pesticides/resources/r/Research_PN0907.pdf

table B.9.2-29 does not include a medium-sized bird category but the ‘large bird’ category does include data from both medium and large birds. As a worst-case it can be assumed that Final Bite granules are equivalent to large seeds, in terms of their attractiveness to birds as a food source. This is a conservative assumption that could be further refined. An exposure level of 116 granules is assumed for the acute exposure scenario for medium-sized birds.

Table B.9.2-28 indicates that consumption of 116 Final Bite granules would be sufficient to exceed the LD₅₀/10 from the [REDACTED] (2008) study for a medium-sized bird. However, 116 granules would be less than the number required to exceed the LD₅₀/10 from the ferrous sulphate heptahydrate study (361 granules) and the LD₅₀/10 from the mammal study (14662 granules). While a precise acute avian LD₅₀ for elemental iron or Final Bite granules cannot be defined from the data available, it is highly unlikely that the ‘true’ LD₅₀ value would be below the ferrous sulphate heptahydrate LD₅₀ and it could be as high as the rodent LD₅₀ for carbonyl iron.

Some consideration is also required for small birds. While they are less likely to consume large granules, they could consume a small number of granules, most likely as fractions rather than whole granules. Based on the 90th percentile consumption per feeding bout for small birds consuming large seeds, an acute risk assessment scenario where a small granivorous bird consumes 11 Final Bite granules can be considered. Again, this level of exposure would exceed the LD₅₀/10 from [REDACTED] (2008) but would be less than the LD₅₀/10 for ferrous sulphate heptahydrate or the LD₅₀/10 for mammals.

Where there are large numbers of granules available on the soil surface following a spill, this represents a worst-case exposure scenario that may not be fully covered by the above comparisons. However, the risk via this scenario can be managed by specifying the following mitigation:

‘SPE 6: To protect birds / wild mammals remove spillages’

It is noted that in addition to elemental iron, Final Bite granules also contain the [REDACTED]

For the consumption of granules as grit scenario, factors which affect the attractiveness of Final Bite granules as a food source are not expected to be relevant, so some further consideration is needed. At first tier it assumed that a bird of 300 g requires 2453 grit particles/day in the acute exposure scenario. For the generic medium-sized bird considered above (390 g bodyweight), this is equivalent to 3189 grit particles per day. If 361 granules are required to exceed the LD₅₀/10 for such a bird (based on extrapolation from ferrous sulphate heptahydrate – see table B.9.2-28), this would mean that 11% of the ingested grit particles would need to be Final Bite granules. Similarly if extrapolating the mammal LD₅₀/10 for carbonyl iron to birds, this is equivalent to 14662 granules, which far exceeds the daily grit requirement. It is also noted that granules have a [REDACTED] (i.e. they are not very hard and would soften further over time in the environment). Section B.3.5 specifies that the force needed to break pellets was 1.45 kg for the proposed product. The likelihood of birds perceiving granules as a grit source is considered to be low. Therefore, it is unlikely that granules would be consumed as grit to a sufficient extent that this would result in an ingested dose that exceeds a dose of concern for the acute risk scenario. The consumption of granules as food is considered the more relevant exposure scenario for the bird risk assessment.

The above theoretical exposure calculations indicate that it is highly unlikely that relevant bird species would consume sufficient Final Bite granules in an acute feeding scenario to exceed their iron homeostatic regulatory capacity and for this to result in mortality. However, it also needs to be considered whether there are any reported data of bird mortalities associated with iron exposure, which could contradict these findings. Given that iron concentrations would not be routinely investigated under the UK Wildlife Incident Investigation Scheme (WIIS), this scheme is not considered an appropriate source of such data and would be only likely to detect major poisoning incidents. Published incidents of toxic effects in birds from iron exposure have been searched for and reviewed. These incidents primarily relate to exotic species, which are not directly relevant for the elemental iron risk assessment. In one publication (Pavone et al., 2014) mortality occurred in blackbird, fieldfare, song thrush and redwing (i.e. potentially relevant species), with this mortality being attributed to a acute

exposure from increased iron in bird diets. This finding raises concern but it does seem at odds with other information available, given the concentration of iron in bird diets in this incident (111 mg Fe/kg) is within the recommended range of iron in commercial bird diets. Also, by way of contrast, in Crissey et al. (1993) starlings were exposed to 3035 ppm iron in their diets without effects on bodyweight or survival. It is unclear whether the captive status of birds, lack of food choice, their overall health status or genetic factors could have played a part in the incident reported in Pavone et al. (2014).

Overall, it is concluded that it has been clearly established that any mortality is unlikely from acute exposure of birds via consumption of Final Bite granules, thus satisfying the surrogate protection goal.

Risks to birds and mammals from consumption of molluscs

No first tier assessment of the risks to birds and mammals from consumption of poisoned molluscs has been conducted. This is due to the absence of data on concentrations of iron in molluscs following consumption of Final Bite granules. Therefore, further consideration is required.

Data on the diets of bird and mammal species that are known to forage within agricultural areas in GB indicates that there are a range of species that can consume significant quantities of molluscs as part of their normal diets. These include song thrushes, fieldfares, corvids, shrews and hedgehogs.

The key line of evidence put forward by the Applicant for evaluating the risk to birds and mammals from consumption of poisoned molluscs relates to the behavioural response of molluscs following ingestion of Final Bite granules. It is proposed that due to the impact of the granule on the ability of the mollusc to feed, they will quickly move away from the area where exposure occurred and retreat below ground to die (mortality occurring within 3-9 days). The availability of slugs/snails that have died below the surface will be greatly reduced for scavenging birds and mammals.

The importance of this behavioural response in mitigating any risk to birds or mammals from consumption of poisoned molluscs has been supported through provision of an expert statement (Whaley, 2022) and the underlying efficacy trials. In all 23 efficacy UK field trials the number of dead slugs found on the soil surface following application of Final Bite granules was negligible. These studies therefore provide strong evidence that molluscs ingesting Final Bite granules move away from the point of exposure before dying, thus greatly reducing the potential for birds or mammals to become exposed to elemental iron via consumption of poisoned molluscs. While it cannot be concluded that there is zero potential for birds and/or mammals to be exposed via consumption of molluscs, exposure via this pathway is expected to be low. It is concluded that exposure of birds and mammals to iron via consumption of poisoned molluscs is a less significant pathway of exposure than direct ingestion of Final Bite granules. Therefore risks to birds and mammals from consumption of molluscs are not further considered.

Long-term/reproductive risk to birds from consumption of granules

When considering the intentional ingestion of granules as a food source scenario, the first tier long-term/reproductive TERs were determined to be 0.0017 for the house sparrow and 0.004 for the partridge. For the ingestion of granules as grit scenario, the first tier long-term/reproductive TER for a medium/large bird was 0.019. These TER values are below the acceptability trigger of ≥ 5 by wide margins.

The NOAEL of 5 mg a.s./kg bw/d used in the first tier risk assessment was derived from data on iron levels in commercial bird diets. It is assumed that iron concentrations in commercial diets do not adversely impact the health of birds. Since this is assumed to be equivalent to a dose at which there are no effects, it is possible and indeed likely that higher doses of iron could also be ingested by birds without impacting their health. The critical NOAEL for birds could be further investigated by conducting a reproductive bird toxicity study, using higher doses of elemental iron than are found in commercial diets. However, this is not recommended as further vertebrate studies should only be conducted where essential.

Given the nature of the information available on iron exposure and its effects on birds (or surrogates), and in light of the very low first tier TERs, it is considered that refinement of the TER calculations is not productive. Instead a qualitative assessment of the overall weight of evidence is required.

Extensive literature reviews and studies are available which consider how iron is absorbed and transported within vertebrates, and how iron levels within such animals are controlled (homeostasis). It is clear that the proportion of iron absorbed from the diet increases where iron levels in the organism are low and decreases where iron levels are high. However, it is also clear that there are limits to the ability of the animal to regulate its iron level that could be exceeded if the amount of iron ingested was critically high. From the literature on iron uptake and transport it is not possible to define a dose range of ingested iron that could be successfully regulated by the bird, and the dose level at which accumulation in organs and damage to the animal could occur. Such a threshold dose would be dependent on a complex range of factors including the iron status of the organism, presence of chemicals that could enhance or reduce absorption in the diet, etc.

It is apparent from the literature on iron toxicity that dietary iron can accumulate in the liver and other organs of birds (haemosiderosis). In fact some accumulation of iron in the livers of birds does not appear to be an uncommon finding. In some cases in the literature this leads to health effects, though this appears less likely (and may not occur at all) in free ranging GB bird species. It is noted that the health effects seen in birds in these studies may be indirectly caused by iron (e.g. by increasing susceptibility to disease), rather than being directly attributable to iron exposure.

To provide insight into the level of long-term risk posed by potential consumption of Final Bite granules, available long-term/reproductive toxicity data from a range of sources have been considered. This includes mammalian data, since such data may also provide an indication of the sensitivity of birds. In the following table long-term/reproductive effects data that are potentially relevant for birds are summarised and these are compared to equivalent exposure level calculations for small and medium-sized birds ingesting granules.

Table B.9.2-30: Contextualising the long-term/reproductive risk to birds from consumption of granules

Concentration (ppm)	Dose (mg Fe/kg bw/d)	Description	Equivalent exposure level for a medium-sized bird (390 g)#	Equivalent exposure level for a small bird (27.7 g)#
35	3.2	No effect level in rats (Whittaker et al., 1996)	Consumption of 1.88 granules per bird per day	Consumption of 0.133 granules per bird per day
50	5*	Lower end of range from commercial poultry diets	Consumption of 2.93 granules per bird per day	Consumption of 0.208 granules per bird per day
80	8.05	Dose received by poultry in commercial diets (Firman, 1991)	Consumption of 4.72 granules per bird per day	Consumption of 0.208 granules per bird per day
111	11.1*	Mortality incidents in captive blackbird, fieldfare, song thrush and redwing (Pavone et al., 2014)	Consumption of 6.51 granules per bird per day	Consumption of 0.462 granules per bird per day
141	14.1*	Upper end of range from commercial poultry diets	Consumption of 8.27 granules per bird per day	Consumption of 0.587 granules per bird per day
350	35	Accumulation of iron in liver and lipid peroxidation in rats (Whittaker et al., 1996)	Consumption of 20.5 granules per bird per day	Consumption of 1.46 granules per bird per day
69.75	19.53	Estimated background dose via normal diet (100% seed consumption)	Consumption of 57 granules per bird per day@	Consumption of 4.07 granules per bird per day@
2000*	200	No effect level in rats (Zhu et al., 2016)	Consumption of 117 granules per bird per day	Consumption of 8.33 granules per bird per day

3035	303.5*	Accumulation of iron in livers of starlings but no mortality or bodyweight effects (Crissey et al., 1993)	Consumption of 178 granules per bird per day	Consumption of 12.6 granules per bird per day
3500	350	20% mortality, cardiomyopathy, splenic and pancreatic atrophy in rats (Whittaker et al., 1996)	Consumption of 205 granules per bird per day	Consumption of 14.6 granules per bird per day

Taking into account standard assessment factor of 5

*Estimated assuming 10-fold difference between the concentration in ppm and dose in mg/kg bw/d (Section 2.3.1.1 of EFSA, 2009)

@ No assessment factor applied

It is noted that the above comparison applies an assessment factor of 5 to calculations. While this assessment factor is relevant where using laboratory study data, it is questionable whether it is appropriate for other sources of data, particularly NOELs extrapolated from iron levels in commercial diets. Without inclusion of this assessment factor, the 8.05 mg a.s./kg bw/d dose from Firman (1991) is equivalent to a medium-sized bird consuming 23.6 granules/d, or a small bird consuming 1.7 granules/d. Without the assessment factor, the upper end of the dose range from commercial poultry diets (14.1 mg a.s./kg bw/d) is equivalent to a medium-sized bird consuming 41 granules/d, or a small bird consuming 2.9 granules/d.

When considering potential exposure levels, a number of factors have been discussed which can impact the likelihood of birds consuming Final Bite granules and the quantity of granules that might be consumed. These avoidance factors are briefly discussed below.

- **Avoidance due to colour** – It has been proposed that the blue colour of granules will make them an unattractive food source to birds. There are some indications from the literature that blue coloured food items can be avoided by birds. However, there is a lack of data on avoidance by relevant GB species when exposed to blue granules under field conditions. While the colour could reduce exposure to birds via consumption of Final Bite granules, this line of evidence has a high degree of uncertainty.
- **Avoidance due to taste** – No data are available that demonstrate the taste of iron inhibits food consumption by birds. This is therefore not a relevant factor to consider.
- **Avoidance due to unfamiliarity with Final Bite granules** – It is proposed that birds would need to be exposed to granules for some time before they become comfortable with them and view them as a potential food source. Given the potential widespread application of granules over an extended time period, and potential year-on-year use, it is not clear that granules would remain an unfamiliar potential food item for birds.
- **Avoidance due to low calorific value** – Optimal foraging strategy posits that higher calorific food items are more likely to be favoured by a foraging animal, all other things being equal. It has been demonstrated that Final Bite granules have a 33-41 % lower calorific content than weed seeds and cereal seeds (used as examples of other seeds which could be available to foraging birds). This is an important factor that can influence the likelihood and level of consumption of granules. Where other, higher calorific food items are abundant it can be expected that birds would largely avoid eating Final Bite granules. However, alternative food items will not always be abundant and thus the likelihood of granules being intentionally consumed for their energy content will vary according to the availability of other food items, which may differ throughout the year (e.g. suitable alternative food items may be more scarce in winter for some species). Additionally, low calorific content would not impact the likelihood of granules being consumed by birds as grit.

Due to the relatively low calorific value and potentially the colour of the granule, it is apparent that granules would not be a favoured food item for birds, where other more favourable food items are available. No information has been submitted on the availability of other food items in fields treated with Final Bite granules. It is noted that these granules can be applied to a wide range of crops and at different timings and it is likely that

the availability of alternative food items in treated fields will vary between crops and timings. Therefore, the availability of alternative food items will be highly variable (in both space and time). As the product can be applied up to 6 times per year, it is unlikely that the availability of alternative food items in treated fields would be low for every application.

While there is a lack of specific supporting evidence representative of the full range of proposed uses of Final Bite granules, it can be concluded that some alternative food items would be available to birds foraging within treated fields. This is concluded on the basis that the presence of Final Bite granules alone would not be sufficient for treated fields to be viewed as a suitable long-term foraging habitat for birds, hence alternative food items must also be available. This has not been accounted for in the first tier risk assessment, where a diet of 100% granules is assumed.

The duration of granule availability has also been considered as a relevant factor in the long-term/reproductive risk assessment. Granules may be degraded over time, potentially becoming less attractive to birds, and they may also be removed by molluscs and hence not be available to birds. However, based on the field trial data, it cannot be assumed that these processes will happen so quickly that no long-term exposure will occur. The trial results also indicated high variability in the rate of degradation of granules and removal of granules by slugs/snails. The first tier risk assessment assumes granules are available over a 21-day period (as standard under EFSA guidance). In light of the data on granule availability and degradation over time, this is considered a reasonable worst-case scenario. It is also noted that the product can be applied up to 6 times per year, depending on mollusc pressures.

In summary, from the factors discussed above it is clear that the first tier screening scenario of birds consuming 100% of their diets as Final Bite granules over a 21-day exposure window is overly conservative and unrealistic. While there are a number of factors likely to reduce the 'true' granule consumption level, the data available do not allow for a robust estimate of how many granules would be expected to be consumed per day by relevant bird species. Since the data do not allow for a simple comparison of exposure and toxicity estimates, instead the following key regulatory questions for the approval decision are considered.

How likely is it that birds consuming Final Bite granules would ingest a dose of elemental iron that exceeds background doses of iron experienced via natural or commercial diets?

No data are available that directly investigate whether birds ingest Final Bite granules at all, and if they do, to what extent. Given that granules have a calorific content, it is possible that birds will consume granules, though they will not be an attractive food source relative to other potential food items. Granules can also be consumed as grit.

Due to the size of the granules, medium or large sized birds are most likely to consume whole granules of Final Bite. HSE has determined an approximate background dietary iron intake value of 19.53 mg a.s./kg bw/d for a granivorous bird, though it is noted there is a high degree of variability associated with this value. This is equivalent to consumption of 57 granules per medium-sized bird per day (not including an assessment factor). Similarly, for captive birds fed commercial diets, the upper end of the recommended concentration in diet would be equivalent to consumption of 41 granules per bird per day.

The consumption of 41 or even 57 granules per day by a medium-sized bird is possible, in that the energy consumed from ingestion of this number of granules would be a small proportion of the daily energy requirement for the bird. A sufficient number of granules would also be available in a small area, given the intended density of application is 60 granules/m². However, the attractiveness of granules as a food source is likely to be limited, particularly where other food items are plentiful.

Data on consumption of large seeds by similar birds indicates a mean consumption value of 12 seeds and 90th percentile of 116 seeds per feeding bout (see table B.9.2-28). Therefore, 41 and 57 food items per day are in the upper part of this range. The representativeness of this data for the long-term risk scenario is however uncertain. It is noted that there could be multiple feeding bouts per day, which could lead to underestimating consumption. Conversely, the assumption that granules are as attractive a food source as large seeds is overly conservative, which would overestimate consumption.

Regarding smaller bird species, granules may be too large for them to consume but they could still ingest fragments of granules. HSE has determined that background exposures to iron via natural diet for a small granivorous bird would be equivalent to 4 granules per bird per day and the upper end of the commercial poultry

diet range is equivalent to 3 granules per bird per day (not including assessment factor). The data on consumption of seeds per feeding bout by small birds shows that a mean of 3 and 90th percentile of 11 large seeds were consumed (see table B.9.2-28).

On balance, HSE consider it rather unlikely that a medium-sized granivorous bird would consume 41 Final Bite granules or more every day for a 21 day period or that a small granivorous bird would consume at least 4 granules every day for a 21 day period. However, due to limitations with the available data, this has not been fully established.

How likely is it that birds consuming Final Bite granules would ingest a dose of elemental iron that could result in reproductive effects?

No laboratory toxicity studies are available investigating reproductive effects in birds exposed to elemental iron or Final Bite granules. Therefore it is not clear whether the active substance or representative formulation can impact reproductive performance. Mammalian data indicates exposure to iron is more likely to impact the functioning of organs such as the liver or heart, rather than directly impact reproductive parameters. However, where ingestion of granules leads to accumulation of iron in organs and this goes on to impact the fitness or survival of the bird (directly or indirectly), this is relevant for the long-term risk assessment.

In a study with rats, there were no effects of exposure to iron at 200 mg a.s./kg bw/d, which is an equivalent dose level to a medium-sized bird consuming 117 granules per day for a 21 day period, or a small bird consuming 8.33 granules per day for a 21 day period (taking into account an assessment factor of 5). These numbers of granules per day are at the extreme upper end of the range of the number of large seeds taken by similar species in single feeding bouts (noting the uncertainties discussed above with the extrapolation of this data).

Additionally, it is expected that wild birds are rather unlikely to be at the upper limit of their homeostatic capacity and hence some ability to cope with elevated iron levels can be assumed. It should also be taken into account that birds will have increased iron requirements during breeding phases, e.g. during pregnancy. Therefore, a higher iron dose may be needed to result in iron overload during breeding periods.

The amount of crop cover will vary during the period of application. Early in the growing season when there is little crop cover the granules would make up a relatively high proportion of the available food in the treated area. This might suggest that the granules may make up a higher proportion of diet at this time of year, although there will also be germinating seed available. While the crop is small and granules might make up a higher proportion of available food for birds within crop it should also be noted that the habitat will be relatively unattractive to birds (unless crop seeds are also present at the soil surface, in which case they are likely to be the preferred food). As crop cover increases the choice of food to birds in the field increases, so it becomes less likely birds will select a high proportion of granules which could offset the habitat being more heavily used. This proposition has not been supported by any data, but it is unlikely there will be long periods during the reproductive period where the treated area is both attractive to birds and has a low proportion of alternative food available.

Overall, HSE consider it unlikely that where granules are scattered across the soil surface, these would be a sufficiently attractive food item that relevant bird species would consume enough granules across a period of multiple days or weeks that this would overload their ability to regulate their absorption of iron, leading to accumulation of iron in organs, impacting the health and survival of the individual. However, it is noted that there is still some uncertainty associated with this conclusion.

Where there are spills of granules and large numbers available on the soil surface, it is more plausible that a fast-eating bird, such as a pigeon, could eat a large number of granules within a short period and may not be aware of any negative impacts of this consumption on their health before it is too late. However, this scenario is less of a concern regarding long-term exposure and it can be managed through appropriate risk mitigation labelling. It is recommended that the following mitigation is applied:

SPe 6: To protect birds / wild mammals remove spillages

For the consumption of granules as grit scenario, factors which influence the attractiveness of Final Bite granules as a food source are not expected to be relevant, so some further consideration is needed. At first tier it is assumed that a bird of 300 g requires 1306 grit particles/day in the long-term exposure scenario. For the generic medium-sized bird considered above (390 g bodyweight), this is equivalent to 1698 grit particles per day. If 117 granules are required to exceed the NOEL/5 for such a bird (extrapolated from mammal data – see table B.9.2-30), this

would mean that 7% of the ingested grit particles would need to be Final Bite granules (34% where not including the assessment factor). It is noted that granules have a [REDACTED] and there is evidence to suggest they significantly degrade over a period of a few days to weeks. Section B.3.5 specifies that the force needed to break pellets was 1.45 kg for the proposed product. Therefore the suitability of Final Bite granules as a grit equivalent for birds is considered relatively low and would decline over time. Overall, the likelihood of birds perceiving granules as a grit source is considered to be low. Therefore, it is unlikely that granules would be consumed as grit to a sufficient extent that this would result in an ingested dose that exceeds a dose of concern for the long-term risk scenario. The consumption of granules as food is considered the more relevant exposure scenario for the bird risk assessment.

Is there evidence of exposure to iron negatively impacting the health of relevant bird species under field conditions?

The literature searches performed have not found any evidence of reproductive effects in individual wild birds or long-term effects on wild bird populations resulting from elevated exposure to iron. It is less clear though whether such impacts would be detected and reported, should they occur. Mortality was recorded in captive blackbird, fieldfare, song thrush and redwing in a publication by Pavone et al. (2014). This was considered a result of acute exposure and is discussed in the acute exposure consideration.

Are there factors which could mitigate any risk to birds at the population level?

While the available information does not allow the risk to be assessed at the population level, it is appropriate to consider that iron is an essential element required to support bird health. The risk assessment conducted above focuses on the potential for birds to be negatively impacted by iron overload. However, individual birds may be deficient in iron, so consumption of iron from Final Bite granules could have a positive effect on the health of these individuals, assuming a critical threshold is not exceeded. Therefore, it is possible that were some individuals in a population to be negatively affected by consumption of Final Bite granules, others could be positively impacted. Without a suitable study or modelling data investigating impacts on bird populations under field conditions, it is not possible to further consider this point.

Overall, it is concluded that it has been established that any mortality or reproductive effects are unlikely from exposure of birds to iron via consumption of Final Bite granules. However, due to uncertainty regarding the likelihood of granules being consumed by birds, a confirmatory data requirement is recommended to validate this conclusion. It is proposed to request that the Applicant provides monitoring data investigating whether birds consume Final Bite granules under field conditions. This could be achieved by observing consumption of granules by birds from a specified area where granules have been applied according to the proposed conditions of use. In light of the above risk assessment and since consumption of granules would only need to be monitored for a short time period, this study would not be expected to result in harm to wild vertebrates.

Long-term/reproductive risk to mammals from consumption of granules

When considering the intentional ingestion of granules as a food source scenario, the first tier long-term/reproductive TER was determined to be 0.0015 for the wood mouse. This TER value is below the acceptability trigger of ≥ 5 by a wide margin.

The first tier TER calculations used the worst-case NOAEL of 3.2 mg a.s./kg bw/d from Whittaker et al. (1996). A second study is available, with a NOAEL of 200 mg a.s./kg bw (Zhu et al., 2016). Both are published studies and while there are limitations regarding their use in regulatory risk assessment, they provide information of relevance for assessing long-term risks. On further examination of the Whittaker et al. (1996) results, clear adverse impacts on the health of test organisms that would influence survival or reproductive success were only observed at 350 mg/kg bw/d and above. When using the NOAEL of 200 mg a.s./kg bw from Zhu et al. (2016) in the first tier risk assessment, the resulting TER for mammals ingesting granules as a food source is 0.095. Therefore, the TER remains below the acceptability trigger of ≥ 5 .

Given the nature of the information available on iron exposure and its effects on mammals, and in light of the very low first tier TERs, it is considered that refinement of the TER calculations is not productive. Instead a qualitative assessment of the overall weight of evidence is required.

As discussed for birds, extensive literature reviews and studies are available which consider how iron is absorbed and transported within vertebrates, and how iron levels within such animals are controlled (homeostasis). However, it is not possible to define a dose range of ingested iron that could be successfully regulated by mammals, and the dose level at which accumulation of iron in organs and damage to the animal could occur based on these publications.

It is apparent from the literature on iron toxicity that dietary iron can accumulate in the liver and other organs of mammals (haemosiderosis). There is little evidence though that this leads to health effects in free ranging GB mammal species.

To provide insight into the level of long-term risk posed by potential consumption of Final Bite granules, available long-term/reproductive toxicity data from a range of sources have been considered. In the following table long-term/reproductive effects data that are relevant for mammals are summarised and these are compared to equivalent exposure level calculations for small mammals ingesting granules.

Table B.9.2-31: Contextualising the long-term/reproductive risk to mammals from consumption of granules

Concentration (ppm)	Dose (mg Fe/kg bw/d)	Description	Equivalent exposure level for a small mammal (21.7 g)#
35	3.2	No effect level in rats (Whittaker et al., 1996)	Consumption of 0.104 granules per mouse per day
350	35	Accumulation of iron in liver and lipid peroxidation in rats (Whittaker et al., 1996)	Consumption of 1.14 granules per mouse per day
69.75	28.95	Estimated background dose via normal diet (100% seed consumption)	Consumption of 4.7 granules per mouse per day@
2000*	200	No effect level in rats (Zhu et al., 2016)	Consumption of 6.53 granules per bird per day
3500	350	20% mortality, cardiomyopathy, splenic and pancreatic atrophy in rats (Whittaker et al., 1996)	Consumption of 11.4 granules per mouse per day

Taking into account standard assessment factor of 5

*Estimated assuming 10-fold difference between the concentration in ppm and dose in mg/kg bw/d (Section 2.3.1.1 of EFSA, 2009)

@ No assessment factor applied

When considering potential exposure levels, a number of factors have been discussed which can impact the likelihood of mammals consuming Final Bite granules and the quantity of granules that might be consumed. These are summarised above for birds. The key aspects relating to mammals are the extent to which the relatively low calorific value of granules makes them an unfavoured potential food item, the availability of other food items in treated fields and the attractiveness of treated fields as a foraging location. It is considered that where alternative food items are abundant, granules are unlikely to be consumed in significant quantities. However, where alternative food items are scarce this may not be the case (though this scarcity would be expected to make fields less attractive as a foraging location). Information is not available on the number of alternative food items that are available per unit area in fields treated with Final Bite granules (noting this number is expected to be highly variable and the range of crops/timings for Final Bite granules is wide). Therefore, the relatively low calorific value of granules will reduce the 'true' level of exposure compared to first tier estimates (which assume a 100% granule diet), but the magnitude of this reduction is unknown and likely to be variable.

The duration of granule availability has also been considered as a relevant factor in the long-term/reproductive risk assessment. The first tier risk assessment assumes granules are available over a 21-day period (as standard under EFSA guidance). In light of the data on granule availability and degradation over time from field trials, this is considered a reasonable worst-case scenario. However, it is noted that dosing of animals in the Whittaker

et al. (1996) and Zhu et al. (2016) studies was maintained over a period of 12-13 weeks. Final Bite granules can be applied up to 6 times, so it cannot be categorically ruled out that where the maximum number of treatments are made and where conditions are such that degradation and removal of granules is particularly slow, granules could be available for a similar time period. However, this is an unlikely scenario and one that would be very uncommon. Therefore, in the majority of cases the duration of dosing in the toxicity studies is overly worst-case compared to the exposure situation in the field. Given the way that elemental iron acts, i.e. as iron accumulates in organs over time, the duration of dosing is likely to impact the nature and magnitude of toxic effects observed in test animals.

In summary, from the factors discussed above it is clear that the first tier screening scenario of mammals consuming 100% of their diets as Final Bite granules is overly conservative and unrealistic. While there are factors likely to reduce the 'true' granule consumption level, the data available do not allow for a robust estimate of how many granules would be expected to be consumed per day by relevant mammal species (e.g. wood mouse). Additionally, the duration of exposure in the toxicity studies is longer than would be expected to occur in the field following use of Final Bite granules, in the majority of cases. Since the data do not allow for a simple comparison of appropriate exposure and toxicity estimates, instead the following key regulatory questions for the approval decision are considered.

How likely is it that mammals consuming Final Bite granules would ingest a dose of elemental iron that exceeds background doses of iron experienced via natural or commercial diets?

No data are available that directly investigate whether mammals ingest Final Bite granules at all, and if they do, to what extent. Given that granules have a calorific content, it is possible that mammals will consume granules, though the calorific content is low, so they will not be an attractive food source relative to other potential food items.

While a range of mammal species could potentially come into contact with Final Bite granules, small omnivorous mammals, such as wood mice, are considered of particular relevance. Wood mice have an omnivorous diet, are known to consume seeds (which granules could be mistaken for) and have a high food intake rate relative to bodyweight. They are also the small mammal species that is most likely to be trapped within a range of agricultural fields.

HSE has determined an approximate background dietary iron intake value of 28.95 mg a.s./kg bw/d for a small omnivorous mammal (e.g. wood mouse) consuming a mixed diet, though it is noted there is a high degree of variability associated with this value. For the small omnivorous mammal this dose is equivalent to consumption of 4.7 Final Bite granules per day.

The consumption of 4.7 granules per day by a small omnivorous mammal is possible, in that the energy consumed from ingestion of this number of granules would be a small proportion of the daily energy requirement. A sufficient number of granules would also be available in a small area, given the intended density of application is 60 granules/m². However, the attractiveness of granules as a food source is likely to be limited, particularly where other food items are plentiful. The likelihood of large numbers of seeds being present in a small area from spills can be reduced through the mitigation discussed in the bird section.

On balance, HSE consider it rather unlikely that a small omnivorous mammal would consume 4.7 Final Bite granules or more every day for a 21 day period. However, due to limitations with the available data, this has not been fully established.

How likely is it that mammals consuming Final Bite granules would ingest a dose of elemental iron that could result in reproductive effects?

When taking into account only parameters that directly impact the survival or reproductive success of an animal, the Whittaker et al. (1996) and Zhu et al. (2016) studies indicate a NOAEL of 200 mg a.s./kg bw/d. At a higher dose of 350 mg a.s./kg bw/d there were effects on the survival of rodents. Therefore, the 'true' maximum no observed adverse effect level may be higher than 200 mg a.s./kg bw/d but it would be expected to be lower than 350 mg a.s./kg bw/d.

The NOAEL of 200 mg a.s./kg bw/d is equivalent to consumption of 6.5 granules per day by a small omnivorous mammal, assuming a standard 5-fold assessment factor to address uncertainties. While this does not seem like a particularly high number of granules, it must be remembered that the small omnivorous mammal has a low

bodyweight (21.7 g) and that this number of seeds would need to be consumed every day for a prolonged period (e.g. 21 days in the standard long-term exposure scenario). The likelihood of a small mammal consuming sufficient seeds will also be further reduced by stipulating that any spills must be buried. To achieve a dose of 350 mg a.s./kg bw (i.e., the LOAEL/5) the small omnivorous mammal would need to consume 11.4 granules per day. It is also noted that these toxicity endpoints may overestimate the toxicity of elemental iron to mammals, given they were derived with a 12-13 week exposure period.

Additionally, it is expected that wild mammals are rather unlikely to be at the upper limit of their homeostatic capacity and hence some ability to cope with elevated iron levels can be assumed. It should also be taken into account that mammals will have increased iron requirements during breeding phases, e.g. during pregnancy. Therefore, a higher iron dose may be needed to result in iron overload during breeding periods.

The amount of crop cover will vary during the period of application. Early in the growing season when there is little crop cover the granules would make up a relatively high proportion of the available food in the treated area. This might suggest that the granules may make up a higher proportion of diet at this time of year, although there will also be germinating seed available. While the crop is small and granules might make up a higher proportion of available food for small mammals within crop it should also be noted that the habitat will be relatively unattractive to small mammals, due to low overall food availability (unless crop seeds are also present at the soil surface, in which case they are likely to be the preferred food) and increased risk of predation resulting from limited crop cover. As crop cover increases the choice of food to mammals in the field increases, so it becomes less likely small mammals will select a high proportion of granules which could offset the habitat being more heavily used. This proposition has not been supported by any data, but it is unlikely there will be long periods during the reproductive period where the treated area is both attractive to small mammals and has a low proportion of alternative food available.

Overall, HSE consider it unlikely that where granules are scattered across the soil surface, these would be a sufficiently attractive food item that relevant mammal species (e.g. wood mouse) would consume enough granules across a period of multiple days or weeks that this would overload their ability to regulate their absorption of iron, thus leading to accumulation of iron in organs, impacting the health and survival of the individual. However, it is noted that there is still some uncertainty associated with this conclusion.

Is there evidence of exposure to iron negatively impacting the health of relevant mammal species under field conditions?

The literature searches performed have not found any evidence of reproductive effects in individual wild mammals or long-term effects on wild mammal populations resulting from elevated exposure to iron. It is less clear though whether such impacts would be detected and reported, should they occur.

Are there factors which could mitigate any risk to mammals at the population level?

While the available information does not allow the risk to be directly assessed at the population level, it is appropriate to consider that iron is an essential element required to support mammal health. The risk assessment conducted above focuses on the potential for mammals to be negatively impacted by iron overload. However, individual mammals may be deficient in iron, so consumption of iron from Final Bite granules could have a positive effect on the health of these individuals, assuming a critical threshold is not exceeded. Therefore, it is possible that were some individuals in a population to be negatively affected by consumption of Final Bite granules, others could be positively affected, thus reducing or even reversing the overall impact on the population.

It is also noted the the relevant focal species used in the risk assessment, the wood mouse, is know to have a high reproductive capacity. Wood mice breed from March through to October (Gurney et al., 1998). The gestation length for this species varies from 19 to 32 d (Niethammer, 1978; Harris and Yalden, 2008), with 4 or more litters produced per year. Litter size varies from 4.3 to 5.4 pups per litter (Clarke, 1985; Harris and Yalden, 2008). Therefore, given the high reproductive output of this species, the capacity for wood mice populations to recover following any impact from exposure to iron via Final Bite granules is high. This ability for wood mouse populations to recover following perturbation has been demonstrated in field populations in studies such as those from the Boxworth Project (Johnson et al., 1991; Grieg-Smith, 1989). The Johnson et al. (1991) study followed wood mouse populations in Cambridgeshire (UK) exposed to pellets containing a non-iron molluscicide (methiocarb). Results showed a decrease in trapping numbers post-treatment in treated fields compared to

controls, with also lower proportions of adults found. However, there were no long-term effects on wood mouse populations. Similar results were seen in Grieg-Smith (1989), with wood mouse populations exposed to methiocarb pellets recovering rapidly following an initial decrease in abundance.

Therefore, there are clear factors that would be expected to reduce the likelihood of any negative effect of iron on the health of individual mice (should that occur) leading to long-term impacts on local populations. It is not possible to further consider/develop these points, in the absence of a suitable monitoring study with exposure to iron or modelling data investigating impacts on mammal populations under field conditions.

Overall, in light of the consideration of the likelihood of small mammals consuming a dose of iron that exceeds background doses or that could result in reproductive/long-term effects, it is concluded that it has been established that any mortality or reproductive effects are unlikely from exposure of mammals via consumption of Final Bite granules. While there remains some uncertainty regarding the likelihood of small mammals consuming Final Bite granules, there is additional information to establish that the actual protection goal that there are ‘no long-term repercussions for abundance and diversity’ would be met.

Secondary poisoning/risk from drinking water

As discussed in the aquatic risk assessment, the active substance is largely insoluble in water and is not considered to be bioavailable to fish when applied according to the requested use. The risk from secondary poisoning via fish is therefore considered to be acceptable, on the basis that the level of iron in fish will not increase as a result of the use of the active substance.

For secondary poisoning via contaminated earthworms, the PEC_{soil} accumulation of the active substance is 12.8 mg a.s./kg soil, compared to a background range of 2000-50,000 mg Fe/kg soil. The addition of iron into the soil from the use of the representative product is not expected to add significantly to the amount of iron already available in earthworms.

The risk from secondary poisoning via earthworms and fish is overall considered to be negligible.

As the active substance is insoluble in water it is considered that the exposure to terrestrial vertebrates via drinking water will be minimal also.

Endocrine Disrupting Properties

Iron ions are considered ubiquitous in the environment and are also important for animal and plant functions. In EFSA Pesticide Peer Review (PPR) meeting 14 (September 2019) the active substance ferric pyrophosphate was discussed and a conclusion was made regarding the data requirement for endocrine disruption, whereby the data requirement could be waived on the basis that the substance is of very low solubility, is ubiquitous in the environment and used as a food additive. The same conclusions can be made for elemental iron.

B.9.3. EFFECTS ON AQUATIC ORGANISMS

B.9.3.1. Acute toxicity to fish, aquatic invertebrates, or effects on aquatic algae and macrophytes

The following data was submitted in support of this application.

Table B.9.3.1-1 – Summary of toxicity data available for the aquatic compartment and elemental iron

Reference	Author	Species	Substance	Endpoint	Value (mg a.s./L)
10.2.1/1	██████ 2008	<i>O. mykiss</i>	Slug and Snail Killer (1 % Iron)	96 h LC50	>0.98 (nom)*
10.2.1/2	██████ 2018	<i>D. magna</i>	Final Bite 040226 (1 % Iron)	48 h EC50	>0.5345 (mm)
10.2.1/3	██████ 2008	<i>D. magna</i>	Slug and Snail Killer (1 % Iron)	48 h EC50	26.873 (nom)*
10.2.1/4	██████ 2018	<i>D. subspicatus</i>	Final Bite 040226 (1 % Iron)	72 h ErC50	>0.529 (mm)
				72 h NOErC	0.529 (mm)
10.2.1/5	██████ 2008	<i>P. subcapitata</i>	Slug and Snail Killer (1 % Iron)	72 h ErC50	>180 (nom)*
				72 h NOErC	32 (nom)*

Nom = nominal concentration

Mm = mean measured concentration

*Study not considered reliable for use in the risk assessment; endpoints presented for informative purposes only.

Slug and Snail Killer Studies

Studies on fish, aquatic invertebrates and algae with the product ‘Slug and Snail Killer’ (1 % iron) were presented by the applicant. Analysis for the concentrations of active substance in the test media was not conducted in any of the studies, and in the case of the study with algae, the validity criterion for coefficient of variation was not met. It is noted that no batch number is provided for the formulation, however, in light of updated consideration in the volume 4, it is concluded that the tested material is representative of the technical specification for elemental iron. The lack of analytical measurement of test concentrations means that the derived endpoints are not reliable for use in the risk assessment. As they cannot be used in the risk assessment, no further evaluation has been conducted and the studies have not been summarised by the UK evaluator. The studies have not been used in the risk assessment or for the purposes of classification. The Applicant text has been presented *verbatim* for informative purposes only in Section B.9.13.2 (end of this document).

B.9.3.1/1 – Acute toxicity to Aquatic Invertebrates

Title: Determination of short term toxicity of Final Bite – 0402206 against *Daphnia magna* STRAUS according to OECD 202 resp EU C.2.

Study code: 17121401G201

Author: [REDACTED] (2018)

Guideline: OECD 202 (2004); OECD guidance document no. 23 'Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures (2000).

GLP: Yes

Materials and Methods:

Test item: Final Bite – 0402206 (Batch no. KM8017420OH)

Active substance: 1 % w/w Iron (nominal)

Reference item: Potassium Dichromate (CAS 7778-50-9), tested separately (2017). The 24 h EC50 was 1.6 mg/L (recommended range 0.6-2.1 mg/L).

Test organism: *Daphnia magna* Straus. In house culture. Between 0 and 24 hours old at the time of the test. The test organisms were placed into a beaker containing dilution water 1 hour before the test to acclimatise. Animals with no apparent damage were selected and used for the test.

Test Conditions:

Test water: Dilution water in line with ISO 6341 (1996) (quoted in OECD 202, 2004).

Test units: 50 mL glass beakers containing 20 ± 5 mL test solution and 5 test organisms (4 replicates per test concentration and control)

Temperature ranged between 20.0-20.8 °C and the light regime was 16 h of light and 8 h of darkness. pH 7.5-8.0, Dissolved Oxygen 8.6-8.8 mg/L.

Test duration: 48 h

Test concentration (s): 100 mg product/L. Untreated control.

Test item was pulverised with a mortar into a fine powder. The water-accommodated fraction (WAF) was prepared by weighing the nominal load of 100.2 mg/L and adding the corresponding amount of dilution water and shaking vigorously for 24 hours. The resulting solution was filtered 0.45 µm PTFE filters and used for the limit test.

Observations:

Test organisms were observed for immobilisation (no movement when the beaker is gently agitated) at 24 and 48 h. The test solutions were analysed for the concentration of the active substance using ICP-OES and the method of analysis was confirmed to be valid by RMS chemistry specialists.

Results:

No immobilisation was noted in the treatment or in the blank control after 24 and 48 hours.

The analysis results indicated that the test solutions contained between 52 and 55 % of the nominal iron concentration. The blank control contained less than 30 % of the LOQ (50 µg/L Fe).

The mean measured concentration of the test item was 534.49 µg a.s./L. The 48 h EC50 was therefore >534.49 µg test item/L.

Validity Criteria (OECD 202):

- Control mortality was no more than 10 % (0 %)
- Dissolved Oxygen concentration was no less than 3 mg/L (8.6 mg/L)

HSE Comments:

The study was conducted to GLP and follows OECD guideline 202 (2004) with no deviations noted. The study meets the validity criteria.

It is noted that no batch number is provided for the iron used in the formulation, however, in light of updated consideration in the volume 4, it is concluded that the tested material is representative of the technical specification for elemental iron.

The agreed endpoint is:

48 h EC50 >534.49 µg a.s./L

B.9.3.1/2 – Toxicity to Algae

Title: Determination of the toxicity of Final Bite – 0402206 against *Desmodesmus subspicatus* according to OECD 201 resp EU C.3

Study code: 17121401G301

Author: (2018)

Guideline: OECD 201 (2006); OECD guidance document no. 23 'Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures (2000).

GLP: Yes

Materials and Methods:

Test item: Final Bite – 0402206 (Batch no. KM8017420OH)

Active substance: 1 % w/w Iron (nominal)

Reference item: Potassium Dichromate (CAS 7778-50-9), tested separately (2017). The 72 h ErC50 was 0.9 mg/L (recommended range 0.6-1.03 mg/L).

Test organism: *Desmodesmus subspicatus*. In house culture originally obtained from MBM Sciencebridge GmbH (Institut für Pflanzenphysiologie of Universität Göttingen).

Five days before the start of the test, an aliquot of the permanent culture was brought into nutrient medium and incubated under continuous lighting for 96 hours. Before usage the pre-culture was checked for the absence of cell aggregates and the cell density of the culture was determined.

Test Conditions:

Test water: OECD 201 growth medium.

Test units: 65 mL glass flasks filled with 45 ± 1 mL of the respective test solution and incubated (covered with a perforated plastic foil acting as a stopper) for 72 h, shaken at 100 rpm on an orbital shaker to keep the algae in suspension. There were 3 replicates for each treatment and 6 replicates for the control.

Temperature ranged between 21.3-23.5 °, light 5000 lux (constant). pH 7.5-7.9

Test duration: 72 h

Test concentration (s): 1, 3.2, 10, 32 and 100 mg test item/L. Untreated control.

Test item was pulverised with a mortar into a fine powder. The water-accommodated fraction (WAF) was prepared by weighing the nominal loads of 1, 3.2, 10, 32 and 100 mg/L and adding the corresponding amount of algal medium and shaking vigorously for 23 h 15 min. The resulting solution was filtered 0.45 µm PTFE filters and used for the limit test.

For each treatment 200 mL of the respective test item solution was mixed with the necessary amount of algal pre-culture (0.363 mL) to achieve a cell concentration of 2×10^3 cells/mL.

Observations:

Test solutions were analysed for cell density at 0, 24, 48 and 72 h using an electronic particle counter. The cells were observed under a microscope for normal and healthy appearance at the end of the test. The test solutions were analysed for the concentration of the active substance at the beginning and end of the test using ICP-OES and the method of analysis was confirmed to be valid by RMS chemistry specialists.

Statistics: Calculations were conducted using Microsoft Excel and ToxRat Professional 3.2.1.

Results:

Table 9.3.1/2-1 Cell numbers/mL

Nominal Concentration in mg/L	Parameter	Cell Number/mL			
		0 h	24 h	48 h	72 h
Blank control	Mean	2040	8610	40710	236700
Blank control	SD	0	778	4374	9780
1	Mean	2040	7587	37333	241367
1	SD	0	571	3636	5191
3.2	Mean	2040	6687	42473	258373
3.2	SD	0	1141	7172	17867
10	Mean	2040	8280	47000	325660
10	SD	0	1065	4089	23991
32	Mean	2040	7873	40880	350240
32	SD	0	1570	6493	55411
100	Mean	2040	6407	25807	172973
100	SD	0	930	3688	15942

SD = Standard Deviation

Table 9.3.1/2-2 Growth rate and Yield

Nominal Concentration in mg/L	Parameter	Growth Rate (0-72h) [day ⁻¹]	Yield (0-72h) [Cell Concentration/mL]
Blank control	Mean	1.58	234660
	SD	0.01	9780
1	Mean	1.59	239327
	SD	0.01	5191
3.2	Mean	1.61	256333
	SD	0.02	17867
10	Mean	1.69	323620
	SD	0.02	23991
32	Mean	1.71	348200
	SD	0.05	55411
100	Mean	1.48	170933
	SD	0.03	15942

SD = Standard Deviation

Table 9.3.1/2-3 Inhibition Values

Nominal concentration in mg/L	% Inhibition	
	Growth Rate (0-72h)	Yield (0-72h)
Blank control	0	0
1	-0.42	-1.99
3.2	-1.82	-9.24
10	-6.69	-37.91
32	-8.08	-48.38
100	6.64	27.16

Negative inhibition values indicate a stimulation of algal growth compared to the blank control.

Table 9.3.1/2-4 Measured iron concentrations

Nominal Concentration Test Item	Nominal Concentration Iron	Measured Iron concentration t = 0 h	Measured Iron concentration t = 72 h	% of nominal iron t = 0 h	% of nominal iron t = 72 h
mg/L	µg/L	µg/L	µg/L		
Blank control	--	< LOQ	< LOQ	--	--
1.0	10	< LOQ	< LOQ	--	--
3.2	32	12.37	< LOQ	39	--
10	100	51.70	12.08	52	12
32	320	205.76	107.03	64	33
100	1000	560.70	496.36	56	50

LOQ (Limit of quantification) = 10 µg/L (= lowest calibration concentration)

The reported 72 hr NOEC was 32 mg test item/L (nominal), and the 72 hour EC50 was >100 mg test item/L. The 72 hr NOEC was 100 mg test item/L (nominal) and the 72 hour EC50 was >100 mg test item/L. No reliable EC10/20 values could be calculated.

Validity Criteria (OECD 201):

- Control cell biomass must increase by a factor of at least 16 over the course of the 72 hour test period (116)
- Mean coefficient of variation of daily growth rates must not exceed 35 % (11.3%)
- Coefficient of variation of average growth rate during the whole test period must not exceed 7 % (0.87 %)

HSE Comments:

The study was conducted to GLP and follows OECD guideline 201 (2006). The results were expressed in terms of nominal concentrations however as they were not maintained within 20 % of the nominal concentration, the geometric mean values should be used to determine the study endpoints (Paragraph 39 OECD 201, 2006).

These are as follows (calculated using MS Excel)

0 h (% nominal)	72 h (% nominal)	Geomean
0	0	0
39	0	0
52	12	24.97999
64	33	45.9565
56	50	52.91503

It is noted that no batch number is provided for the iron used in the formulation, however, in light of updated consideration in the volume 4, it is concluded that the tested material is representative of the technical specification for elemental iron.

The agreed endpoints are:

72 h NOErC = 529.1 µg a.s./L

72 h ErC50 >529.1 µg a.s./L

B.9.3.2. Additional long-term and chronic toxicity studies on fish, aquatic invertebrates and sediment dwelling organisms

No additional data was submitted.

B.9.3.3. Further testing on aquatic organisms

No further data was submitted.

B.9.4. RISK ASSESSMENT FOR AQUATIC ORGANISMS

The following toxicity data is available and considered reliable for use in the risk assessment for aquatic organisms.

Reference	Author	Species	Substance	Endpoint	Value (mg a.s./L)
10.2.1/2	██████ 2018	<i>D. magna</i>	Final Bite-040226 (1 % Iron)	48 h EC50	>0.5345 (mm)
10.2.1/4	██████ 2018	<i>D. subspicatus</i>	Final Bite-040226 (1 % Iron)	72 h ErC50	>0.529 (mm)
				72 h NOErC	0.529 (mm)

The toxicity studies with the representative formulation 'Final Bite-040226' (1 % iron) showed no effects of the test item on algae or aquatic invertebrates, represented by *D. subspicatus* and *D. magna*, respectively. No reliable data was available for the representative product or the active substance and fish. A study with the surrogate formulation 'slug and snail killer (1 % iron)' was submitted in support of this application but the study did not analyse the test medium for concentrations of the test item, and is therefore not reliable for the purposes of aquatic risk assessment or hazard classification. This would normally be considered a data gap, especially for the purposes of hazard classification, however as discussed below it is considered that fish, like other water dwelling organisms, are capable of metabolising the additional iron in surface water from the proposed uses of Final Bite with no toxic symptoms. In the interest of reducing vertebrate testing it is acceptable to waive the data requirement for fish toxicity in this case.

For the purposes of the UK the risk to the aquatic compartment is considered from two routes of exposure. Firstly, spray-drift, whereby the waterbody is exposed to the intact formulation as well as the active substance and metabolites. Secondly, drainflow, which assesses the risk from the active substance and metabolites only, as the formulation is not considered to remain intact during the transition from the field soil to the waterbody.

In Part B Section 8 (Vol 3CP) the risk from spray-drift was discounted due to the nature of the product and the requested GAP - 'Final Bite ®' is a ready-to-use granular bait that will be scattered by mechanical applicator or by hand to the soil surface. Elemental iron has no metabolites and so only the risk from drainflow of the active substance needs consideration. The following worst-case PEC_{sw} values for elemental iron were calculated based on the requested GAP for the representative product. The text below in *italics* is copied from Part B section 8 also (for further details regarding background levels of iron in water, please refer to that document).

Table 3CP B.8.5-2 PEC_{sw} and PEC_{sed} via drainflow of the iron Fe²⁺ and Fe³⁺ ions following applications of 'Final Bite ®' at 1x 480 g a.s./ha (0% interception) using the first tier EXCEL 'PEC sw-sed (drainflow)' spreadsheet

Compartment	Fe ²⁺	Fe ³⁺
PEC _{sw} (µg/L)	70.154	0.295
PEC _{sed} (µg/kg)	323.787	1.363

The surface water exposure assessment for Fe²⁺ represents an extreme worst-case scenario of applications of 'Final Bite ®' to anaerobic soils and 100% conversion of Fe⁰ to Fe²⁺ ions, followed by transport via drainflow to the surface water body. Under normal environmental conditions, in aerobic soils over a pH range 4-9, most iron will be in the oxidised form Fe³⁺. Furthermore, any Fe²⁺ is likely to oxidise and hydrolyse to Fe³⁺ ions and insoluble compounds upon entering the surface water compartment which would then precipitate to the sediment compartment. Under circumneutral, oxic surface waters Fe²⁺ is expected to oxidise to Fe³⁺ ions within minutes to hours (WFD, 2012). The HSE considers that granular applications of elemental iron will be applied to the soil surface under aerobic conditions, as agricultural crops are not grown under prolonged anaerobic conditions. Most of the iron in the bait which is not consumed will come into contact with oxygen and water and essentially rust, converting to Fe³⁺ ions.

Background levels of iron in surface waters vary greatly worldwide but is considered naturally abundant in the aquatic environment. In the UK, concentrations have been found ranging from 17.0-11700 µg/L total iron (3397 samples from 1617 sites – WFD, 2012). In terms of Fe²⁺ and Fe³⁺ ions, the mean concentrations are estimated to be 10.406 µg Fe²⁺/L and 2474.58 µg Fe³⁺/L in the UK. In the sediment the background levels range from 600-358000 µg/kg total iron (FOREGS database, 2005), far in excess of the values calculated for the uses of iron requested under this application (see table B.8.5-2 above). Further text from part B8 :

The soil exposure assessment under section 3CP B.8.2 demonstrated that the initial PEC_{soil} arising from applications of 'Final Bite ®' is negligible compared to natural background levels in soil. The PEC_{soil}

accumulation based on 20 consecutive years without tillage is 12.8 mg/kg which is considerably less than the range naturally observed in UK and European soils (140- 22300 mg/kg). The HSE considers that in situations where large amounts of iron naturally transport via drainflow or runoff from soils into adjacent water bodies, the use of 'Final Bite ®' will not impact significantly on this. The surface water exposure assessment represents a worst-case scenario for the mobile Fe^{2+} ions, which are more prevalent under anaerobic conditions. The HSE considers that it is highly unlikely 'Final Bite ®' granules will be applied during flooding events in which anaerobic conditions may prevail. Furthermore, in situations where the soil is under prolonged anaerobic conditions, most of the mobile Fe^{2+} ions would be naturally occurring. The HSE does not consider the addition of iron from 'Final Bite ®' would impact on the natural existing environmental processes.

Overall, it is considered that the elemental iron, used as requested under this application, will not add to the amount of iron already bio-available to aquatic organisms in either the surface water or the sediment. The available toxicity data (covering algae and aquatic plants) shows that no adverse effects occur at the highest tested concentration (0.529 mg a.s./L mean measured).

Classification and Labelling

The relative insolubility of elemental iron means that the toxicity of this active substance to aquatic invertebrates and algae technically qualifies for the highest degree of acute classification, with acute mean measured endpoints equivalent to >0.5 mg a.s./L. However as discussed above, iron occurs naturally in the aquatic environment but is not usually bioavailable to aquatic organisms, which is reflected in the results of the first tier aquatic studies available, with no effects being noted but a low mean measured endpoint being derived. Overall, the low solubility of this active substance means that it is mostly unavailable to aquatic organisms and so represents a low hazard. The data gap noted for reliable acute fish toxicity data can be waived for the purposes of classification for the same reason i.e. its insoluble nature means that it will largely be unavailable to fish. Overall, no environmental classification **for aquatic hazard** is proposed for Elemental iron.

B.9.5. EFFECTS ON ARTHROPODS**B.9.5.1. Effects on bees**

No formulation data was submitted.

B.9.5.2. Effects on non-target arthropods other than bees

Report:	CP 10.3.2.2/01. [REDACTED] 2018
Title:	Final Bite - 0402206: Effects on the Reproduction of Rove Beetles <i>Aleochara bilineata</i> - Extended Laboratory Study - Dose Response Test -
Report no.:	Study No. 127511071 (R-37825)
Guidelines:	Grimm <i>et al.</i> 2000: A test for evaluating the chronic effects of plant protection products on the rove beetle <i>Aleochara bilineata</i> Gyll. (Coleoptera: Staphylinidae) under laboratory and extended laboratory conditions.
GLP:	Yes, certified
Published:	No

MATERIALS AND METHODS**A. MATERIALS:**

- Test material:** Final Bite - 0402206
Batch no.: KM8017420OH
Purity: 10 g/kg elemental iron (nominal); 1.21% w/w (analysed)
Description: Solid blue pellets
- Reference material:** BAS 152 11 I
Ref. concentration: dimethoate, nominal: 400 g/L
- Vehicle:** deionised water
- Test substrate:** LUFA 2.1 soil
Test units: Plastic boxes (18.3 cm x 13.6 cm x 6 cm; length, width, height), covered with perforated plastic lids. The height of the moistened soil was approximately 4 cm and soil surface area was 190 cm².

Emergence containers: The parasitized fly pupae were placed in a funnel which was placed on a glass beaker; the bottom of the funnel was perforated with holes (diameter approximately 2 mm) through which the emerged beetles fall into the glass beaker below. The pupae remained in the funnel.

- Test organisms:** Rove beetle (Coleoptera, Staphylinidae)
Species: *Aleochara bilineata* Gyll
Age: Adults 2-5 days old
Source: De groene Vlieg, Duivenwaardsedijk 1; NL- 3244 LG - Nieuwe Tonge
Diet: Frozen midge larvae (commercial food for aquarium fish; *Chironomus sp.*) every 2 - 3 days *ad libitum*.
- Treatment:** Control, 8, 16, 32, 48 and 100 kg product/ha; corresponding to 1, 2, 5, 7 and 14 pellets per test unit (190 cm²), respectively; corresponding to 53, 105, 263, 368 and 737 pellets/m², respectively (as calculated by HSE using the number of pellets per arena and the arena area of 190 cm²).
 Reference item: 4000 mL/ha.
- Environmental conditions:**
Temperature: 17.0 – 21.0 °C
pH of soil: 4.9
Water content of soil: max 32.5 %
Photoperiod: 16 h light: 8 h dark; light intensity: 310 - 990 lux

B. STUDY DESIGN AND METHODS

- In-life phase:** 27 Sept. – 11 Dec. 2017
- Test organism assignment and treatment**

The beetles were exposed to the presence of Final Bite - 0402206 pellets (i.e. 1, 2, 5, 7 or 14 individual pellets) on natural soil (LUFA 2.1). On the day of treatments, 10 pairs of beetles were transferred to each prepared test unit. The control group was left untreated and the reference item was sprayed via laboratory spray applicator on the soil surface before introduction of the beetles. Exposure of the beetles in the reference item group (dimethoate) was reached via treated natural soil LUFA 2.1.

Each replicate contained 10 female and 10 male beetles; 4 replicates per treatment. The beetles were exposed to untreated control soil, test item pellets and spray residues of the reference item for 28 days. On days 7, 14 and 21 *ca.* 500 pupae of *Delia antiqua* were buried into the soil of each replicate to be parasitized by the larvae of the beetles. On day 28 the adults were separated from the soil and the soil with the pupae was allowed to dry for seven days. On day 35 the pupae were sieved out of the natural soil and transferred into an emergence container. The emergence of the F1-generation of beetles was observed from day 37 - 75.

3. Dose preparation

The appropriate amounts of pellets per test item treatment group were added to the soil surface of the units (*i.e.* 1, 2, 5, 7 or 14 individual pellets). Pellets were roughly equidistant from each other and were not within 1 cm of the wall of the exposure unit. Afterwards the beetles were transferred to the already prepared test units. The applied amount in this study was: 8, 16, 32, 48 and 100 kg product/ha; corresponding to 1, 2, 5, 7 and 14 pellets per test unit (190 cm²), respectively; corresponding to 53, 105, 263, 368 and 737 pellets/m², respectively (as calculated by HSE using the number of pellets per arena and the arena area of 190 cm²).

4. Measurements and observations

The reproduction efficiency was assessed by counting the total number of beetles emerged from the fly pupae until the emerging of the F1-generation was finished. Emerging beetles were counted and removed from the emergence containers at least 3 times per week; emergence of the F1-generation was monitored until the control treatment fell below a rate of two beetles per replicate per day.

5. Statistics

Reproduction data were tested for normal distribution and homogeneity of variance using Shapiro-Wilk's test ($\alpha = 0.05$) and Levene's test ($\alpha = 0.05$). Because reproduction data were normally distributed and homogenous, Dunnett's multiple t-test (test item group) or Student pairwise t-test (reference item group), one-sided smaller, $\alpha = 0.05$, was used. The determination of the NOER was based on the results of the statistical evaluation. As effect on reproduction in all test item treatment groups was < 50 %, the LR₅₀ can be considered as > the highest tested rate (100 kg product/ha).

The software used to perform the statistical analysis was ToxRat Professional, Version 3.2.1, © ToxRat Solutions GmbH.

II. RESULTS AND DISCUSSION

A. Mortality and abnormal responses of beetles

There were no effects on reproduction in any of the test item groups compared to the control. The numbers of emerged beetles in each group are summarised in the table below.

The reference item had a significant effect on the reproduction of beetles with a 99.2% reduction in beetles emerging compared to the control.

Table B.9.5.2-1: Effects on reproduction of staphylinid beetles (*Aleochara bilineata*) exposed to Final Bite - 0402206 in an extended laboratory trial

	Rate ¹	Reproduction Efficiency [mean number of emerged beetles ± Standard Deviation]	Effect on Reproduction ² [%]
Final Bite - 0402206	8 kg/ha (1 pellet per test unit) (53 pellets/m ²)	742 ± 37 (n.s.)	- 2.5
	16 kg/ha (2 pellets per test unit) (105 pellets/m ²)	675 ± 73 (n.s.)	+ 6.8
	32 kg/ha (5 pellets per test unit) (263 pellets/m ²)	745 ± 51 (n.s.)	- 3.0
	48 kg/ha (7 pellets per test unit) (368 pellets/m ²)	705 ± 84 (n.s.)	+ 2.6
	100 kg/ha (14 pellets per test unit) (737 pellets/m ²)	700 ± 62 (n.s.)	+ 3.3
NOER Test Item	≥ 100 kg product/ha (14 pellets per test unit) (737 pellets/m²)	ER₅₀	> 100 kg product/ha (14 pellets per test unit) (737 pellets/m²)
Control	-	724 ± 61	-
Reference Item	4.0 L/ha	6 ± 5 (*)	+ 99.2

¹ Test item was applied as pellets per test unit and added on the soil surface of the test units; Reference item was applied in 400 L water/ha;

² Effect on reproduction according to the following formula: $(1 - R_t/R_c) \times 100\%$ calculated on the exact raw data (negative values represent an increased, positive values a decreased reproduction compared to the control)
Statistic: * = statistically significantly difference compared to the control; n.s. = not statistically significantly difference compared to the control;

Test Item: Dunnett's multiple t-test; Reference Item: Student pairwise t-test, one-sided smaller, $\alpha = 0.05$

NOER: based on Dunnett's multiple t-test.

B. Analytical verification

None conducted.

C. Validity criteria

The study met the validity criteria according to Grimm *et al.* (2000).

Mean Number of Emerged Beetles in the Control Group: 724 beetles, i.e. above validity criterion of 400 minimum.

Reduction of Reproduction in the Reference Item compared to the Control: 99.2 %, i.e. above the validity criterion of 50% minimum.

III. CONCLUSION

No effects on the reproduction capacity of the rove beetle *Aleochara bilineata* were observed after exposure of the beetles to the presence of 8, 16, 32, 48 and 100 kg Final Bite - 0402206/ha, corresponding to 1, 2, 5, 7 and 14 pellets per test unit (190 cm²), respectively, corresponding to 53, 105, 263, 368 and 737 pellets/m², respectively. The reduction of reproduction capacity of the rove beetle *Aleochara bilineata* exposed to Final Bite - 0402206 at all test item rates was below 7 %.

The ER₅₀ was estimated to be > 100 kg product/ha (737 pellets/m²). The NOER (no observed effect rate) for mortality was 100 kg product/ha (737 pellets/m²).

HSE Comments:

It is noted that no batch number is provided for the iron used in the formulation, however, in light of updated consideration in the volume 4, it is concluded that the tested material is representative of the technical specification for elemental iron.

The study was carried out largely in accordance with the agreed guideline and in compliance with GLP. However, it is noted that the granules were left whole and placed on the surface of the test soil. The test guideline states that

'If the test item is a granule..., it is incorporated directly into the substrate...'. The test item was not incorporated, therefore the impact of this should be considered further at risk assessment in the context of all other toxicity data. Furthermore, as the granules were broadcast onto the surface of the soil, it is not considered appropriate to report the endpoints only as 'kg product/ha'; therefore, HSE calculated the endpoints in terms of 'pellets/m²'.

The reference item was applied as a spray to the surface so the reference only indicates that the cohort of test species is suitably sensitive, rather than the test set up is appropriate for testing the toxicity of the product. This should be considered further in the context of the risk assessment.

There were no effects observed, therefore the endpoint for consideration in regulatory risk assessment is **NOER = 100 kg product/ha (737 pellets/m²), noting the concerns raised above.**

Report:	CP 10.3.2.2/02. [REDACTED] 2018
Title:	Final Bite - 0402206: Effects on the Carabid beetle <i>Poecilus cupreus</i> L. - Extended Laboratory Study --Dose Response Study
Report no.:	127511007
Guidelines:	<ul style="list-style-type: none"> - Auswirkungen von Pflanzenschutzmitteln auf Imagines von <i>Poecilus cupreus</i> L. als Vertreter der Familie Carabidae (= Laufkäfer) im Laboratorium, (Heimbach 1991) - A method for testing effects of plant protection products on the carabid beetle <i>Poecilus cupreus</i> (Coleoptera, Carabidae) under laboratory and semi-field conditions, Heimbach U., Dohmen P., Barrett K.L., Brown K., Kennedy P.J., Kleiner R., Römbke J., Schmitzer S., Schmuck R., Ufer A., Wilhelmy H. (2000).
GLP:	Yes, certified
Published:	No

I. MATERIALS AND METHODS

A. MATERIALS:

- Test material:** Final Bite - 0402206
Batch no.: KM80174200H
Purity: 10 g/kg elemental iron (nominal); 1.21% w/w (analysed)
Description: Solid blue pellets
- Reference material:** BAS 152 11 I
Ref. concentration: dimethoate: 400.0 g/L (nominal), 405.2 g/L (analytical) According to certificate of analysis.

Tested: 1200 mL/ha BAS 152 11 I in 400 L deionised water/ha (corresponding to 3.00 mL (3.22 g) BAS 152 11 I/L).

- Vehicle:** None
- Test substrate:** LUFA 2.1; 250 ± 1 g dry soil - The substrate surface was about 175 cm² and the height of the moistened soil was approximately 1 cm.
Test units: Plastic boxes (18.3 cm x 13.6 cm x 6 cm; length, width, height). The substrate surface was about 175 cm² and the height of the moistened soil was approximately 1 cm.
- Test organisms:** Carabid beetle
Species: *Poecilus cupreus* L.
Age: about 5-6 weeks old
Source: Bio-Test Labor GmbH, Sagerheide, Germany
Diet: Punctured and frozen fly pupae (*Calliphora spec.*) at day 0 before application) and at days 2, 4, 7 and 10 after application, at a rate of 1 pupa per viable beetle. First feeding was done shortly before application
- Treatment:** 8, 16, 32, 48 and 100 kg product/ha; corresponding to 1, 2, 4, 6 and 13 pellets per test unit (175 cm²); corresponding to 57, 114, 229, 343 and 743 pellets/m² (as calculated by HSE using test arena area of 175 cm²).
- Environmental conditions:**
Temperature: 18-21 °C
pH of soil: 4.9

-
- Relative humidity:** 64 % - 79 %;
Photoperiod: 16 h light : 8 h dark; light intensity: 860 - 1100 lux
- B. STUDY DESIGN AND METHODS**
- 1. In-life phase:** 06 Nov. – 20 Nov. 2017
- 2. Test organism assignment and treatment**

For acclimatisation, beetles were kept in the laboratory at room temperature for 10 days and then 3 days before test start they were put under test conditions in the prepared test units. At this point, the individuals for use in the test were chosen.

Five replicates per treatment group with 6 individuals per unit (3 females and 3 males per unit, 30 individuals per treatment group) were transferred to the test units. On the day of application, the beetles were temporarily removed, then the pellets were introduced to the test units and then the beetles were re-introduced to the test units (with pellets) and exposure was started.

For the reference item group, a single application was made with laboratory-spraying equipment. The control units were applied with deionised water.

3. Dose preparation

The appropriate number of pellets per test item treatment group was added to the soil surface of the units (i.e. 1, 2, 4, 6 or 13). Pellets were roughly equidistant from each other and were not within 1 cm of the wall of the exposure unit.

4. Measurements and observations

The number of living and dead beetles was counted *ca.* 2 hours after application and 1, 2, 4, 7, 10 and 14 days after the application; at the last assessment the soil was searched for missing beetles. Damaged beetles were placed at one corner of the trays and were counted as dead, if they were still there 24 h later (only reference item).

To measure the food consumption, the number of fly pupae consumed or untouched were counted at days 2, 4, 7, 10 and 14 after application; missing pupae were assumed consumed; at the last assessment the soil was searched for remaining untouched food.

Further sublethal symptoms were noted by the number of damaged beetles (*e.g.* behavioural abnormalities, uncoordinated movements, lying on the back) counted *ca.* 2 hours and 1, 2, 4, 7, 10 and 14 days after application.

5. Statistics

Mortality: Test item: since no mortality occurred in the test item group, no statistical analyses were performed for these parameters; Ref. Item: Fisher's Exact Test, $\alpha = 0.05$; one-sided greater; NOER: Fisher's Exact Test, $\alpha = 0.05$; one-sided greater;

Food consumption: Dunnett's (Test Item) or Student t-test (Reference Item); $\alpha = 0.05$; one-sided smaller. The software used to perform the statistical analysis was ToxRat Professional, Version 3.2.1, © ToxRat Solutions GmbH.

II. RESULTS AND DISCUSSION

A. Mortality and abnormal responses of beetles

No test item related mortalities or behavioural abnormalities occurred at any time for the duration of the experiment. In the reference item tested, 20 of the 30 beetles (66.7 %) died during the experiment (statistically significantly different to the control, Fisher Exact Test; $\alpha=0.05$; one-sided greater).

A similar food consumption was found in all Final Bite - 0402206/ha treatment groups, when compared to the control group. There was no statistically significant difference compared to the control according to Dunnett's t test ($\alpha = 0.05$; multiple comparison, one-sided smaller).

Table B.9.5.2-2: Effects on the mortality and food consumption of *Poecilus cupreus* in an extended laboratory toxicity test

	Rate	¹⁾ Mortality [%]	²⁾ corrected Mortality [%]	³⁾ Pupae consumed per beetle ⁴⁾	Food consumption ⁵⁾ [%]
Final Bite - 0402206	8 kg/ha (1 pellet per test unit) (57 pellets/m ²)	0.0	0.0	2.9 (n.s.)	98 %
	16 kg/ha (2 pellets per test unit) (114 pellets/m ²)	0.0	0.0	3.2 (n.s.)	106 %
	32 kg/ha (4 pellets per test unit) (229 pellets/m ²)	0.0	0.0	3.3 (n.s.)	109 %
	48 kg/ha (6 pellets per test unit) (343 pellets/m ²)	0.0	0.0	3.0 (n.s.)	99 %
	100 kg/ha (13 pellets per test unit) (743 pellets/m ²)	0.0	0.0	2.9 (n.s.)	96 %
NOER	100 kg product/ha (13 pellets per test unit; 743 pellets/m²)			LR₅₀: > 100 kg product/ha (> 743 pellets/m²)	
Control	--	0.0	--	3.0	100 %
Reference Item	1.2 L	66.7 (*)	66.7	1.8 (*)	58 %

¹⁾ Test item was applied as pellets per test unit and added on the soil surface of the test units; Reference item was applied in 400 L water/ha;

²⁾ Mortality: mean of 5 replicates with 6 beetles/replicates, 30 beetles on total

³⁾ corrected mortality according to Abbott, 1925

⁴⁾ rounded values

⁵⁾ Food consumption as a percentage of food consumed in the control group.

n.s. = not statistically significant difference compared to the control; (*) = statistically significant difference compared to the control

B. Analytical verification

No analytical verification was performed.

C. Validity criteria

The study met the validity criteria according to Grimm et al. (2000):

Control Mortality:	0.0 %, i.e. less than maximum of 2 beetles for a 2 week test stated in the validity criteria
Reference Item Mortality:	66.7 % corrected mortality, i.e. above the minimum of 65 ± 35 % mortality stated in the validity criteria

III. CONCLUSION

No effects on mortality, food consumption or behaviour of the ground dwelling predator *Poecilus cupreus* were observed after exposure of the beetles to the presence of 8, 16, 32, 48 and 100 kg Final Bite - 0402206/ha, corresponding to 1, 2, 4, 6 and 13 pellets per test unit (175 cm²), corresponding to 57, 114, 229, 343 and 743 pellets/m² (as calculated by HSE using test arena area of 175 cm²).

The LR₅₀ was concluded to be > 100 kg product/ha (> 743 pellets/m²).

The NOER (no observed effect rate) for mortality was concluded to be 100 kg product/ha (743 pellets/m²).

HSE Comments:

It is noted that no batch number is provided for the iron used in the formulation, however, in light of updated consideration in the volume 4, it is concluded that the tested material is representative of the technical specification for elemental iron.

The study was carried out in accordance with the agreed guideline and in compliance with GLP; and in particular the granules were left whole and placed on the surface of the test soil. Whilst this followed the guideline it does introduce some uncertainty as to whether the test organisms were adequately exposed to the active substance. This will be considered further in the risk assessment.

As the granules were broadcast onto the surface of the soil, it is not considered appropriate to report the endpoints only as 'kg product/ha'; therefore, HSE calculated the endpoints in terms of 'pellets/m²'.

The reference item was applied as a spray to the surface so the reference only indicates that the cohort of test species is suitably sensitive, rather than the test set up is appropriate for testing the toxicity of the product. This should be considered further in the context of the risk assessment.

There were no effects observed, therefore the endpoint for consideration in regulatory risk assessment is **NOER = 100 kg product/ha (743 pellets/m²), noting the concerns raised above.**

Report:	CP 10.3.2.2/03. [REDACTED] 2018
Title:	Final Bite - 0402206: An extended laboratory test to determine effects on spiders of the genus <i>Pardosa</i> (Araneae, Lycosidae) when exposed to granules applied to a natural soil substrate
Report no.:	MAK-18-3 (R-39833)
Guidelines:	Heimbach et al. (2000)
GLP:	Yes, certified
Published:	No

MATERIALS AND METHODS

A. MATERIALS:

- Test material:** Final Bite - 0402206
Batch no.: KM80174200H
Purity: 10 g/kg elemental iron (nominal), 1.21% w/w (measured)
Description: Solid blue pellets
- Reference material:** BAS 152 11 I
Ref. concentration: dimethoate: 400.0 g/L (nominal), 429 g/L (analytical)
 Tested: 1500 mL/ha BAS 152 11 I in 400 L deionised water/ha
 (corresponding to 1.875 mL BAS 152 11 I/L)
- Vehicle:** None
- Test substrate:** LUFA 2.1; organic matter content (0.68 ± 0.07%)
Test units: Plastic pots (46 mm tall, internal diameter of 84 mm). Total surface area of test soil: approximately 55.42 cm²
- Test organisms:**
Species: *Pardosa* (Lycosidae)
Age: adult/sub-adult spiders
Source: Collected from untreated grassy meadow

Diet: provided with (3rd/4th-instar) pea aphids (*Acyrtosiphon pisum* (Harris)) for food reared on dwarf broad beans (*Vicia faba* var. The Sutton)

6. Treatment: 24.2, 48.4, 96.7 kg product/ha; corresponding to 1, 2 or 4 pellets per test unit; corresponding to 180, 361 and 722 pellets/m² (calculated by HSE based on test arena area of 55.42 cm²).

7. Test Environmental conditions:

Temperature: 20.4-21.4°C

Relative humidity: 71-77%

30 ± 5% of its WHC (5 days before achieved application)

50 ± 5% of the WHC (3 days before application)

Photoperiod: 16 h light : 8 h dark; light intensity: 500 - 1100 lux

B. STUDY DESIGN AND METHODS

1. In-life phase:

2. Test organism assignment and treatment

Adult spiders of the genus *Pardosa* (Araneae, Lycosidae) were collected from an untreated grassy meadow at Chilworth Manor, Southampton, UK (OS Map ref. SU403185). They were collected by hand and stored individually in plastic pots. The spiders were collected on the 26 April, 8 May and 9 May 2018. They were stored in plastic pots in a controlled environment maintained at 13.8-16.6°C, under a 16 h photoperiod of 600-900 lux with drinking water and provided aphids for food.

Prior to treatment application, the moisture content of the soil was determined by placing a 100 g sample in an oven set at 80°C and then after 5 hours re-weighing the sample to determine the amount of water lost. The maximum water-holding capacity (WHC) of the soil was also determined by placing 100 g of the undried soil into a glass funnel, the stem of which was first blocked with a small piece of damp cotton wool. The weight of the funnel, soil and cotton wool bung was recorded before water was slowly added until the soil was fully saturated. The obtained WHC was not reported. The apparatus was then left to stand so that any excess water could drain away. The funnel, wet soil and cotton wool bung were reweighed and the gain in weight, together with the amount of water initially present in the 100 g of undried soil, was taken to be equivalent to the 100% water-holding capacity of the soil.

There were thirty-four replicate arenas per treatment, each containing one spider. There were equal numbers of female and male spiders in each treatment.

Four days before application, the spiders were weighed to confirm that they are within the range 12-35 mg. Any that are outside of this range were discarded. Prior to being weighed, any egg sacs being carried by the females were removed.

Three days prior to the application of treatments, the spiders were placed into holding pots (plastic pots identical to the test arenas, but without soil) with drinking water and transferred to the test room, maintained at 20.4-21.4°C and 71-77% RH, under a 16 h photoperiod of 500-1100 lux for pre-conditioning. The spiders were deprived of food from this time until the start of the test. The ventilated lids were fitted to the pots.

3. Dose preparation

For the three test-item treatment rates, intact pellets from the sample provided were counted out in batches of either 34, 68 or 136 pellets. These were then weighed to confirm that they have a mean weight of 13.4 ± 0.75 mg/pellet.

For each test-item treatment, the appropriate number of pellets were added to the surface of the soil in each pot, being distributed in an even pattern where appropriate. The spiders were then introduced, and the ventilated lids were fitted to the pots.

The reference item was sprayed on to the surface of the test soil.

4. Measurements and observations

The condition of the spiders were assessed approximately 1-3 h after they are introduced to the treatment residues and then 1, 2, 3, 4, 7, 10 and 14 DAT. The symptoms displayed by 'affected' spiders were categorised and noted. Any spiders that escaped from their arenas and were not returned to the test vessel but were recorded as escapees and were eliminated from the data analysis.

To assess any changes in the feeding activity of the spiders, five food items (i.e. pea aphids) were placed in each arena approximately 1-3 h after test initiation and again after 1, 2, 3, 7 and 10 days. In each case, an assessment of the numbers of items consumed were made after approximately 24 h (i.e. at 1, 2, 3, 4, 8 and 11 DAT) and any remains (live or dead aphids) were removed.

Since the feeding activity of spiders may be reduced in the period prior to moulting, and their sensitivity to effects of plant protection products may be increased in the period after moulting, the presence of any moulted skins in the pots was recorded at the same time that any of the other assessments were being made. The skins were then removed. In addition, the development of egg sacs by females was recorded.

If, within the test item treatment, three or more spiders (having adjusted this value for any control treatment deaths using Abbott's formula (Abbott, 1925)) had died within the second week of the test, and this was the same or more as had died in the first week, the bioassay was to be extended for a further week to allow detection of any slow-acting treatment effects. Similarly, if feeding activity in the test item treatment was reduced by > 50%, relative to the control during the second week of the bioassay, the test would have been extended for a further week. These criteria for extending the test were not met.

5. Statistics

All values presented throughout this report were calculated using the original raw data and were not based on rounded values, as presented in summary tables. Statistical analyses were performed using validated computer software (SPSS, 2016).

The results have been expressed in terms of:

- i) percentage mortality of spiders at 14 DAT, both before and after correction of the data for any control losses using Abbott's formula (Abbott, 1925).
- ii) the mean number of food items consumed per spider per 24-h assessment period and the percentage difference in numbers taken when compared to the control treatment.

The mortality in each treatment after 14 days was compared to that in the control using Fisher's Exact Test ($\alpha = 0.05$) (Sokal and Rohlf, 1981). The *median lethal rate* (LR50) of the test item was also estimated by simple extrapolation from the results.

The feeding activity of spiders in the individual test-item treatments was compared to that seen in the control. Analysis was made on a date-by-date basis by Mann-Whitney *U*-test ($\alpha = 0.05$) (Fowler *et al.*, 1998).

II. RESULTS AND DISCUSSION

A. Mortality and abnormal responses of spiders

The percentage mortality of spiders after 14 days was 5.9% in the control treatment, compared with 2.9%, 2.9% and 0.0% in the 24.2, 48.4 and 96.7 kg product/ha (180, 361 and 722 pellets/m²) application rates of Final Bite®, respectively. The results for the three treatment rates did not differ significantly from the control (Fisher's Exact Test, $\alpha = 0.05$), indicating that, in terms of spider survival, the NOER was 96.7 kg product/ha (722 pellets/m²). In the toxic reference treatment 94.1% mortality (93.8% corrected mortality) was observed.

Table B.9.5.2-3: Summary of the percentage mortality of spiders (n = 34 per treatment) at 14 DAT

Treatment	Test Item Rate (kg product/ha)/ (pellets/m ²)	% Mortality ^{a)}	Corrected Mortality ^{b)} [%]
Control	-	5.9	-
Final Bite	24.2/180	2.9	-3.1
	48.4/361	2.9	-3.1
	96.7/722	0.0	-6.3
Reference Item ^{c)}	-	94.1*	93.8

a) Mortality in individual treatments was compared to that in the control using Fisher's Exact Test ($\alpha = 0.05$). An asterisk indicates where differences were significant.

b) Calculated using Abbott's formula. A positive value indicates an increase and a negative value indicates a decrease in mortality, relative to the control.

c) Dimethoate (EC formulation containing nominally 400 g a.s./L, applied at a rate of 1.5 L prod./ha).

Statistically, feeding activity was significantly reduced in the Final Bite treatments, relative to the control, on a few occasions (during the six assessment dates). For the 96.7 kg product/ha treatment (722 pellets/m²), this was at 3 DAT, whilst for the 48.4 kg product/ha treatment (361 pellets/m²), it was at 3 and 11 DAT (Mann-Whitney *U*-test, $\alpha = 0.05$). For the 24.2 kg product/ha treatment (180 pellets/m²), there were no significant differences. Any significant reductions were not consistent or dose related. The overall percentage change in feeding activity in each treatment, relative to the control, was < 10%. Also, for some assessment dates the feeding was greater in these two test item treatments than in the control treatment (at DAT 11 for the 96.7 kg product/ha treatment (722 pellets/m²) and at DAT 4 for the 48.4 kg product/ha (361 pellets/m²) treatment). Furthermore, the feeding activity varied across the course of the study and the test item groups did not exceed this range. Therefore, it was considered that any significant differences were outliers and so the NOER with respect to feeding activity should be considered 96.7 kg product/ha (722 pellets/m²).

Table B.9.5.2-4: The mean numbers of pea aphids eaten per living spider over six 24-h assessment periods. Five aphids were provided per spider on each occasion.

Treatment	Test Item Rate (kg product/ha)/ (pellets/m ²)	Day 1	Day 2	Day 3	Day 4	Day 8	Day 11	Mean	% Change ^{a)}
Control	-	3.4	2.8	3.1	2.1	3.6	4.3	3.2	-
Final Bite	24.2/180	3.4	2.8	2.5	2.4	4.1	4	3.2	0.4
	48.4/361	3.4	2	2.2*	2.9	3.4	3.7*	2.9	9.5
	96.7/722	3.0	2.5	2.5*	2.1	3.4	4.4	3	7.2
Reference Item ^{b)}	-	3.3	4.5	3.5	4	4.5	4.5	4.1	-25.9

* For each assessment date, pair-wise comparisons were made of data from each treatment and the control using Mann-Whitney *U*-tests ($\alpha = 0.05$). An asterisk indicates daily treatment means that differed significantly from the control.

a) Change in feeding activity relative to the control. A positive value indicates a decrease and a negative value indicates an increase.

b) Dimethoate (EC formulation containing nominally 400 g a.s./L, applied at a rate of 1.5 L prod./ha.).

B. Analytical verification

No analysis is required for this test.

C. Validity criteria

According to the guideline of Heimbach *et al.* (2000), for a bioassay to be considered valid, control mortality should not exceed 9% (i.e. 3 spiders from 34) during the initial 14 days or 15% (i.e. 5 spiders from 34) if the test is extended to 21 days. Both criteria were met.

The reference-item treatment should result in 65 ± 35 % corrected mortality by 14 days after treatment; this was met at 93.8 % corrected mortality.

III. CONCLUSION

Under extended laboratory test conditions, the 14-day LR₅₀ for Final Bite to spiders of the genus *Pardosa* (Araneae, Lycosidae) was estimated to be > 96.7 kg product/ha (>722 pellets/m²). The NOER with respect to both spider survival and side-effects on their feeding activity was 96.7 kg product/ha (722 pellets/m²).

HSE Comments:

It is noted that no batch number is provided for the iron used in the formulation, however, in light of updated consideration in the volume 4, it is concluded that the tested material is representative of the technical specification for elemental iron.

The study was carried out in accordance with the agreed guideline and in compliance with GLP. However, it is noted that the granules were left whole and placed on the surface of the test soil and whilst in line with the guideline does not necessarily represent worst case exposure to the active substance. This will be considered further in the risk assessment.

As the granules were broadcast onto the surface of the soil, it is not considered appropriate to report the endpoints only as 'kg product/ha'; therefore, HSE calculated the endpoints in terms of 'pellets/m²'.

The reference item was applied as a spray to the surface so the reference only indicates that the specimens of the test species were suitably sensitive, rather than the test set up is appropriate for testing the toxicity of the product. This should be considered further in the context of the risk assessment.

The spiders were collected in late April and early May, this should be considered when using the endpoints in regulatory risk assessment as per the guideline.

There were no effects observed, therefore the endpoint for consideration in regulatory risk assessment is **NOER = 96.7 kg product/ha (722 pellets/m²), noting the concerns raised above.**

B.9.6. RISK ASSESSMENT FOR ARTHROPODS

B.9.6.1. Risk Assessment for Bees

No toxicity data was submitted for the active substance or the representative formulation. Regulation EU No 283-2013 specifies that toxicity data is required when bees are likely to be exposed. The representative product is formulated as a ready to use granular bait applied to the ground to reach the target species, and is therefore not available for bees foraging on flowers. The routes of exposure to bees are therefore limited to dust drift and potentially contaminated pollen/nectar via systemic absorption of the active substance by flowering plants.

In the case of dust drift there is no guidance currently available to assess the risk from this route of exposure. There is also evidence that the risk of dust-drift exposure will be low - as shown in Vol 3CP 2.8.5/01, the representative product was found to be almost dust-free (optical dust factor 1.33).

Plants have been shown to be capable of regulating the uptake of iron from the soil¹⁵ which has high background levels of iron. The background levels are unlikely to be significantly increased by application of the representative formulation. UK Fate and Behaviour evaluators concluded background levels ranging from 2000-50000 mg iron/kg soil, and a maximum PEC_{soil} from the proposed uses of elemental iron to be 12.8 mg/kg. Pollen is also highly nutrient dense with levels of iron as high as 2872.89 mg/kg found in *Helianthus annuus* (common sunflower)¹⁶; the same study showed that unifloral bee pollen from the same plant species contained only 2742 mg iron/kg, a reduction which was thought to be due to dilution of the flower pollen by honeybee saliva. Honeybee pollen collected from European countries can contain up to 136.1 mg iron/kg pollen (See footnote 3). It is considered that the use of elemental iron according to the proposed GAP will not significantly increase the level of iron normally available to bees.

Overall, the exposure of bees to iron from the proposed uses of the representative formulation is considered to be negligible and no toxicity studies are required. An acceptable risk to bees is concluded.

B.9.6.2. Risk assessment for non-target arthropods other than bees

Three toxicity studies on soil-dwelling non-target arthropod species were submitted. The toxicity endpoints are summarised in Table B.9.6.2-1.

Table B.9.6.2-1: Summary of endpoints for non-target arthropods

Test species – Life stage	Test substance	Time scale (test type)	Endpoint (kg product/ha)	Endpoint (pellets/m ²) ⁴	Data point Author, year
<i>Aleochara bilineata</i>	Final Bite - 0402206	75 days Laboratory test ¹⁾	Reproduction: ER ₅₀ > 100 kg product/ha NOER = 100 kg product/ha (1210 g a.s./ha) ³	Reproduction: ER ₅₀ > 737 pellets/m ² NOER = 737 pellets/m ²	CP 10.3.2.2/01. [REDACTED] 2018
<i>Poecilus cupreus</i>	Final Bite - 0402206	14 days Laboratory test	Survival & feeding: L/ER ₅₀ > 100 kg product/ha NOER = 100 kg product/ha (1210 g a.s./ha) ³	Survival & feeding: L/ER ₅₀ > 743 pellets/m ² NOER = 743 pellets/m ²	CP 10.3.2.2/02. [REDACTED] 2018

¹⁵ Connorton, J., J. Balk and J. Rodriguez-Celma. 2017. 'Iron homeostasis in plants – a brief overview'. *Metallomics* 9, 813-823.

¹⁶ Stanciu, O., L. Marghitas, D. Dezmirean and M. Campos. 2011. 'A comparison between the mineral content of flower and honeybee collected pollen of selected plant origin (*Helianthus annuus* L. and *Salix* sp.)'. *Romanian Biotechnological Letters* 16, No. 4, 6291-6296.

Test species – Life stage	Test substance	Time scale (test type)	Endpoint product/ha (kg)	Endpoint (pellets/m ²) ⁴	Data point Author, year
<i>Pardosa</i> sp.	Final Bite - 0402206	14 days Laboratory test ²⁾	Survival & feeding: L/ER ₅₀ > 96.7 kg product/ha NOER = 96.7 kg product/ha (1170.07 g a.s./ha) ³	Survival & feeding: L/ER ₅₀ > 722 pellets m ² NOER = 722 pellets m ²	CP 10.3.2.2/03. [REDACTED] 2018

¹⁾ Test item applied whole to the surface of the test area, rather than mixed in.

²⁾ Test specimens collected in late April and early May.

³⁾ Toxicity endpoint converted to active substance using tested weight for weight percentage content of the test item, i.e. 1.21 %.

⁴⁾ Toxicity endpoints calculated based on reported arena area and number of pellets applied to each test arena.

Some uncertainties were identified during the study evaluation phase, namely:

- The reference item used in the toxicity studies was sprayed onto the test arenas, therefore the reference only indicates that the specimens of the test species were suitably sensitive, rather than the test set up is appropriate for testing the toxicity of the product. This issue has been considered above when discussing the fact that the pellets were broadcast over the soil surface.
- The spiders for use in the *Pardosa* toxicity test were collected in late April/early May. The testing guideline states:

‘if the intended application timing for the plant protection products is both in spring/summer and in autumn, the test should be performed using the more sensitive spring-collected spider (i.e. over-wintered animals).’

The proposed uses are all through the year, therefore the spiders used in the test were the most appropriate animals for the proposed uses.

The standard risk assessment scheme for non target arthropods as proposed under ESCORT II guidance covers the off-field and in-field risk from use of the product. The representative product is formulated as a ready to use granular bait applied to the ground to reach the target species, therefore the potential exposure to standard foliar dwelling species is considered negligible and no data is provided for *T. pyri* and *A. rhopalosiphi*. The risk to the off-field environment is considered to be limited to dust drift. There is no guidance currently available to assess the risk from this route of exposure. There is also evidence that the risk of dust-drift exposure will be low - as shown in Vol 3CP 2.8.5/01, the representative product was found to be almost dust-free (optical dust factor 1.33). Overall, the off-field exposure is considered to be negligible and the risk assessment will focus on the in-field environment, in particular soil-dwelling species.

Elemental iron is a naturally occurring element in soil (UK Fate and Behaviour specialists estimate background levels of 2000-50000 mg/kg soil), indicating that the element is present in all soils. The calculated PEC_{soil, accumulation} is 12.8 mg a.s./kg based on the proposed use of the product. This PEC, resulting from the worst case use of the product is significantly lower than even the lowest background level; therefore, no quantitative risk assessment for exposure to the active substance has been carried out.

Soil organisms will be exposed directly to the product, ‘Final Bite’, which contains iron and other co-formulants. The toxicity endpoints have been determined in terms of the test item, i.e. the product. Three sub-lethal toxicity studies were submitted for non-target arthropods other than bees.

A worst case in-field exposure rate was calculated assuming all applications are made at once, i.e. MAF = 6. Iron does not degrade in the same way that a standard organic chemistry compound would, therefore the standard MAFs, which take account of degradation, are not applicable for this product. The resulting exposure rate is summarised in Table B.9.6.2-2.

Table B.9.6.2-2: In-field PER values for ‘Final Bite’

Test substance	Max application rate single	No. of applications	MAF	In-field PER
‘Final Bite’	60 pellets/m ²	6	6.0	360 pellets/m ²

The measured ER50 values gathered for NTA are over double the maximum possible number of pellets/m² following six consecutive applications, which makes the conservative assumption that no pellets degrade or are consumed between applications. Therefore the risk to ground-based non-target arthropods from the intact pellet is considered acceptable.

Arthropods are not considered likely to directly consume the intact pellet (it being too large at 2.5 mm). Another potential route of exposure is from ‘hotspots’ of the product in the immediate vicinity of the partially broken-down pellet. There is also the possibility of consuming prey contaminated with the product. Without a dedicated granule-based toxic reference item, the ability of the test systems used in this application to demonstrate the toxicity of the representative product is uncertain. The endpoints are therefore best used in the risk assessment in terms of pellets/m², which is considered to cover the risk from the intact pellet. It is uncertain if this covers the risk from ‘hotspots’ of the partially broken down product.

However, data is available with the soil dwelling macroorganisms *H. aculeifer* and *F. candida* (see Section B.9.7, below) where the formulation was ground up and mixed homogeneously into the soil. According to SANCO/10329/2002 rev 2 final (Guidance Document on Terrestrial Ecotoxicology), page 24:

“The standard approach is not appropriate for...plant protection products such as granules, seed treatments and pellets. In these cases it is recommended that studies are conducted with Hypoaspis aculeifer or Folsomia candida as proposed by EPPO (2002a). If deemed appropriate, studies with Aleochara sp. might be conducted, e.g. at tier 2.”

It is therefore possible to follow the SANCO guidance document and use data with *H. aculeifer* and *F. candida* as first-tier surrogates for soil dwelling non-target arthropods.

As demonstrated in section B.9.7 of this document, the risk of the formulated product to the above species when used according to the proposed GAP is low. The TER values for *H. aculeifer* and *F. candida* were 48.48 and 18.04, respectively, exceeding the long term trigger value of 5 for these species with a margin of safety. This margin of safety is considered to mitigate the uncertainty involved with extrapolating between these species and the larger soil dwelling arthropods tested with the intact pellet only.

Overall the risk of ‘hotspots’ of the broken down product to soil-dwelling non-target arthropods is considered addressed and a low risk is concluded.

B.9.7. EFFECTS ON NON-TARGET SOIL MESO- AND MACROFAUNA**B.9.7.1. Earthworms****B.9.7.1.1. Sub-lethal toxicity tests**

Report:	CP 10.4.1.1/01. [REDACTED] 2018a
Title:	Final Bite - 0402206: Effects on Reproduction and Growth of Earthworms <i>Eisenia andrei</i> in Artificial Soil
Report no.:	127511022 (R-37822)
Guidelines:	OECD, Guideline for the testing of chemicals No. 222, Earthworm, Reproduction Test (adopted July 29, 2016) ISO-Guideline 11268-2, Soil quality - Effects of pollutants on earthworms - Part 2: Determination of effects on reproduction of <i>Eisenia fetida</i> / <i>Eisenia andrei</i> , International Organization for Standardization, 2012.
GLP:	Yes, certified
Published:	No

I. MATERIALS AND METHODS**A. MATERIALS:**

1. **Test material:** Final Bite - 0402206
Batch no.: KM8017420OH
Purity: content of a.s.: 1.21% w/w (analysed)
Description: Solid blue pellets
Reference material: Carbendazim
Ref. concentration: 600 g/L SC (600 g/L nominal)
2. **Test organism:** Earthworms (Annelida: Oligochaeta) *Eisenia andrei*
Age: adults - Approximately 7 months, with well-developed clitellum, age range between test individuals not differing by more than 4 weeks
Body weight: 314 mg to 581 mg
Source: Bred under standardised conditions in ibacon laboratories in a breeding medium of cattle manure, peat, sand, calcium carbonate and straw, fed with cattle manure, stored at room temperature
3. **Treatment:** Control, 17.15, 30.87, 55.57, 100.0, 180.0, 324.1, 583.3 and 1050 mg Final Bite - 0402206/kg soil (dry weight of artificial soil) (nominal)
4. **Test vessels:** Plastic boxes (18.3 cm x 13.6 cm x 6 cm, tapered towards the bottom, with a soil surface of approximately 16.5 cm x 11.5 cm = 189.75 cm²) with perforated transparent lids to enable exchange of air, to minimise evaporation from the artificial soil and to prevent the worms from escaping. Each container was filled with 686.9 g of the prepared soil (500 g dry weight plus deionised water). The height of the soil layer in the containers was approximately 5 cm.
Soil: 10 % Sphagnum peat, 20 % kaolin clay, 69.6% fine quartz sand, 0.4 % calcium carbonate
Diet: Finely ground cattle manure was used as food. 5 g/container was scattered on the soil surface at day of application and was moistened with 5 g deionised water; 5 g/container (moistened with 2 g deionised water) was added each week for the first 4 weeks of the experiment, when the food of the previous week had almost been consumed. If the food was not quite fully consumed, the added amount of food was adjusted to replace the visually estimated consumption. Four weeks after application, the food was mixed into the substrate following removal of the adult worms.
5. **Environmental conditions:**
Temperature: 18 °C to 22 °C
pH: 5.6 to 5.7

Moisture content: 37.2% to 39.8% (51.7% to 55.3% of the maximum WHC) (start) – 36.0% to 41.6% (49.9% to 57.7% of the maximum WHC) (end of the test)

Photoperiod: 16 h light : 8 h dark, light intensity: within the range of 400 lux to 800 lux.

B. STUDY DESIGN AND METHODS

1. Experimental dates: Start 17 October 2017; completion 05 February 2018

2. Test organism assignment and treatment

All worms were rinsed with tap water, dried with dry papertowels, weighed individually and randomly assigned to batches of 10 worms. The different batches were sorted into four classes based on the total weight and one batch of each weight class was assigned to each treatment group (two batches for the control) to ensure weights were homogeneous. There were four replicates for the treatment groups and eight for the control group.

The earthworms were placed on the surface of the artificial soil after application.

3. Dose preparation

Final Bite - 0402206 was weighed separately for each concentration using an analytical balance and 20 g fine quartz sand was added to each weighing.

After using a pestle and mortar to reach a homogeneous distribution of the test item within the sand the mixture was added to artificial soil equivalent to 2080 g dry weight.

4. Measurements and observations

Number of dead adult earthworms were counted at day 28 after application (including any missing ones). Number of affected adult earthworms (*e.g.* lack of movement, rigidity) counted at day 28 after application to measure morphological and behavioural abnormalities. Also measured cumulative amount of food added to each test container during the test period and body weights were determined at start (day 0) and 28 days after application. Finally, the number of juveniles were counted on day 56 after application.

5. Statistics

Mortality data were analysed for significance by using the Fisher's Exact Binomial Test (multiple comparison, with Bonferroni Correction, $\alpha = 0.05$, one-sided greater).

The body weight change and reproduction data were tested for normal distribution and homogeneity of variance ($\alpha = 0.05$) using the Shapiro-Wilk's test and the Levene's test, respectively. As the data for body weight changes were normally distributed but heterogeneous, Bonferroni-Welch t-test was used to compare treatment and control values (multiple comparison, two-sided). As data for reproduction were normally distributed and homogeneous, Williams t-test was used to compare treatment and control values (multiple comparison, one-sided smaller for reproduction, $\alpha = 0.05$).

The EC values and their 95% confidence limits for reproduction were calculated by applying Probit-Analysis. The software used to perform the statistical analysis was ToxRat Professional, Version 3.2.1, ToxRat® Solutions GmbH.

II. RESULTS AND DISCUSSION

A. Mortality and abnormal responses of earthworms

Final Bite - 0402206 did not show effects on mortality of the earthworms up to and including the concentration of 180.0 mg test item/kg soil. At the concentration of 324.1 mg test item/kg soil and above mortality was biologically and statistically significantly increased compared to the control group (Fisher's Exact Test, $\alpha = 0.05$, one-sided greater).

The body weight change of the earthworms after 4 weeks exposure to Final Bite - 0402206 were not statistically significantly different compared to the control up to the highest test concentration of 1050 mg test item/kg soil (Bonferroni-Welch t-test, $\alpha = 0.05$, two-sided).

No statistically significant effects on reproduction were observed up to concentration of 100.0 mg test item/kg soil but in the test item concentration of 180.0 mg test item/kg soil and above reproduction was statistically significantly reduced compared to the control group (Williams t-test, $\alpha = 0.05$, one-sided smaller). No behavioural abnormalities were observed in any of the treatment groups and the feeding activity in all the treated groups was comparable to the control up to and including the concentration of 324.1 mg/kg soil. The food intake at the concentration of 583.3 mg/kg soil and higher was reduced.

Table B.9.7.1-1: Mortality, body weight, food consumption and reproduction effects after exposure to ground and homogenously mixed in 'Final Bite'

Final Bite - 0402206 [mg/kg soil dry weight]	Control	17.15	30.87	55.57	100.0	180.0	324.1	583.3	1050
Mortality (day 28) [%]	0	0	0	0	0	2.5	30.0	62.5	85.0
Statistical Significance ¹⁾	-	n.s.	n.s.	n.s.	n.s.	n.s.	*	*	*
Body weight change (day 28) [%]	35.8	36.0	37.4	43.7	37.5	36.9	43.0	32.8	40.3
Statistical Significance ²⁾	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Mean No. of juveniles (day 56)	248	224	238	238	247	207	114	28	7
Statistical Significance ³⁾	-	n.s.	n.s.	n.s.	n.s.	*	*	*	*
Reproduction in [%] of control (day 56)	-	90.1	95.9	95.8	99.3	83.2	45.7	11.3	2.7
Food consumption [g]	25.0	25.0	25.0	25.0	25.0	25.0	23.0	17.5	14.3
Endpoints [mg/kg soil dry weight]									
NOEC (day 28 mortality)	180.0								
LOEC (day 28 mortality)	324.1								
LC Values (mortality) ⁴⁾	LC ₁₀		LC ₂₀			LC ₅₀			
	224.5		295.2			498.8			
95% confidence limits	170.7 – 271.3		239.4 – 345.8			431.7 – 582.5			
NOEC (day 28 weight)	1050								
LOEC (day 28 weight)	>1050								
NOEC (day 56 reproduction)	100.0								
LOEC (day 56 reproduction)	180.0								
EC Values (reproduction) ⁴⁾	EC ₁₀		EC ₂₀			EC ₅₀			
	147.1		188.6			303.7			
95% confidence limits	108.0 – 177.3		150.7 – 218.0			270.5 – 341.0			

The results represent rounded values calculated on the exact raw data.

- = not applicable

n.s. = not significantly different compared to the control

* = significantly different compared to the control

¹⁾ Fisher's Exact Test, $\alpha = 0.05$, one-sided greater

²⁾ Bonferroni-Welch t-test, $\alpha = 0.05$, two-sided

³⁾ Williams t-test, $\alpha = 0.05$, one-sided smaller

⁴⁾ Probit analysis

B. Analytical verification

No analytical verification was conducted.

C. Validity criteria

All validity criteria were met:

Control Mortality:	Control mortality was 0% and so this validity criterion was met.
Reproduction of Control:	The number of juvenile worms per replicate was greater than 30 (186 to 315 individuals) and so this validity criterion was met.
Coefficient of Variation of Reproduction in Control:	Was 15.3% and so this validity criterion was met.

III. CONCLUSION

In an earthworm reproduction and growth study with Final Bite - 0402206 the No Observed Effect Concentration (NOEC) for mortality of the earthworm *Eisenia andrei* was determined to be 180.0 mg test item/kg soil and the Lowest Observed Effect Concentration (LOEC) was determined to be 324.1 mg test item/kg soil. The NOEC for weight changes was determined to be 1050 mg test item/kg soil, the highest tested concentration, and the LOEC

was estimated to be >1050 mg test item/kg soil. The NOEC for reproduction was determined to be 100.0 mg test item/kg soil and the LOEC was determined to be 180.0 mg test item/kg soil.

The EC₁₀ based on reproductive effects was determined to be 147.1 mg test item /kg soil (95% confidence limits of 108.0 to 177.3 mg test item /kg soil), the EC₂₀ was determined to be 188. 6 mg test item/kg soil (95% confidence limits of 150.7 to 218.0 mg test item /kg soil) and the EC₅₀ was determined to be 303.7 mg test item/kg soil (95% confidence limits of 270.5 to 341.0 mg test item /kg soil).

HSE Comments:

It is noted that no batch number is provided for the iron used in the formulation, however, in light of updated consideration in the volume 4, it is concluded that the tested material is representative of the technical specification for elemental iron.

The study was conducted in accordance with the agreed regulatory guideline and in compliance with GLP. There were clear dose responsive effects on mortality and reproduction, with the first effects on reproduction occurring at lower concentrations than mortality. The key endpoints for consideration in regulatory risk assessment are those derived for reproductive effects: EC₁₀ = 147.1 mg test item /kg soil (95% confidence limits of 108.0 to 177.3 mg test item /kg soil); EC₂₀ = 188. 6 mg test item/kg soil (95% confidence limits of 150.7 to 218.0 mg test item /kg soil) and NOEC = 100.0 mg test item/kg soil.

Report:	CP 10.4.1.1/02. [REDACTED] 2018b
Title:	Final Bite - 0402206: Effects on Reproduction and Growth of Earthworms <i>Eisenia andrei</i> in Artificial Soil
Report no.:	127512022 (R-39839)
Guidelines:	OECD, Guideline for the testing of chemicals No. 222, Earthworm, Reproduction Test (adopted July 29, 2016) ISO-Guideline 11268-2, Soil quality - Effects of pollutants on earthworms - Part 2: Determination of effects on reproduction of <i>Eisenia fetida</i> / <i>Eisenia andrei</i> , International Organization for Standardization, 2012.
GLP:	Yes, certified
Published:	No

I. MATERIALS AND METHODS

A. MATERIALS:

1. **Test material:** Final Bite - 0402206
Batch no.: KM80174200H
Purity: content of a.s.: 10 g/kg elemental iron (nominal) 1.21% w/w, according to Certificate of Analysis
Description: Solid blue pellets
Reference material: Carbendazim
Ref. concentration: 600 g/L SC (600 g/L nominal)
2. **Test organism:** Earthworms (Annelida: Oligochaeta) *Eisenia andrei*
Age: adults - Approximately 8 months, with well-developed clitellum, age range between test individuals not differing by more than 4 weeks
Body weight: 308 mg to 598 mg
Source: Bred under standardised conditions in ibacon laboratories in a breeding medium of cattle manure, peat, sand, calcium carbonate and straw, fed with cattle manure, stored at room temperature
3. **Treatment:** Control, 334, 484, 702, 1018, 1476, 2140, 3103 and 4500 mg Final Bite - 0402206/kg soil (nominal), corresponding to 662, 959, 1391, 2017, 2924, 4240, 6148 and 8916 pellets/m², respectively (calculated by HSE based on single pellet weight of 13.3 mg and test arena area of 189.75 m²)

-
- 4. Test vessels:** Plastic boxes (18.3 cm x 13.6 cm x 6 cm, tapered towards the bottom, with a soil surface of approximately 16.5 cm x 11.5 cm = 189.75 cm²) with perforated transparent lids to enable exchange of air, to minimise evaporation from the artificial soil, and to prevent the worms from escaping. Each container was filled with 689.1 g of the prepared soil (500 g dry weight plus deionised water). The height of the soil layer in the containers was approximately 5 cm.
- Soil:** 10 % Sphagnum peat, 20 % kaolin clay, 69.6% fine quartz sand, 0.4 % calcium carbonate
- Diet:** Finely ground cattle manure was used as food. 5 g/container was scattered on the soil surface at day 1 after application and was moistened with 5 g deionised water; 5 g/container (moistened with 2 g deionised water) was added each week for the first 4 weeks of the experiment, when the food of the previous week had almost been consumed. If the food was not quite fully consumed, the added amount of food was adjusted to replace the visually estimated consumption. Four weeks after application, the food was mixed into the substrate following removal of the adult worms.
- 5. Environmental conditions:**
- Temperature:** 18.4 – 20.2 °C
- pH:** 5.8 to 5.9 (start) 5.8 to 6.2 (end)
- Moisture content:** 38.4% to 39.6% (53.3% to 55.0% of the maximum WHC) (start) – 36.3% to 40.8% (50.4% to 56.7% of the maximum WHC (end of the test))
- Photoperiod:** 16 h light : 8 h dark, light intensity: within the range of 400 lux to 800 lux.

B. STUDY DESIGN AND METHODS

- 1. In-life phase:** 04 January – 02 March 2018

2. Test organism assignment and treatment

All worms were rinsed with tap water, dried with dry paper towels, weighed individually and randomly assigned to batches of 10 worms. The different batches were sorted into four classes based on the total weight and one batch of each weight class was assigned to each treatment group (two batches for the control) to ensure weights were homogeneous. There were four replicates for each treatment group and eight replicates for the control group.

The earthworms were placed on the surface of the artificial soil prior to the application. Four weeks after application, the artificial soil was transferred to a tray and adult worms were counted, removed and weighed per replicate after being rinsed under tap water and dried on paper towels. The remaining soil (without the adult worms) was then returned to the respective test containers.

After an additional 28 days, juveniles were removed by placing the test units in a water bath at 50 – 60°C and counting all emerging worms. In addition, the soil of each container was emptied out onto a tray and checked visually for any remaining juvenile worms.

3. Dose preparation

The pellets of Final Bite - 0402206 were spread over the soil surface instead of mixing them into the soil.

The test item was weighed separately for each test container using an analytical balance, the pellets were then scattered on top of the soil surface of each replicate as homogeneously as possible.

The application was performed after earthworms had burrowed into the soil. There were no significant deviations to the nominal target concentration (< 5%). The control was left untreated.

The dose in terms of pellets/m² was calculated by HSE using an individual pellet weight of 13.3 mg (as per other toxicity studies carried out with the same product), the dry weight of soil per container was 0.5 kg and the test area was 189.75 cm².

4. Measurements and observations

Number of dead adult earthworms were counted at day 28 after application (including any missing ones). Number of affected adult earthworms (*e.g.* lack of movement, rigidity) counted at day 28 after application to measure morphological and behavioural abnormalities. Also measured cumulative amount of food added to each test container during the test period and adult body weights were determined at start (day 0) and 28 days after application. Finally, the number of juveniles were counted on day 56 after application.

5. Statistics

Mortality data were analysed for significance by using the Fisher's Exact Binomial Test (multiple comparison, with Bonferroni Correction, $\alpha = 0.05$, one-sided greater).

The body weight change and reproduction data were tested for normal distribution and homogeneity of variance ($\alpha = 0.05$) using the Kolmogorov-Smirnov-test and the Levene's test, respectively. As the data for body weight changes and reproduction were normally distributed but heterogeneous, Bonferroni-Welch t-test was used to compare treatment and control values (multiple comparison, one-sided smaller, $\alpha = 0.05$).

The LC and EC (for reproduction) values and their 95% confidence limits were calculated by applying Probit-Analysis. The software used to perform the statistical analysis was ToxRat Professional, Version 3.2.1, ToxRat® Solutions GmbH.

II. RESULTS AND DISCUSSION

A. Mortality and abnormal responses of earthworms

Final Bite - 0402206 did not show effects on mortality of the earthworms up to and including the concentration of 484 mg test item/kg soil dry weight (959 pellets/m²). At the concentration of 702 mg test item/kg soil dry weight (1391 pellets/m²) and above mortality was statistically significantly increased compared to the control group (Fisher's Exact Test, $\alpha = 0.05$, one-sided greater).

The body weight changes of the earthworms after 4 weeks exposure to Final Bite - 0402206 were not statistically significantly different compared to the control at the concentration of 334 mg test item/kg soil dry weight (662 pellets/m²). At the concentration of 484 mg test item/kg soil dry weight (959 pellets/m²) and above body weight changes were biologically and statistically significantly reduced when compared to the control except for the test concentration of 3103 mg test item/kg soil dry weight (6148 pellets/m²) (Bonferroni-Welch t-test, $\alpha = 0.05$, one-sided smaller). But since at this concentration worms lost weight (-15.1%) during the exposure period of 28 days compared to earthworms in the control where there was an increase of weight (28.4%) observed, it is then considered that the test item at this concentration had a significant effect on body weight of earth worms.

Biologically and statistically significant effects on reproduction were observed at the lowest test concentration of 334 mg test item/kg soil dry weight (662 pellets/m²) (Bonferroni-Welch t-test, $\alpha = 0.05$, one-sided smaller). The effect was dose responsive. No behavioural abnormalities were observed in any of the treatment groups. The feeding activity was comparable to the control in the 334 and 484 mg/kg soil dry weight groups (662 and 959 pellets/m², respectively), however the food intake was reduced at 702 mg/kg soil dry weight and higher (1391 pellets/m²). The effect was dose responsive.

HSE calculated the endpoints in terms of pellets/m². The individual pellet weight was taken to be 13.3 mg (as per other toxicity studies carried out with the same product), the dry weight of soil per container was 0.5 kg and the test area was 189.75 cm². HSE agreed endpoints are included in the endpoint summary Table B.9.7.1-2.

Table B.9.7.1-2: Mortality, body weight, food consumption and reproduction effects after exposure to surface broadcast 'Final Bite'

Final Bite - 0402206 [mg/kg soil dry weight]/ (pellets/m ²)	Control	334/ 662	484/ 959	702/ 1391	1018/ 2017	1476/ 2924	2140/ 4240	3103/ 6148	4500/ 8916
Mortality (day 28) [%]	0	0	0	22.5	35.0	40.0	57.5	82.5	70.0
Statistical Significance ¹⁾	-	n.s.	n.s.	*	*	*	*	*	*
Body weight change (day 28) [%]	28.4	26.1	14.7	-12.9	-16.8	-28.2	-22.7	-15.1	-42.0
Statistical Significance ²⁾	-	n.s.	*	*	*	*	*	n.s.	*
Mean No. of juveniles (day 56)	294	154	130	16	5	0	1	0	0
Statistical Significance ²⁾	-	*	*	*	*	*	*	*	*
Reproduction in [%] of control (day 56)	-	52.4	44.4	5.4	1.7	0.0	0.2	0.1	0.0
Food consumption [g]	25.0	25.0	24.5	17.3	16.0	10.8	9.5	8.3	7.0
Endpoints [mg/kg soil dry weight]/[pellets/m ²]									
NOEC (day 28 mortality)	484/959								
LOEC (day 28 mortality)	702/1391								
LC Values (mortality) ³⁾	LC ₁₀		LC ₂₀		LC ₅₀				
	559.7/1109		841.2/1667		1833.8/3633				
95% confidence limits	256.8 – 823.8/ 509 – 1632		486.2 – 1155.9/ 963 – 2290		1355.2 – 2688.1/ 2685 – 5326				
NOEC (day 28 weight)	334/662								
LOEC (day 28 weight)	484/959								
NOEC (day 56 reproduction)	<334/<662								
LOEC (day 56 reproduction)	334/662								
EC Values (reproduction) ³⁾	EC ₁₀		EC ₂₀		EC ₅₀				
	n.d.		232.4/460		375.1/743				
95% confidence limits	n.d.		224.3 – 240.0/ 444 – 475		369.0 – 381.0/ 731 – 755				

The results represent rounded values calculated on the exact raw data.

- = not applicable

n.s. = not significantly different compared to the control

* = significantly different compared to the control

¹⁾ Fisher's Exact Test, $\alpha = 0.05$, one-sided greater

²⁾ Bonferroni-Welch t-test, $\alpha = 0.05$, two-sided, one-sided smaller

³⁾ Probit analysis

In the most recent test with the reference item Carbendazim 600 g/L SC (performed under ibacon Study Number 105683022 from July to September 2017), there were statistically significant effects on reproduction at a concentration of 1.44 mg a.s./kg soil and higher, which is in line with the guideline OECD 222 (effects should be observed between 1 and 5 mg a.s./kg soil). The EC₅₀ for reproduction was calculated as 1.47 mg a.s./kg soil.

B. Analytical verification

No analytical verification was conducted.

C. Validity criteria

All validity criteria were met:

Control Mortality:	Control mortality was 0% and so this validity criterion was met.
Reproduction of Control:	The number of juvenile worms per replicate was greater than 30 (211 to 415) and so this validity criterion was met.
Coefficient of Variation of Reproduction in Control:	Was 21.8% and so this validity criterion was met.

III. CONCLUSION

In an earthworm reproduction and growth study with Final Bite - 0402206 the No Observed Effect Concentration (NOEC) for mortality of the earthworm *Eisenia andrei* was determined to be 484 mg test item/kg soil dry weight (959 pellets/m²) and the Lowest Observed Effect Concentration (LOEC) was determined to be 702 mg test item/kg soil dry weight (1391 pellets/m²). The NOEC for weight changes was determined to be 334 mg test item/kg soil dry weight (662 pellets/m²) and the LOEC was determined to be 484 mg test item/kg soil dry weight (959 pellets/m²). The NOEC for reproduction was determined to be <334 mg test item/kg soil dry weight (<662 pellets/m²) and the LOEC was determined to be 334 mg test item/kg soil dry weight (662 pellets/m²).

The LC₁₀ (mortality) was determined to be 559.7 mg test item/kg soil dry weight (1109 pellets/m²) (lower - upper 95% confidence level: 256.8 – 823.8 mg test item/kg soil dry weight (509 – 1632 pellets/m²)); the LC₂₀ (mortality) was determined to be 841.2 mg test item/kg soil dry weight (1667 pellets/m²) (lower - upper 95% confidence level: 486.2 – 1155.9 mg test item/kg soil dry weight (963 – 2290 pellets/m²)) and the LC₅₀ (mortality) was determined to be 1833.8 mg test item/kg soil dry weight (3633 pellets/m²) (lower - upper 95% confidence level: 1355.2 – 2688.1 mg test item/kg soil dry weight (2685 – 5326 pellets/m²)).

The EC₁₀ (reproduction) could not be determined, the EC₂₀ (reproduction) was determined to be 232.4 mg test item/kg soil dry weight (460 pellets/m²) (95% confidence limits of 224.3 to 240.0 mg test item/kg soil dry weight (444 – 475 pellets/m²)) and the EC₅₀ (reproduction) was determined to be 375.1 mg test item/kg soil dry weight (743 pellets/m²) (95% confidence limits of 369.0 to 381.0 mg test item/kg soil dry weight (731 – 755 pellets/m²)).

HSE Comments:

It is noted that no batch number is provided for the iron used in the formulation, however, in light of updated consideration in the volume 4, it is concluded that the tested material is representative of the technical specification for elemental iron.

The study was conducted largely in accordance with the agreed regulatory guideline; however, the test item was broadcast over the surface of the soil, rather than being homogeneously mixed into the test soil. As the granules were broadcast onto the surface of the soil, it is not considered appropriate to report the endpoints only as 'kg product/ha'; therefore, HSE calculated the endpoints in terms of 'pellets/m²'. The study was conducted in compliance with GLP. There were clear dose responsive effects on mortality, bodyweight, reproduction and food consumption. Effects on reproduction occurred at the lowest tested concentration, therefore no NOEC could be calculated. Furthermore, no EC₁₀ value could be determined for this parameter.

LC₁₀ and LC₂₀ values were determined, however, it is noted that the confidence intervals are very wide for these values.

The lowest available endpoint for consideration in a regulatory risk assessment is the **EC₂₀ (reproduction) = 232.4 mg test item/kg soil dry weight, or 460 pellets/m²**. The lack of EC₁₀ for this most sensitive parameter should be considered in the context of the risk assessment.

B.9.7.1.2. Earthworm Field Studies

Reference:	CP 10.4.1.1/01
Report author:	Axmann, S.
Report year:	2019

Report title:	Final Bite - Blue – A Field Study to Investigate Effects on the Earthworm Fauna in Central Europe
Report No:	Eurofins Study Code - S18-02597
Document No:	ADAMA Study code - R-39838
Guidelines followed in study:	ISO Guideline 11268-3, 2014; ISO Guideline 23611-1, 2006
GLP/Officially recognised testing facilities:	Yes, conducted under GLP

I. MATERIALS AND METHODS

A. MATERIALS

- Test Item:** Final Bite - Blue, granules (2.5 mm)
Batch no.: KM8017420OH
Active substance / Purity: Iron 1.21% w/w (analysed)
Appearance: Blue granules
Expiry date: 31 May 2019
- Reference item:** Carbomax 500 SC
Batch no: 0002-16-14400T/B
Active substance / content: 500 g/L (490 g/L analysed)
- Test Species:** Naturally occurring field populations of earthworms (juveniles and adults)
 Pre-treatment sampling confirmed that the test site contained representatives of the major earthworm groups at a number recommended in the test guidelines. Density, diversity and homogeneity of earthworm population was determined.

B. STUDY DESIGN AND METHODS

- In-life phase:** Field activity took place 17 April 2018 to 24 May 2019
 Test item applied between 24 April to 19 May 2018
- Test conditions**
Test field site: Near Bodelshausen, Baden-Württemberg, Southern Germany (48.406572°N, 8.992258°E).
 Last crop was Apr 2016- Sept 2017 (Maize)
 Last fertiliser was Feb 2018 (25 m³/ha compost)
 Last pesticide use was May 2017 (bromoxynil, mesotrione, s-metolachlor, nicosulfuron)
 Most recent cultivation measure April 2018: rotary harrow to 3cm depth (whole field site), seed drill 20th April (only areas surrounding the plots).
 On 6th and 26th June, and 9th August 2018, the whole field site was mowed.
Test facility: Eurofins GmbH, Niefern-Öschelbronn, Germany
Test soil: Natural arable soil: soil type, particle size distribution, pH, cation exchange capacity, total carbon, total organic carbon and water holding capacity determined
 Mixed soil sample prepared from 10 soil cores (50 mm wide x 20 cm deep) taken from across test site before first test item application

	Characterised as silt loam (USDA soil texture) with pH 7.49 Organic matter 4.4% (2.88% total carbon) Water holding capacity 50.76 g/100 g dry weight
Treatment:	Test item (Final Bite – Blue) applied six times at 8 kg product/ha (96.8 g iron/ha, equivalent to 606060 granules/ha) with 5 days interval Toxic reference (Carbomax 500 SC) applied once at 20 L product/ha (10 kg carbendazim/ha) Control: Water treated plots
Test design:	Field study, randomised block design
Number of replicates:	4 replicates (plots) per treatment group
Number of sampling areas:	4 sub-samples per replicate (12 x 10 m plot with 3 m space between); 1 plot out of 4 rejected following initial screening of the sampling results of the first earthworm sampling (pre-application sampling).
Number of sampling occasions:	5 (7 DBA1, 34 DAA1, 56 DAA1, 204 DAA1, 366 DAA1) DBA – Days Before Application DAA – Days After Application
Test duration:	April 2018 to May 2019
Temperature:	Mean monthly air temperature 0.6 to 5.6°C Lowest recorded temperature: -11.8°C Highest recorded temperature: 36.4°C
Total rainfall:	540.9 mm (60% of long-term average) Artificial irrigation (105.1 mm in total) applied using a sprinkler system on 16 occasions over April, May, July and August 2018. Overall precipitation deficit including artificial irrigation 28.5% (see Results section)

3. Administration of the test item

Dosing and application

Final Bite - Blue was applied six times to bare soil field plots, seeded with a grass-clover mixture, at a rate of 8.0 kg product/ha (equivalent to 96.8 g iron/ha) with an interval of 5 days. The applications of the test item were made with a modified spreader for granules and seeds and were performed in spring. The control plots remained untreated. The toxic reference item plots were treated once at the first application with 10 kg a.s./ha carbendazim. The spray applications for the toxic reference were made with a boom sprayer calibrated to apply a spray volume of 300 L/ha. For dose verification the numbers of granules on the soil surface were counted in pre-defined areas after the first application to the test item plots. Before and after applications 1 to 6, pictures of defined soil surface areas were taken at each test item plot to count the number of newly applied granules per area.

4. Measurements and observations

Earthworm surface monitoring

Monitoring for direct mortality of earthworms was performed on the two days after each application in the control and test item plots. The soil surface was checked for earthworms by visual observation of two monitoring areas per plot in all replicates of the control and test item plots. The monitoring areas of 1.0 m² (total 2 m² per replicate) were placed in pre-assigned central locations within each plot. Assessments were always conducted in the same areas of each plot. The number of alive, moribund and dead earthworms was counted.

Earthworm population sampling

The field site was sampled to assess the earthworm population prior to the first application (7 DBA1), at approx. 1 month after the first application (34 DAA1), 1 month after the sixth application (56 DAA1), 6 months (204 DAA1) and 12 months (366 DAA1) after the first application.

In total 16 samples (four per plot) were taken from each treatment on each of the five sampling occasions. Earthworms were extracted by hand-sorting with subsequent formalin sampling in the excavated hole (50 cm x 50 cm width, 20 cm depth). The duration of the formalin was 30 minutes at sampling 1-3 and 45 minutes at sampling 4. Collected earthworms were placed in containers with water covering their bases. At the end of the sampling period the water was poured away and the worms were placed in watertight containers containing 80% alcohol.

Identification of adult earthworms to species level was made by means of the relevant identification literature and nomenclature. For juvenile worms that were difficult to identify, a distinction between tanylobous and epilobous was made. Before weighing, the preserved worms were put onto filter paper to remove adhering liquid. The weight of the earthworms was recorded on the basis of species and age. Earthworm species were grouped in the main key ecological groups. Where worms were incomplete, only front ends were counted.

Climatic conditions

During the application, the earthworm surface monitoring and earthworm samplings the temperature, air humidity, soil moisture and soil temperature were recorded with portable devices. These weather data were recorded under GLP. For the whole field phase climatic data were recorded continuously by a data logging weather station (non-GLP) near the field site. The following environmental parameters were recorded – mean, max. and min. air temperature, daily mean wind speed, daily mean soil temperatures and soil moisture at depth of approx. 5 cm and 20 cm, and daily rainfall. Long term precipitation and air temperature data were taken from the weather station of the Deutscher Wetterdienst in Hechingen (3.6 km distance to field site) covering the period 1981-2010.

A mixed soil sample was prepared from 10 soil cores (40 mm wide by 20 cm deep) taken across the field site in the guard rows before the first application on 24 April 2018.

5. Data evaluation

The statistical analysis methods were applied to both earthworm counts and weights, whether for individual taxa or for population totals. For both counts and weights, the sum of the four sample areas per plot (each 0.25 m² in size) was calculated, which gives the counts/weight per square metre. Taxa with low abundances (threshold < 5 ind./m² at two or more of the sampling occasions in the control plots) and earthworm fragments were not considered within this detailed analysis but were part of grouped and total earthworm numbers/weights.

All statistical analyses were performed using SAS, version 9.4. Graphs were generated with the data outputs from SAS using Excel 2010.

The normality of the distributions of abundance and weight were tested using the Shapiro-Wilk test. Homogeneity of variance was tested with the Levene's test. Both tests were evaluated at the $p < 0.05$ level. If the data set was homoscedastic and distributed normally, an ANOVA was calculated. The ANOVA was followed by Dunnett's tests (left-sided) to identify statistically significant ($p < 0.05$) pair-wise differences between the test item treatment or the toxic reference on one hand and the control on the other.

In all other cases (non-normal data, heteroscedastic data, or both), a Kruskal-Wallis test was calculated, followed by Bonferroni-U-tests (left-sided) to identify statistically significant ($p < 0.05$) pair-wise differences between the test item treatment or the toxic reference on one hand and the control on the other. For the Bonferroni-U-Test (left-sided) no p-values were calculated if the data of the treatment (T or R) are similar or higher than the data of the respective control (C).

To evaluate the effect of the treatments on species diversity, the mean number of species per plot and sampling date was calculated based on the total number of different earthworm species found in each individual plot. Statistics were calculated on these data for earthworm abundance and biomass.

In addition, the minimum detectable differences (MDD) were calculated in order to determine the difference between the means of a treatment and the control that must exist to detect a statistically significant effect. The MDD can be calculated a posteriori for the statistical method used (Dunnett's test), considering the actual test design (replication, selected type-I error level α) and the sample variation.

As there is no guidance available yet to classify the calculated MDDs, the MDD classes proposed in the Aquatic Guidance Document (EFSA PPR Panel 2013) were used (Table 1). The MDDs were only accurate if Dunnett's tests were used for detection of differences between means. For the non-parametric Kruskal-Wallis test, the MDDs will differ, but could not be calculated exactly.

Table 1: MDD classes according to EFSA PPR PANEL (2013)

MDD	MDD range	Comment
0	>100%	No effects can be determined statistically
I	>90-100%	Only large effects can be determined statistically
II	>70-90%	Large to medium effects can be determined statistically
III	>50-70%	Medium effects can be determined statistically
IV	≤50%	Small effects can be determined statistically

II. RESULTS AND DISCUSSION

A. APPLICATION VERIFICATION

The application rates were verified by weighing the initial and the remaining amount of test item used for each plot at each application. All deviations from the target rate were within the application tolerance of $\pm 10\%$.

Additionally, photographs of predefined areas ($2 \times 1 \text{ m}^2$) were taken before and after each application. The number of granules applied was calculated by subtracting the number of granules before application from the number of granules after application. As the vegetation cover was increased at the last two applications the mean number of granules applied varied from the results of the first four applications (Table 33).

Table 33: Results of photo documentation

Application	Plot	Area	Number of granules before application	Number of granules after application	Number of granules applied at application
1	Ta	M1	0	81	81
		M2	0	56	56
	Tb	M1	0	46	46
		M2	0	64	64
	Tc	M1	0	62	62
		M2	0	46	46
	Td	M1	0	99	99
		M2	0	76	76
mean		0	66.3	66.3	
2	Ta	M1	33	87	54
		M2	17	58	41
	Tb	M1	11	51	40
		M2	19	75	56
	Tc	M1	13	83	70
		M2	14	77	63
	Td	M1	31	127	96
		M2	14	97	83
mean		19.0	81.9	62.9	
3	Ta	M1	77	156	79
		M2	37	128	91
	Tb	M1	34	136	102
		M2	50	134	84
	Tc	M1	42	96	54
		M2	39	97	58
	Td	M1	90	149	59
		M2	43	107	64
mean		51.5	125.4	73.9	
4	Ta	M1	106	169	63
		M2	64	117	53
	Tb	M1	57	113	56
		M2	53	122	69
	Tc	M1	35	99	64
		M2	43	109	66
	Td	M1	87	141	54
		M2	32	109	77
mean		59.6	122.4	62.8	
5	Ta	M1	109	142	33
		M2	55	73	18
	Tb	M1	61	126	65
		M2	61	104	43
	Tc	M1	58	104	46
		M2	78	123	45
	Td	M1	95	151	56
		M2	56	103	47
mean		71.6	115.8	44.1	

mean=n/m²

Table 33 (continued): Results of photo documentation

Application	Plot	Area	Number of granules before application	Number of granules after application	Number of granules applied at application
6	Ta	M1	6	52	46
		M2	3	46	43
	Tb	M1	7	40	33
		M2	5	32	27
	Tc	M1	10	44	34
		M2	13	63	50
	Td	M1	6	42	36
		M2	2	37	35
	mean		6.5	44.5	38.0

mean=n/m²

Table 2: Mean number of granules applied (photo documentation)

Application	1	2	3	4	5	6
Mean number of granules [n/m ²]*	66.3	62.9	73.9	62.8	44.1	38.0
Standard deviation [n]	18.3	19.6	17.6	8.2	14.1	7.6

* sum of mean number of granules/m² prior application minus mean number of granules/m² after application of eight areas (four replicate plots with two areas per plot)

Climatic conditions during application of treatment:

Application no. (date)	Air Temp (° C)	Air Humidity (%)	Wind speed (m/s)	Cloud cover (%)	Ground cover (%)	Soil temperature (° C)	Soil condition
1 (24/04/18)	14.7-16.3	69.8-74.5	2.1-2.5	30-50	~1	13.6-15.0	Moist
2 (29/04/18)	18.9-23.0	41-45	1.5-2.5	0-10	1	14.5-15.7	Dry
3 (04/05/18)	13.7-16	57.2-63.5	1.8-2.9	30-100	5	12.2-12.7	Moist
4 (09/05/18)	21.2-23.8	38.6-41.6	1.3-2.0	0	5	16.7-17.6	Dry
5 (14/05/18)	12.9-14	93.6-94	0-1.2	100	~10	14.6-14.9	Moist
6 (19/05/18)	13.4	80.2-81.2	1.2-2.3	90-95	~15	14.3-14.6	Moist

Table 30: Monthly values of air temperature, soil temperature, precipitation, soil moisture and wind speed at the field site at Bodelshausen, Germany

Month	Mean air temp. ^{a)}	Max. air temp. ^{a)}	Min. air temp. ^{a)}	Soil temp. ^{a)}		Precipitation ^{a)}	Soil moisture 5 cm ^{a)}		Wind Speed ^{a)}
				5 cm	20 cm		5 cm	20 cm	
	°C	°C	°C	°C	°C	mm	Vol. %	Vol. %	m/s
Apr 2018	13.7 ^{b)}	28.8 ^{b)}	-0.3 ^{b)}	15.7 ^{c)}	14.4 ^{c)}	10.7 ^{b)}	30.0 ^{c)}	23.9 ^{c)}	1.8 ^{cd)}
May 2018	15.2	28.5	4.3	17.4	16.4	74.8	32.1	29.5	1.1
Jun 2018	17.6	30.0	3.3	19.2	19.9	22.0	28.3	28.0	0.8
Jul 2018	19.7	36.4	7.7	20.2	19.7	55.0	25.8	24.3	0.8
Aug 2018	19.7	35.1	5.3	20.5	20.4	80.8	26.1	23.1	1.0
Sep 2018	15.3	30.5	-0.9	16.4	16.6	39.4	26.8	24.8	1.0
Oct 2018	10.1	27.4	-3.8	11.5	12.0	36.8	25.2	24.7	1.0
Nov 2018	4.6	17.3	-6.5	6.3	6.9	13.2	29.8	27.5	0.6
Dec 2018	3.0	13.5	-7.8	4.3	4.6	64.2	33.5	31.0	1.8
Jan 2019	-0.4	7.2	-11.8	1.7	2.1	42.0	34.6	32.0	1.8
Feb 2019	3.8	20.3	-8.1	3.7	2.8	14.2	34.2	31.6	1.3
Mar 2019	6.3	20.1	-5.7	11.1	6.0	48.6	34.5	31.8	2.6
Apr 2019	8.8	25.0	-3.5	18.1	9.1	39.2	32.7	30.1	1.0

^{a)} data data from Eurofins weather station at the field site (non-GLP)

^{b)} data from 01 Apr 2018 – 17 Apr 2018 from LTZ weather station Unterjesingen (13.6 km distance to field site)

^{c)} no data available for the period 01 Apr 2018 – 17 Apr 2018

The period of April 2018 to the end of April 2019 was much warmer and drier than the long-term average from 1981-2010 for the nearest official weather station at Hechingen (Deutscher Wetterdienst 2015). Only one month had lower mean air temperatures than the long-term mean (January 2019, -0.5 °C, compared to long term mean). The mean monthly air temperature for all other months during the field phase was between 0.6 °C (Nov 2018) and 5.6 °C (April 2018) above the corresponding monthly long-term average temperatures. The lowest recorded air temperature was -11.8 °C (January 2019) and the highest was 36.4 °C (July 2018). Air temperatures above 25 °C were recorded for every month from April to October 2018 and again in April 2019.

The total rainfall during the reported period (01 April 2018 to 30 April 2019) without artificial irrigation was 540.9 mm, which corresponds to 59.9 % of the long term average during that period (903 mm). Every month between April and November 2018 with the exception of August 2018, had a moderate to strong precipitation deficit compared to the long term average. Additional artificial irrigation during the months of April, May, July and August 2018 (total 105.1 mm) helped to reduce the precipitation deficit during the critical phase of the study, before and during the application period.

Table 22: Timing and amount of water by irrigation and precipitation

Timing		Mean Irrigation (mm)	Precipitation* (mm)
24 Apr 2018	0DAA1	7.0	0.2
25 Apr 2018	1DAA1	2.9	0.4
26 Apr 2018	2DAA1	10.1	2.4
30 Apr 2018	6DAA1	2.5	0.2
01 May 2018	7DAA1	4.6	0.0
02 May 2018	8DAA1	2.0	0.0
03 May 2018	9DAA1	5.1	0.0
05 May 2018	11DAA1	4.8	0.0
07 May 2018	13DAA1	6.1	0.0
09 May 2018	15DAA1	4.5	0.0
29 May 2018	35DAA1	9.3	12.6
30 May 2018	36DAA1	8.9	2.4
30 Jul 2018	97DAA1	8.8	0.0
31 Jul 2018	98DAA1	15.5	1.6
09 Aug 2018	107DAA1	13.0	0.2

DAA1 = day(s) after first application

* = values taken from EAS weather station near to the field site

During the second half of the study (winter 2018/2019 and spring 2019) the rainfall deficit compared to long-term averages was less than during the first half of the study period, but there still was a rainfall deficit in most months. Therefore even though rainfall sufficed to maintain mean soil moisture levels in the topsoil layers above 30 % Vol. throughout the second half of the study period (low evapo-transpiration during the cold season), water supplies in the deeper soil layers could not be replenished. Overall for the reported period the precipitation deficit (considering additional irrigation) compared to the long-term average was approximately 28.5 % (see table 32).

Table 32: Monthly precipitation (incl. irrigation, long-term precipitation)

Month	Precipitation ^{a)} (=Pcp) [mm]	Irrigation [mm]	Sum of Pcp and irrigation [mm]	Long-term Pcp ^{c)} [mm]	Deviation Pcp to long- term Pcp [mm]	Deviation sum of Pcp and Irrigation to long- term Pcp [mm]	Deviation sum of Pcp to long-term [%]
Apr 18	10.7 ^{b)}	22.5	33.2	64	-53.3	-30.8	-48.1
May 18	74.8	45.3	120.1	104	-29.2	+16.1	15.5
Jun 18	22.0	0.0	22.0	96	-74.0	-74.0	-77.3
Jul 18	55.0	24.3	79.3	101	-46.0	-21.7	-21.5
Aug 18	80.8	13.0	93.8	80	+0.8	+13.8	17.3
Sep 18	39.4	0.0	39.4	66	-26.6	-26.6	-40.3
Oct 18	36.8	0.0	36.8	68	-31.2	-31.2	-45.9
Nov 18	13.2	0.0	13.2	56	-42.8	-42.8	-76.4
Dec 18	64.2	0.0	64.2	61	+3.2	+3.2	+5.2
Jan 19	42.0	0.0	42.0	45	-3.0	-3.0	-6.7
Feb 19	14.2	0.0	14.2	42	-27.8	-27.8	-66.2
Mar 19	48.6	0.0	48.6	56	-7.4	-7.4	-13.2
Apr 19	39.2	0.0	39.2	64	-24.8	-24.8	-38.8
Sum	540.9	105.1	646.0	903	-362.1	-257.0	-28.5

^{a)} data from Eurofins weather station at the field site (non-GLP)^{b)} data from 01 Apr 2018 – 17 Apr 2018 from LTZ weather station Unterjesingen^{c)} data from DEUTSCHER WETTERDIENST (2015), weather station Hechingen, covering the period of 1981-2010

B. BIOLOGICAL DATA

Surface monitoring

Surface monitoring on the first two days after each application showed that there was no acute primary effect on earthworms caused by the test item. No alive, moribund or dead earthworms were found in any of the monitoring areas in all untreated control and test item treatment plots at all monitoring dates.

Abundance and biomass

For earthworm abundance and biomass, the total values per m² were calculated prior to any statistical analysis. Taxa with low abundances (threshold <5 earthworms per m² for at least two sampling occasions in the control plots) and tanylobous and epilobous front ends (fragments) are not presented within the detailed analysis but are part of grouped and total earthworm numbers.

The abundance and biomass results are laid out as follows:

- 1) Community – the proportion of different types of earthworms in the total population
- 2) Total juveniles, adults and total earthworm population – results for abundance and biomass
- 3) Endogenic earthworms- results for abundance and biomass
- 4) Anecic Earthworms – results for abundance and biomass
- 5) Juvenile Earthworms- results for abundance and biomass

1. Community

Seven different adult taxa were observed and identified over the course of the study. Table 7 provides a list of all taxa identified in this study and their ecological grouping in terms of niche occupied.

Table 7: List of identified species

Species	Adult/Juvenile	Ecological group
<i>Aporrectodea caliginosa</i>	Adult	endogeic
<i>Allolobophora chlorotica</i> *	Adult	endogeic
<i>Aporrectodea longa</i>	Adult	anecic
<i>Aporrectodea rosea</i>	Adult	endogeic
<i>Lumbriculus terrestris</i>	Adult	anecic
<i>Octolasion lacteum</i> *	Adult	endogeic
<i>Murchieona minuscula</i> *	Adult	endogeic
Tanylobous	Juvenile	-
Epilobous	Juvenile	-
Tanylobous	Front ends	-
Epilobous*	Front ends	-

*Not analysed (characterised as ‘Other’ in Figure 3) due to low abundance (< 5 individuals/m² at two or more sampling occasions in the control plots)

The taxa were grouped to obtain total abundances as follows:

- Total juvenile earthworms: comprising all juvenile earthworms (tanylobous and epilobous juveniles).
- Total adults: comprising all adults (*Aporrectodea caliginosa*, *Allolobophora chlorotica*, *Aporrectodea longa*, *Aporrectodea rosea*, *Lumbriculus terrestris*, *Octolasion lacteum* and *Murchieona minuscula*).
- Total: comprising all taxa (*Aporrectodea caliginosa*, *Allolobophora chlorotica*, *Aporrectodea longa*, *Aporrectodea rosea*, *Lumbriculus terrestris*, *Octolasion lacteum*, *Murchieona minuscula*, tanylobous juveniles, epilobous juveniles, tanylobous and epilobous front ends).

- Endogeic earthworms: comprising the taxa *Aporrectodea caliginosa*, *Allolobophora chlorotica*, *Aporrectodea rosea*, *Octolasion lacteum* and *Murchieona minuscula*. Endogeic earthworm species live in the mineral top soil (0-20 cm soil depth). Depending on the soil conditions (seasonal dynamics in weather, i.e. moisture) endogeic earthworms may move to slightly deeper soil layers. They mainly construct horizontal burrows and, as secondary decomposer, they feed from organic substance in the soil.
- Anecic earthworms (*Aporrectodea longa* and *Lumbricus terrestris*). Anecic earthworms live in permanent deep vertical borrows. They are primary decomposer and feed from organic substance (i.e. litter and leaves) from the soil surface which they take into the burrows. Whilst being on the soil surface and searching for food they can be directly exposed to high concentrations of pesticides (directly after application).

The mean earthworm abundance was 232.9 earthworms/m² across all plots at the start of the trial. The adult: juvenile ratio was 0.97 (equivalent to 47.5% adults). The initial earthworm population as % of adult earthworms of the field site was characterised by 81.9% endogeic and 18.1% anecic earthworms. The dominant endogeic species at trial start was *Aporrectodea caliginosa* (43.0 earthworms/m², 18.5% of total earthworms, 38.8% of adult earthworms) followed by *Aporrectodea rosea* (38.8 earthworms/m², 16.7% of total earthworms, 35.1% of adult earthworms). The dominant anecic earthworm species was *Lumbricus terrestris* (12.4 earthworms/m², 5.3% of total earthworms, 11.2% of adult earthworms).

The trend of the earthworm population at the field site during the test period was as follows. The mean earthworm abundance (mean values from control plots) was 225.3 (± 17.4) earthworms/m² at trial start (7 DBA1), increased to 233.8 (± 6.6) earthworms/m² at 34 DAA1, decreased to 144.8 (± 47.0) earthworms/m² at 56 DAA1 and increased to 196.3 (± 27.6) earthworms/m² at 204 DAA1 and 202.0 (± 30.0) earthworms/m² at 366 DAA1. The changes in the abundance and standard deviation of the earthworm population follow the natural dynamics influenced by the environmental conditions (seasonal weather).

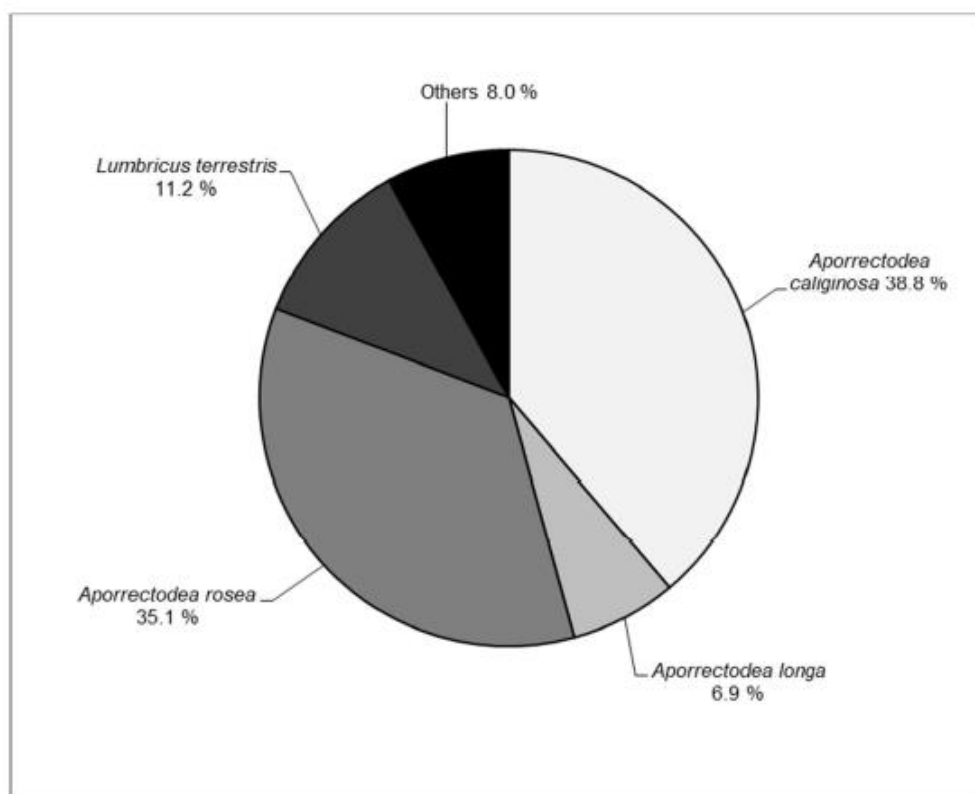


Figure 3: Adult earthworm species distribution (percentage of number of adult earthworms across all plots at trial start)

2. Total Juveniles, Adults and Total Earthworms

Abundance

The total abundances of juveniles, adults and overall earthworm populations are detailed in the table below, copied from the full study report.

Table 12: Mean number (n/m^2) standard deviation and percentage change of total earthworm abundance in the test item treatment (T) and toxic reference treatment (R) relative to the control (C)

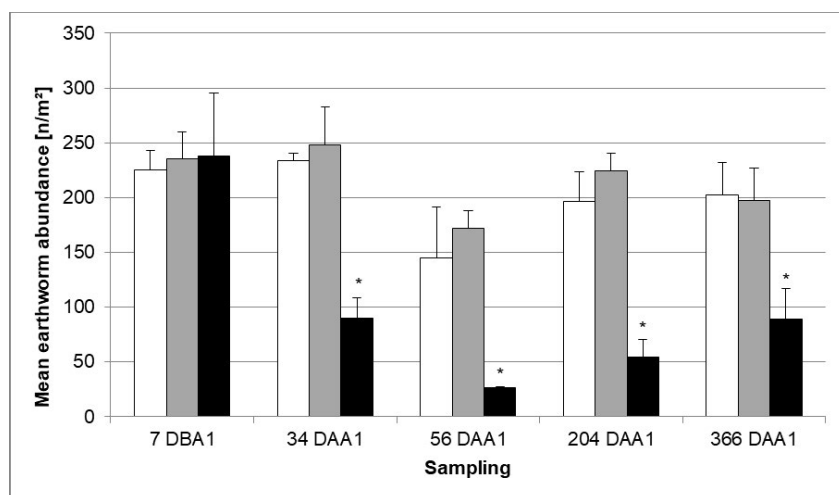
Species / group	Timing	Mean number (n/m^2) \pm standard deviation and deviation from control (%) ^{a)}		
		C (untreated)	Final Bite – Blue (T)	Carbomax (R)
Total Juveniles	7 DBA1	101.5 \pm 19.0	113.0 \pm 15.6 (11.3)	129.3 \pm 43.7 (27.3)
	34 DAA1	129.8 \pm 14.9	136.5 \pm 21.9 (5.2)	57.5 \pm 15.9 * (-55.7)
	56 DAA1	96.3 \pm 29.5	107.5 \pm 11.7 (11.7)	20.3 \pm 3.1 * (-79.0)
	204 DAA1	94.0 \pm 7.9	109.3 \pm 25.1 (16.2)	39.3 \pm 12.3 * (-58.2)
	366 DAA1	134.3 \pm 28.2	122.5 \pm 18.4 (-8.8)	67.0 \pm 14.8 * (-50.1)
Total Adults	7 DBA1	117.3 \pm 13.6	112.8 \pm 10.2 (-3.8)	102.3 \pm 14.8 (-12.8)
	34 DAA1	98.5 \pm 11.5	100.0 \pm 19.3 (1.5)	28.8 \pm 4.9 * (-70.8)
	56 DAA1	45.5 \pm 16.1	62.3 \pm 6.3 (36.8)	6.0 \pm 3.7 * (-86.8)
	204 DAA1	101.3 \pm 21.1	112.8 \pm 12.9 (11.4)	15.0 \pm 4.5 * (-85.2)
	366 DAA1	61.8 \pm 5.3	67.5 \pm 12.9 (9.3)	19.8 \pm 12.6 * (-68.0)
Total	7 DBA1	225.3 \pm 17.4	235.5 \pm 24.3 (4.6)	238.0 \pm 57.1 (5.7)
	34 DAA1	233.8 \pm 6.6	247.8 \pm 35.3 (6.0)	90.0 \pm 18.9 * (-61.5)
	56 DAA1	144.8 \pm 47.0	171.8 \pm 16.5 (18.7)	26.3 \pm 1.0 * (-81.9)
	204 DAA1	196.3 \pm 27.6	224.0 \pm 16.6 (14.1)	54.5 \pm 15.6 * (-72.2)
	366 DAA1	202.0 \pm 30.0	197.0 \pm 29.5 (-2.5)	89.3 \pm 27.3 * (-55.8)

^{a)} negative values (-) indicate lower earthworm numbers compared to the control; positive values (+) indicate higher earthworm numbers compared to the control

DBA1= days before application 1, DAA1= days after application 1

*significantly different from control ($p \leq 0.05$)

The initial mean total earthworm abundance (7 DBA1) in the control plots and the test item treatment plots were comparable and low standard deviations showed a homogenous distribution of the total earthworm population throughout all control and test item treatment plots (Figure 1, below). This was unchanged at the first earthworm population sampling 34 days after the first application (34 DAA1). At the second post-application earthworm population sampling (56 DAA1) the mean total earthworm abundance of control and treatment plots decreased to the minimum level of the test period whilst the standard deviation in the untreated control plots increased to a maximum (± 47.0) and reflects high differences of the earthworm abundance between the replicate control plots. An increase of the mean total earthworm abundance of control and treatment plots was observed at the third post-application sampling in autumn 2018 (204 DAA1). At the last post-application sampling (366 DAA1) the mean total abundance and the standard deviations did not differ between the control plots and the test item treatment plots and were in the range of the initial abundance, again. The differences in the mean total abundance of the test item treatment plots compared to the untreated control plots were positive at the first (34 DAA1), second (56 DAA1) and third (204 DAA1) post-application samplings and in the range of the control (-2.5%) at the last post-application sampling (366 DAA1).



* significantly different to control ($p \leq 0.05$)

Figure 1: Graphical results of mean numbers of total earthworms. White = untreated control, grey = treatment (6x 96.8 g iron/ha with 5 day interval), black = reference item (single application 10 kg carbendazim/ha)

During the whole study period the earthworm abundance of the total adults and total juveniles in the test item treatment plots were not statistically significant different to the control data. Deviations of mean total earthworm abundance in the test item treatment plots were between -8.8 to 16.2% for the group total juveniles and between -3.8 to 36.8% for the group total adults (Table 12, above).

Statistically significant reductions >50% in the total abundance of the toxic reference item treated plots at all post-application samplings showed that the toxic standard worked and that the study design and site was appropriately sensitive.

The minimum detectable differences (MDDs) of total earthworm groups (total, total adults and total juveniles) were <50% throughout the whole study period (Table 4).

% MDD	Abundance				
Taxon	7 DBA1	34 DAA1	56 DAA1	204 DAA1	366 DAA1
Total Juveniles	44 (IV)	21 (IV)	30 (IV)	28 (IV)	24 (IV)
Total Adults	17 (IV)	21 (IV)	35 (IV)	22 (IV)	27 (IV)
Total	25 (IV)	15 (IV)	31 (IV)	16 (IV)	22 (IV)

Biomass

The mean biomass of juveniles, adults and overall earthworm populations are detailed in the table below, copied from the full study report.

Table 15: Mean biomass (g/m²), standard deviation and percentage change of total earthworm biomass in the test item treatment (T) and toxic reference treatment (R) relative to the control (C)

Species / group	Timing	Mean biomass (g/m ²) ± standard deviation and deviation from control (%) ^{a)}		
		C (untreated)	Final Bite – Blue (T)	Carbomax (R)
Total Juveniles	7 DBA1	27.5±10.0	30.2±2.6 (9.8)	26.1±1.3 (-5.1)
	34 DAA1	23.6±8.4	28.4±6.3 (20.1)	7.5±3.4 * (-68.1)
	56 DAA1	12.3±4.3	14.7±9.0 (20.1)	2.1±2.5 * (-82.8)
	204 DAA1	8.3±1.3	9.5±1.7 (15.2)	4.8±2.5 * (-41.6)
	366 DAA1	10.3±4.1	11.1±2.3 (7.2)	5.6±2.1 * (-45.9)
Total Adults	7 DBA1	71.3±6.2	71.3±20.8 (0.0)	68.3±17.9 (-4.2)
	34 DAA1	49.2±17.1	51.9±15.5 (5.4)	9.7±5.2 * (-80.3)
	56 DAA1	16.3±5.1	28.8±5.1 (76.0)	1.5±1.3 * (-90.6)
	204 DAA1	86.5±23.5	116.3±6.3 (34.4)	11.6±2.6 * (-86.7)
	366 DAA1	63.1±5.6	58.1±22.5 (-7.8)	15.1±7.5 * (-76.1)
Total	7 DBA1	99.4±9.5	103.1±18.2 (3.7)	95.1±18.3 (-4.4)
	34 DAA1	73.5±26.0	81.1±21.7 (10.4)	17.7±7.5 * (-76.0)
	56 DAA1	29.0±8.1	43.8±13.9 (51.0)	3.6±1.7 * (-87.4)
	204 DAA1	94.9±24.2	126.6±5.5 (33.4)	16.4±3.7 * (-82.7)
	366 DAA1	74.2±1.3	71.3±21.5 (-3.8)	20.9±8.1 * (-71.8)

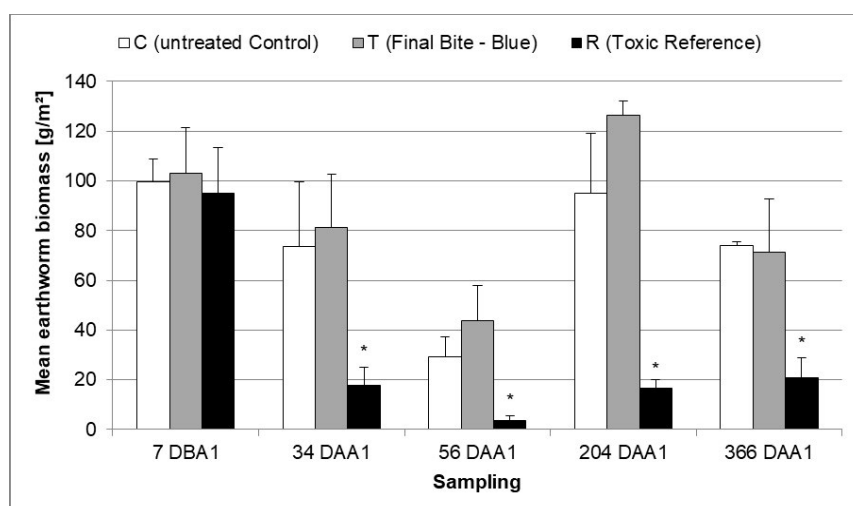
^{a)} negative values (-) indicate lower earthworm numbers compared to the control; positive values (+) indicate higher earthworm numbers compared to the control

DBA1= days before application 1, DAA1= days after application 1

*significantly different from control ($p \leq 0.05$)

The total earthworm biomass in the control plots was 99.4 g/m² at the initial sampling (7 DBA1), decreased to 73.5 g/m² (34 DAA1) at the first post-application sampling and to 29.0 g/m² at the second post-application sampling in June 2018 (56 DAA1) (Figure 3 below, Table 15 above). At the third post-application sampling (204 DAA1) the total earthworm biomass was 94.9 g/m² and in the range of the initial biomass. 12 months after the first application (366 DAA1) the total earthworm biomass in the untreated control plots was 74.2 g/m². Low earthworm biomass at the second post-application sampling was observed as a result of the low earthworm abundance at this time-point.

At the initial earthworm population sampling the total earthworm biomass in the test item treatment plots was at the same level as the respective control data, increased to a maximum relative difference of +51.0% (56 DAA1) and was +33.4% at the third post-application sampling (204 DAA1). The biomass of total earthworms at the third post-application sampling (204 DAA1) was higher compared to the respective biomass in the untreated control plots and only with a slight difference of -3.8% at the fourth post-application sampling (366 DAA1). As the biomass of the group 'total juveniles' and 'total adults' were not reduced compared to the control data, except the slight reduction in the group 'total adults' at the last post-application sampling (-7.8%, 366 DAA1), there was no statistically significant reduction detected.



* significantly different to control ($p \leq 0.05$)

Figure 2: Graphical results of mean biomass of total earthworms White = untreated control, grey = treatment (6x 96.8 g iron/ha with 5 day interval), black = reference item (single application 10 kg carbendazim/ha)

The minimum detectable differences (MDDs) of these groupings indicate the possibility to determine statistically small effects with exception of the second post-application sampling (56 DAA1) for total juveniles (determination of statistically large to medium effects) and total earthworms (determination of statistically medium effects) as shown in Table 6 (below). The data for earthworm biomass reflects the respective earthworm abundance.

The initial biomass values for total earthworm biomass (7 DBA1) and the group total adults and total juveniles of the toxic reference item treatment were at the same level as the respective biomass values in the untreated control plots. The biomass for the group total juveniles of the toxic reference was significantly reduced at the first, second and third post-application sampling (34 DAA1, 56 DAA1 and 204 DAA1). The biomass for the group's total adults and total earthworms of the toxic reference were significantly reduced compared to the respective control data at all post-application sampling times. These reductions were up to -87.4% for total earthworms at the second post-application sampling (56 DAA1), a time-point with low earthworm biomass in general.

Table 6: MDDs calculated for biomass a posteriori for Dunnett's test (3 treatment groups: control, T, R, $\alpha = 0.05$, 4 replicates per treatment group, left sided test)

% MDD	Biomass				
Taxon	7 DBA1	34 DAA1	56 DAA1	204 DAA1	366 DAA1
Total Juveniles	34 (IV)	41 (IV)	75 (II)	36 (IV)	45 (IV)
Total Adults	35 (IV)	43 (IV)	40 (IV)	25 (IV)	34 (IV)
Total	25 (IV)	42 (IV)	50 (IV)	24 (IV)	28 (IV)

(MDD classification (EFSA PPR Panel (2013)): 0: >100%, I: >90-100%, II: >70-90%, III: >50-70%, IV: ≤50%)

3. Endogeic Earthworms

Abundance

The results in terms of abundance of the endogeic Taxa *Aporrectodea caliginosa* and *Aporrectodea rosea*, and the total grouping of endogeic earthworms, are detailed in Table 8, and figures 4 and 5 below (copied from the full study report).

Table 8: Mean number (n/m²), standard deviation and percentage change of endogeic species earthworm abundance in the test item treatment (T) and toxic reference treatment (R) relative to the control (C)

Species / group	Timing	Mean number (n/m ²) ± standard deviation and deviation from control (%) ^{a)}		
		C (untreated)	Final Bite – Blue (T)	Carbomax (R)
<i>Aporrectodea caliginosa</i>	7 DBA1	47.8±11.3	44.3±14.1 (-7.3)	37.0±7.9 (-22.5)
	34 DAA1	30.8±7.5	29.8±11.1 (-3.3)	3.0±2.6 * (-90.2)
	56 DAA1	17.5±10.3	25.0±2.4 (42.9)	1.0±1.2 * (-94.3)
	204 DAA1	28.0±3.2	30.3±9.1 (8.0)	1.0±1.4 * (-96.4)
	366 DAA1	23.0±6.3	27.0±15.2 (17.4)	3.3±5.2 * (-85.9)
<i>Aporrectodea rosea</i>	7 DBA1	39.5±4.4	38.5±6.0 (-2.5)	38.5±6.6 (-2.5)
	34 DAA1	39.5±13.8	46.0±6.0 (16.5)	20.0±4.1 * (-49.4)
	56 DAA1	18.5±6.5	22.0±5.0 (18.9)	3.5±2.6 * (-81.1)
	204 DAA1	43.8±13.6	43.0±7.9 (-1.7)	10.3±2.9 * (-76.6)
	366 DAA1	23.5±4.9	24.3±2.5 (3.2)	13.0±8.2 (-44.7)
Endogeic	7 DBA1	96.5±13.1	91.8±17.7 (-4.9)	83.8±10.0 (-13.2)
	34 DAA1	78.8±17.1	82.0±13.9 (4.1)	24.5±3.7 * (-68.9)
	56 DAA1	37.0±15.4	49.3±5.5 (33.1)	5.0±3.2 * (-86.5)
	204 DAA1	79.5±16.5	79.8±12.4 (0.3)	11.5±4.4 * (-85.5)
	366 DAA1	51.5±7.3	58.0±17.0 (12.6)	17.8±12.6 * (-65.5)

^{a)} negative values (-) indicate lower earthworm numbers compared to the control; positive values (+) indicate higher earthworm numbers compared to the control

DBA1= days before application 1, DAA1= days after application 1

*significantly different from control ($p \leq 0.05$)

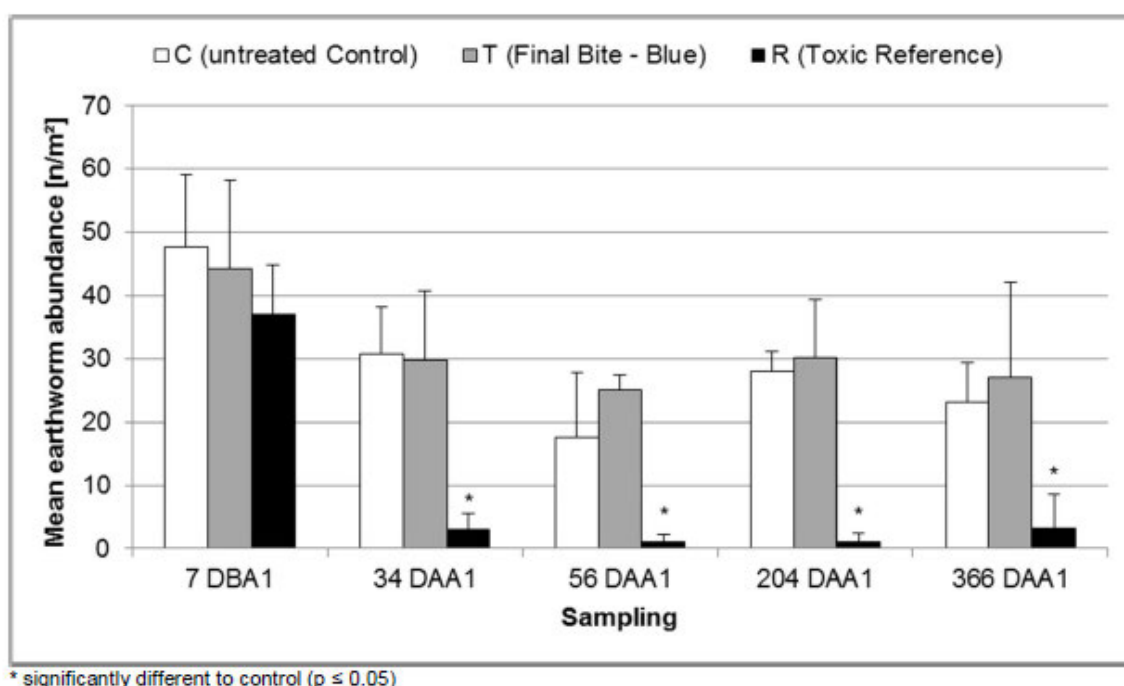


Figure 4: Graphical results of mean abundance of *A. caliginosa*. White = untreated control, grey = treatment (6x 96.8 g iron/ha with 5 day interval), black = reference item (single application 10 kg carbendazim/ha)

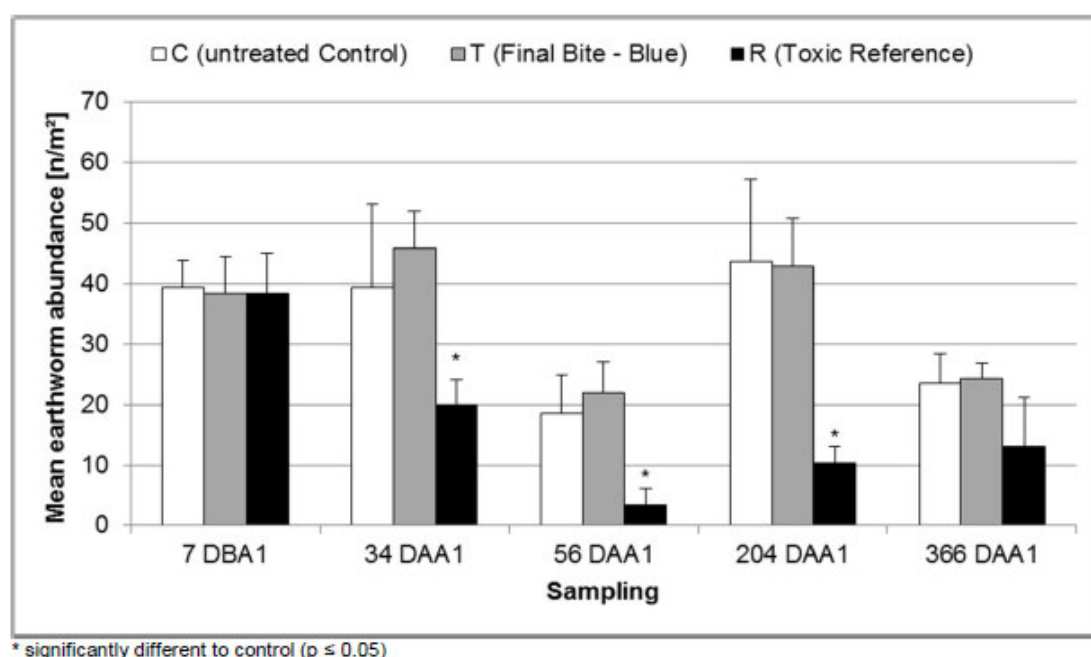


Figure 5: Graphical results of mean abundance of *A. rosea*. White = untreated control, grey = treatment (6x 96.8 g iron/ha with 5 day interval), black = reference item (single application 10 kg carbendazim/ha)

The abundances of the endogeic taxa *Aporrectodea caliginosa* and *Aporrectodea rosea*, in the test item treated plots were not significantly different from the respective control data at any earthworm population sampling. Both taxa, *Aporrectodea caliginosa* and *Aporrectodea rosea*, represented 73.9% of the initial adult earthworm population at the field site. The maximum deviations were +42.9% for the taxon *Aporrectodea caliginosa* and

+18.9% for *Aporrectodea rosea*, both occurring at the second post-application sampling (56 DAA1). At the last earthworm population sampling, 12 months after the first application of the test item (366 DAA1), the deviations were +17.4% for the taxon *Aporrectodea caliginosa* and +3.2% for *Aporrectodea rosea*. The taxa *Aporrectodea caliginosa* and *Aporrectodea rosea* belong to the ecological group endogeic earthworms. Endogeic earthworms live in the upper mineral soil horizon (i.e. top 20 cm) and construct mainly horizontal borrows. They present the majority of the earthworm species and abundance of adult earthworms at the field site used for the study. The abundance of all endogeic earthworms in the test item treated plots was not statistically different from the control at any earthworm population sampling. The maximum deviation to the respective control data was +33.1% and occurred at the timing with the least abundance in all treatments (56 DAA1).

The MDDs of endogeic earthworms were <50 % throughout the whole study period except from the data for *A. caliginosa* at the third sample (56 DAA1; highlighted in **bold** in table 9).

Table 9: MDDs calculated for abundance a posteriori for Dunnett's test (3 treatment groups: control, T, R, $\alpha = 0.05$, 4 replicates per treatment group, left sided test)

% MDD	Abundance				
Taxon	7 DBA1	34 DAA1	56 DAA1	204 DAA1	366 DAA1
<i>Aporrectodea caliginosa</i>	37 (IV)	40 (IV)	54 (III)	31 (IV)	67 (III)
<i>Aporrectodea rosea</i>	22 (IV)	35 (IV)	41 (IV)	32 (IV)	37 (IV)
Endogeic	22 (IV)	25 (IV)	40 (IV)	24 (IV)	39 (IV)

(MDD classification (EFSA PPR Panel (2013)): 0: >100%, I: >90-100%, II: >70-90%, III: >50-70%, IV: ≤50%)

Biomass

The biomass of the endogeic taxa *Aporrectodea caliginosa* and *Aporrectodea rosea* in the test item treated plots were not significantly different from the control data at any earthworm population sampling. The maximum deviations were +23.6% for the taxon *Aporrectodea caliginosa* at the fourth post-application sampling (366 DAA1) and +13.1% for the taxon *Aporrectodea rosea* at the first post-application sampling (34 DAA1). The biomass of the group endogeic earthworms in the test item treated plots was not statistically different from the control at any earthworm population sampling. The maximum deviation to the respective control data was +22.9% and occurred at the timing with the least biomass in all treatments (56 DAA1). For brevity the results have not been presented graphically, as they reflect the results for abundance of these species.

The MDDs calculated for the biomass endogeic earthworms were low on most sampling occasions. Class IV MDDs were calculated for all three groupings on the first, second and fourth sampling occasions. Class III MDDs were calculated on the third sampling occasion. On the final sampling, the MDDs ranged from Class II to Class IV.

Table 10: MDDs calculated for biomass a posteriori for Dunnett's test (3 treatment groups: control, T, R, $\alpha = 0.05$, 4 replicates per treatment group, left sided test)

% MDD	Biomass				
Taxon	7 DBA1	34 DAA1	56 DAA1	204 DAA1	366 DAA1
<i>Aporrectodea caliginosa</i>	31 (IV)	37 (IV)	70 (III)	22 (IV)	77 (II)
<i>Aporrectodea rosea</i>	28 (IV)	39 (IV)	50 (III)	35 (IV)	60 (III)
Endogeic	29 (IV)	37 (IV)	58 (III)	25 (IV)	45 (IV)

(MDD classification (EFSA PPR Panel (2013)): 0: >100%, I: >90-100%, II: >70-90%, III: >50-70%, IV: ≤50%)

4. Anecic Earthworms

Abundance

The group anecic earthworms is represented at the field site used for the study by the taxa *Lumbricus terrestris* and *Aporrectodea longa*. Anecic earthworms live in vertical burrows which reach into deeper (<20 cm) mineral soil horizons. They feed from organic material which they collect from the soil surface and pull deep in the burrows. In the study they can potentially get direct contact with the test item whilst they are collecting organic material (i.e. litter and leaves) on the soil surface.

Table 11: Mean number (n/m²), standard deviation and percentage change of Anecic earthworm abundance in the test item treatment (T) and toxic reference treatment (R) relative to the control (C)

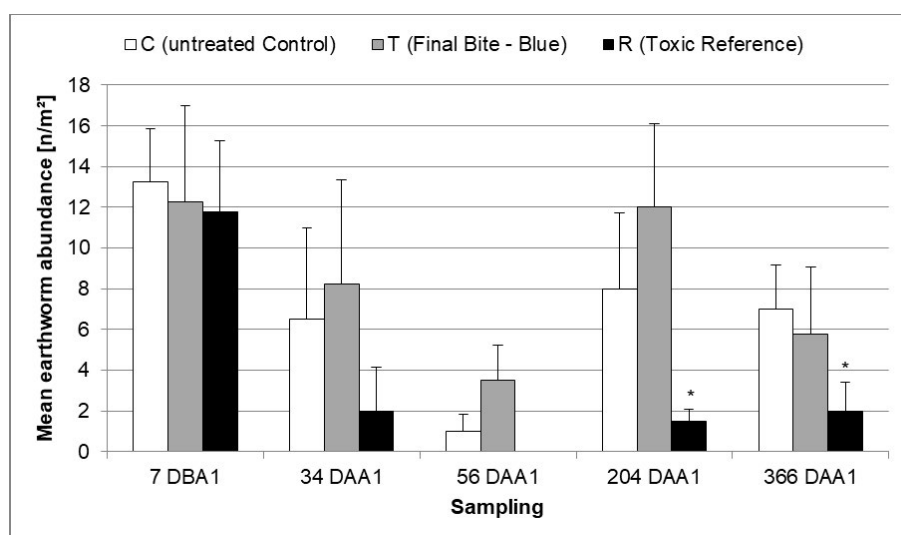
Species / group	Timing	Mean number (n/m ²) ± standard deviation and deviation from control (%) ^{a)}		
		C (untreated)	Final Bite – Blue (T)	Carbomax (R)
<i>Aporrectodea longa</i>	7 DBA1	7.5±1.0	8.8±4.6 (16.7)	6.8±1.7 (-10.0)
	34 DAA1	13.3±5.7	9.8±4.7 (-26.4)	2.3±2.1 * (-83.0)
	56 DAA1	7.5±2.6	9.5±4.8 (26.7)	1.0±1.2 * (-86.7)
	204 DAA1	13.8±4.6	21.0±5.8 (52.7)	2.0±2.2 * (-85.5)
	366 DAA1	3.3±1.9	3.8±2.1 (15.4)	0.0±0.0 * (-100.0)
<i>Lumbricus terrestris</i>	7 DBA1	13.3±2.6	12.3±4.7 (-7.5)	11.8±3.5 (-11.3)
	34 DAA1	6.5±4.5	8.3±5.1 (26.9)	2.0±2.2 (-69.2)
	56 DAA1	1.0±0.8	3.5±1.7 (250.0)	0.0±0.0 (-100.0)
	204 DAA1	8.0±3.7	12.0±4.1 (50.0)	1.5±0.6 * (-81.3)
	366 DAA1	7.0±2.2	5.8±3.3 (-17.9)	2.0±1.4 * (-71.4)
Anecic	7 DBA1	20.8±3.1	21.0±8.8 (1.2)	18.5±5.1 (-10.8)
	34 DAA1	19.8±8.7	18.0±7.1 (-8.9)	4.3±3.2 * (-78.5)
	56 DAA1	8.5±3.3	13.0±3.5 (52.9)	1.0±1.2 * (-88.2)
	204 DAA1	21.8±5.9	33.0±1.8 (51.7)	3.5±1.7 * (-83.9)
	366 DAA1	10.3±2.2	9.5±4.9 (-7.3)	2.0±1.4 * (-80.5)

^{a)} negative values (-) indicate lower earthworm numbers compared to the control; positive values (+) indicate higher earthworm numbers compared to the control

DBA1= days before application 1, DAA1= days after application 1

*significantly different from control ($p \leq 0.05$)

The taxon *Lumbricus terrestris* is the dominant species of the group anecic earthworms at 11.2% of total adult earthworms. The mean abundance of *Lumbricus terrestris* in the untreated control plots was 13.3 n/m² at the initial earthworm population sampling (7 DBA1), decreased to 6.5 n/m² (34 DAA1) and 1.0 n/m² (56 DAA1). At the third post-application sampling (204 DAA1) the abundance of *Lumbricus terrestris* was 8.0 n/m² and decreased to 7.0 n/m² at the last earthworm population sampling (366 DAA1). In the test item treated plots, the abundance of *Lumbricus terrestris* was 12.3 n/m² at the initial earthworm population sampling (7 DBA1), decreased to 8.3 n/m² (34 DAA1) and 3.5 n/m² (56 DAA1). At the third post-application sampling (204 DAA1) the abundance (12.0 n/m²) was comparable with the initial abundance in the test item treatment plots before the application and decreased to 5.8 n/m² at the fourth post-application sampling (366 DAA1). Graphical results of mean numbers of *Lumbricus terrestris* are presented in Figure 6.



* significantly different to control ($p \leq 0.05$)

Figure 6: Graphical results of mean numbers of *Lumbricus terrestris* adults. White = untreated control, grey = treatment (6x 96.8 g iron/ha with 5 day interval), black = reference item (single application 10 kg carbendazim/ha)

Statistical analysis of the abundance of the taxon *L. terrestris* did not reveal any statistically significant effect in the test item treatment plots at any sampling time after the applications. Low numbers, especially in the untreated control plots, at the second post-application sampling in Jun 2018 (56 DAA1) caused a deviation of +250% in the test item treated plots compared to the respective control data. A high MDD (**bold text in table 12**) reflects these low numbers at the second post-application sampling (56 DAA1).

Table 12: MDDs calculated for abundance a posteriori for Dunnett's test (3 treatment groups: control, T, R, $\alpha = 0.05$, 4 replicates per treatment group, left sided test)

% MDD	Abundance				
Taxon	7 DBA1	34 DAA1	56 DAA1	204 DAA1	366 DAA1
<i>Aporrectodea longa</i>	59 (III)	51 (III)	66 (III)	50 (IV)	77 (II)
<i>Lumbricus terrestris</i>	43 (IV)	98 (I)	170 (0)	62 (III)	53 (III)
Anecic	46 (IV)	53 (III)	52 (III)	26 (IV)	49 (IV)

(MDD classification (EFSA PPR PANEL (2013)): 0: >100%, I: >90-100%, II: >70-90%, III: >50-70%, IV: ≤50%)

The anecic taxon *Aporrectodea longa* was present in the test item treatment plots with a mean abundance of 8.8 n/m² up to 9.8 n/m² at the initial and the first two post-applications and increased to 21.0 n/m² at the third post-application sampling in autumn 2018 (204 DAA1) whilst the respective abundance in the control varied between 7.5 n/m² (7 DBA1, 56 DAA1) and 13.8 n/m² (204 DAA1). At the fourth earthworm population sampling (366 DAA1) *A. longa* was present in lower numbers in both the test item treated plots (3.8 n/m²) and in the untreated control plots (3.3 n/m²).

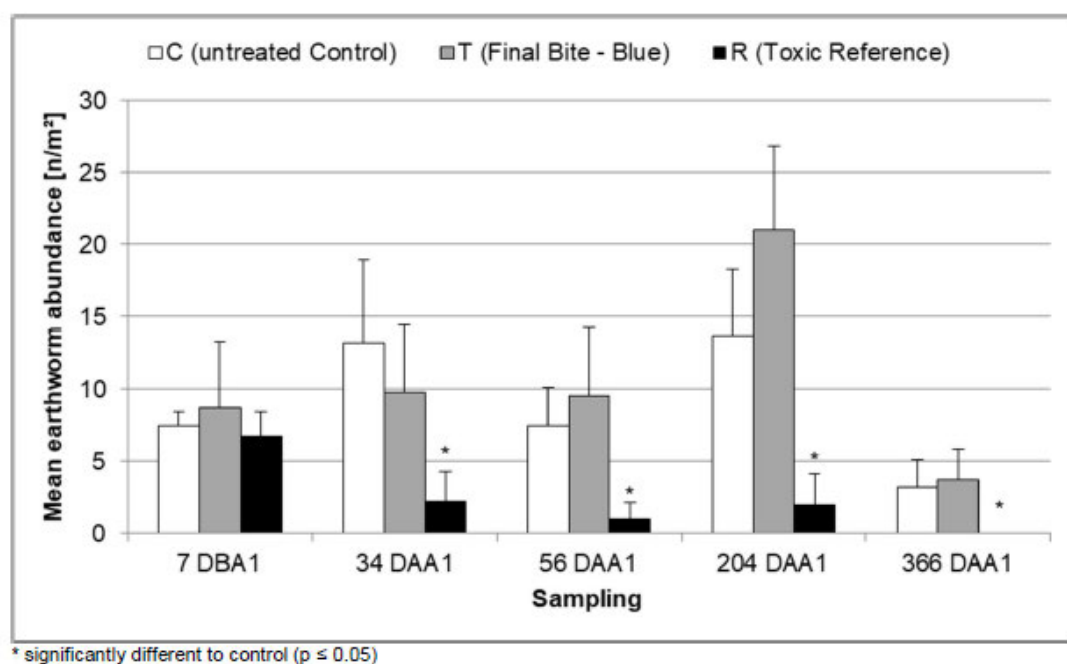


Figure 7: Graphical results of mean numbers of *A. longa* adults. White = untreated control, grey = treatment (6x 96.8 g iron/ha with 5 day interval), black = reference item (single application 10 kg carbendazim/ha)

Deviations in abundance of anecic earthworms reflect the abundance of the taxa *Lumbricus terrestris* and *Aporrectodea longa*. Especially the differences in earthworm abundances of the taxon *Lumbricus terrestris* compared to the untreated control at the second (56 DAA1) and third (204 DAA1) post-application sampling led to these high (relative) deviations in the abundance of anecic earthworms.

Biomass

Low earthworm biomass occurred for the anecic taxa *Lumbricus terrestris* and *Aporrectodea longa* in the control and test item treated plots at the second post-application sampling (56 DAA1). At the last post-application sampling (366 DAA1) the biomass of *Lumbricus terrestris* in the test item treatment plots was at the level of the respective biomass in the untreated control plots (Figure 7).

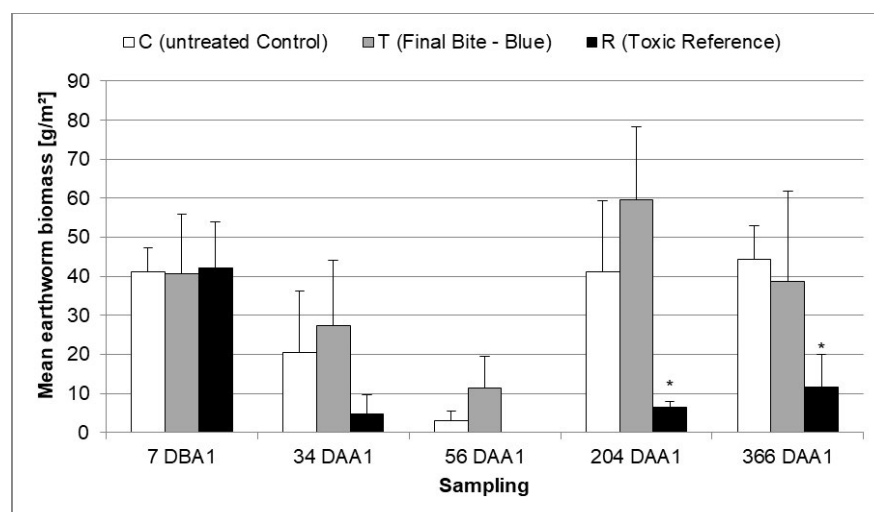


Figure 7: Graphical results of mean biomass of *Lumbricus terrestris* adults. White = untreated control, grey = treatment (6x 96.8 g iron/ha with 5 day interval), black = reference item (single application 10 kg carbendazim/ha)

Compared to the control the biomass of the group anecic earthworms in the test item treated plots was higher at the second (+99.9%, 56 DAA1) and third (+45.1%, 204 DAA1) post-application sampling. At the fourth post application sampling (366 DAA1) the relative difference from the untreated control data was -12.1%. Deviations in the biomass of anecic earthworms reflect the results of earthworm abundances.

Table 13 details the MDD values for biomass of anecic earthworms. As highlighted in bold text, the MDD values were class 0 on the second and third sampling occasion for the species *L. terrestris*.

Table 13: MDDs calculated for biomass a posteriori for Dunnett's test (3 treatment groups: control, T, R, $\alpha = 0.05$, 4 replicates per treatment group, left sided test)

% MDD	Biomass				
Taxon	7 DBA1	34 DAA1	56 DAA1	204 DAA1	366 DAA1
<i>Aporrectodea longa</i>	71 (II)	49 (IV)	55 (III)	47 (IV)	75 (II)
<i>Lumbricus terrestris</i>	44 (IV)	101 (0)	243 (0)	57 (III)	52 (III)
Anecic	47 (IV)	67 (III)	49 (IV)	26 (IV)	49 (IV)

(MDD classification (EFSA PPR Panel (2013)): 0: >100%, I: >90-100%, II: >70-90%, III: >50-70%, IV: ≤50%)

5. Juvenile Earthworms

Abundance

No statistically significant differences in the abundance of the juvenile groups tanylobous juveniles and epilobous juveniles in the test item treated plots compared to the respective control abundance were detected at any earthworm population sampling. With deviations of +5.7% (34 DAA1) to +15.4% (204 DAA1) and -9.0% (366 DAA1) the abundance of epilobous juvenile earthworms was in the range of the respective control data throughout the field phase. Tanylobous juvenile earthworms were present in low numbers. The maximum abundances were 7.8 n/m² (7 DBA1, 34 DAA1) in the untreated control and 7.5 n/m² (34 DAA1) in the test item treated plots, only. At the third post-application sampling in (204 DAA1) autumn 2018 no tanylobous juvenile earthworms were present in the untreated control plots. Also, at the fourth earthworm population sampling (366 DAA1) the abundance of tanylobous juvenile earthworms was low in the test item treated plots (1.0 n/m²) and did not differ from the respective abundance in the untreated control plots (0.8 n/m²).

Table 14: Mean number (n/m²), standard deviation and percentage change of juvenile earthworm abundance in the test item treatment (T) and toxic reference treatment (R) relative to the control (C)

Species / group	Timing	Mean number (n/m ²) ± standard deviation and deviation from control (%) ^{a)}		
		C (untreated)	Final Bite – Blue (T)	Carbomax (R)
Tanylobous juveniles	7 DBA1	7.8±5.3	7.3±3.9 (-6.5)	6.5±1.3 (-16.1)
	34 DAA1	7.8±3.6	7.5±3.4 (-3.2)	2.5±1.9 * (-67.7)
	56 DAA1	1.8±1.0	2.3±3.9 (28.6)	0.8±1.5 (-57.1)
	204 DAA1	0.0±0.0	0.8±1.0 (-)	0.3±0.5 (-)
	366 DAA1	0.8±1.0	1.0±1.2 (33.3)	0.3±0.5 (-66.7)
Epilobous juveniles	7 DBA1	93.8±14.6	105.8±17.3 (12.8)	122.8±44.8 (30.9)
	34 DAA1	122.0±15.4	129.0±20.4 (5.7)	55.0±16.3 * (-54.9)
	56 DAA1	94.5±29.6	105.3±14.0 (11.4)	19.5±2.5 * (-79.4)
	204 DAA1	94.0±7.9	108.5±25.7 (15.4)	39.0±12.6 * (-58.5)
	366 DAA1	133.5±28.2	121.5±18.7 (-9.0)	66.8±14.7 * (-50.0)

^{a)} negative values (-) indicate lower earthworm numbers compared to the control; positive values (+) indicate higher earthworm numbers compared to the control

DBA1= days before application 1, DAA1= days after application 1

*significantly different from control ($p \leq 0.05$)

Table 15: MDDs calculated for abundance a posteriori for Dunnett's test (3 treatment groups: control, T, R, $\alpha = 0.05$, 4 replicates per treatment group, left sided test)

% MDD	Abundance				
Taxon	7 DBA1	34 DAA1	56 DAA1	204 DAA1	366 DAA1
Tanylobous juveniles	77 (II)	61 (III)	216 (0)	-	188 (0)
Epilobous juveniles	48 (IV)	22 (IV)	31 (IV)	28 (IV)	25 (IV)

(MDD classification (EFSA PPR PANEL (2013)): 0: >100%, I: >90-100%, II: >70-90%, III: >50-70%, IV: ≤50%)

Biomass

No significant differences in the biomass were detected for tanylobous or epilobous juveniles compared to the respective untreated control at any sampling occasion. The deviations in biomass of the group epilobous juveniles varied from +4.4% (56 DAA1) to +22.7% (34 DAA1) at the post-application samplings. Tanylobous juvenile earthworms were present in low biomass of 0.0 g/m² in the control plots and 0.3 g/m² in the test item treated plots at the third post-application sampling (204 DAA1) in autumn 2018.

Table 16: MDDs calculated for biomass a posteriori for Dunnett's test (3 treatment groups: control, T, R, $\alpha = 0.05$, 4 replicates per treatment group, left sided test)

% MDD	Biomass				
Taxon	7 DBA1	34 DAA1	56 DAA1	204 DAA1	366 DAA1
Tanylobous juveniles	82 (II)	75 (II)	267 (0)	-	252 (0)
Epilobous juveniles	46 (IV)	29 (IV)	37 (IV)	36 (IV)	32 (IV)

(MDD classification (EFSA PPR Panel (2013)): 0: >100%, I: >90-100%, II: >70-90%, III: >50-70%, IV: ≤50%)

Except for the anecic taxon *Lumbricus terrestris* and the tanylobous juvenile earthworms the earthworm biomass of all taxa, epilobous juveniles and the ecological groups endogeic and anecic earthworms were significantly reduced in the toxic reference item plots on at least two post-application samplings compared to the respective control data. *Lumbricus terrestris* biomass was statistically significantly reduced in the toxic reference item plots at the third (204 DAA1) and at the final (366 DAA1) post-treatment sampling occasion, but was markedly lower than the control (between 73.9 and 100% reduction) on all post treatment sampling occasions.

The calculated MDDs for the data of the earthworm biomass were low (Table 6), mainly class IV and III according to the classification of EFSA PPR panel (2013). High MDDs were calculated for tanylobous juvenile earthworms. The MDD values of the taxon *Lumbricus terrestris* were low, and therefore small to medium effects can be statistically determined at the third (204 DAA1) and final (366 DAA1) earthworm population sampling. Due to the high MDDs for *Lumbricus terrestris* (class 0) at 34 DAA1 and 56 DAA1, which are related to the low biomass values, it is not possible to detect significant differences of the toxic reference compared to the untreated control.

Table 7: Mean biomass (g/m²), standard deviation and percentage change of species earthworm biomass in the test item treatment (T) and toxic reference treatment (R) relative to the control (C)

Species / group	Timing	Mean biomass (g/m ²) ± standard deviation and deviation from control (%) ^{a)}		
		C (untreated)	Final Bite – Blue (T)	Carbomax (R)
<i>Aporrectodea caliginosa</i>	7 DBA1	6.3±1.4	5.9±1.4 (-6.3)	5.1±0.9 (-18.1)
	34 DAA1	4.1±0.9	4.1±1.5 (-0.2)	0.3±0.3 * (-91.7)
	56 DAA1	2.8±2.1	3.4±0.6 (20.8)	0.1±0.2 * (-95.0)
	204 DAA1	5.8±0.5	5.6±1.3 (-4.3)	0.2±0.2 * (-96.9)
	366 DAA1	4.1±1.5	5.1±3.0 (23.6)	0.7±1.2 (-82.9)
<i>Aporrectodea longa</i>	7 DBA1	9.8±1.2	13.3±7.3 (36.3)	9.2±2.4 (-6.1)
	34 DAA1	14.5±5.7	10.7±5.2 (-25.9)	2.1±1.9 * (-85.8)
	56 DAA1	8.2±1.8	11.1±4.6 (35.7)	1.0±1.1 * (-88.1)
	204 DAA1	29.6±8.5	43.0±12.7 (45.4)	3.1±3.0 * (-89.5)
	366 DAA1	7.7±5.4	7.1±3.7 (-7.4)	0.0±0.0 * (-100.0)
<i>Aporrectodea rosea</i>	7 DBA1	4.8±0.9	4.9±0.8 (2.2)	5.0±1.0 (3.8)
	34 DAA1	4.6±1.8	5.2±0.7 (13.1)	2.4±0.5 (-47.6)
	56 DAA1	1.8±0.9	1.7±0.3 (-5.8)	0.3±0.3 * (-83.3)
	204 DAA1	5.8±1.8	5.5±1.0 (-4.0)	1.7±0.8 * (-89.8)
	366 DAA1	3.2±0.7	3.2±0.4 (-0.2)	2.4±2.0 (-26.0)
<i>Lumbricus terrestris</i>	7 DBA1	41.2±6.0	40.7±15.3 (-1.3)	42.2±11.8 (2.4)
	34 DAA1	20.5±15.7	27.4±16.6 (33.3)	4.7±5.1 (-77.2)
	56 DAA1	3.1±2.4	11.4±8.1 (270.3)	0.0±0.0 (-100.0)
	204 DAA1	41.1±18.3	59.5±18.7 (44.9)	6.5±1.4 * (-84.2)
	366 DAA1	44.4±8.4	38.7±23.1 (-12.9)	11.6±8.4 * (-73.9)
Tanylobous juveniles	7 DBA1	13.9±10.2	13.4±7.1 (-3.4)	12.8±2.9 (-7.4)
	34 DAA1	14.5±8.5	17.2±8.0 (18.5)	4.5±3.6 (-88.8)
	56 DAA1	2.9±2.1	5.0±8.1 (70.7)	1.3±2.7 (-54.5)
	204 DAA1	0.0±0.0	0.3±0.5 (-)	0.0±0.0 (-)
	366 DAA1	1.0±1.8	1.1±1.8 (17.4)	0.5±0.9 (-51.7)
Epilobous juveniles	7 DBA1	13.7±2.5	16.8±5.6 (23.2)	13.3±3.7 (-2.9)
	34 DAA1	9.1±1.8	11.2±2.2 (22.7)	3.0±0.7 * (-86.9)
	56 DAA1	9.3±3.7	9.8±1.1 (4.4)	0.8±0.3 * (-91.7)
	204 DAA1	8.3±1.3	9.2±1.8 (11.3)	4.8±2.5 * (-41.7)
	366 DAA1	9.3±2.7	9.9±0.7 (6.2)	5.1±2.0 * (-45.3)
Endogeic	7 DBA1	20.3±2.3	17.3±4.2 (-14.9)	16.9±4.7 (-16.8)
	34 DAA1	14.2±4.0	13.8±4.3 (-2.9)	3.0±0.3 * (-79.1)
	56 DAA1	5.1±2.8	6.2±1.6 (22.9)	0.6±0.4 * (-88.8)
	204 DAA1	15.8±3.8	13.7±2.0 (-13.6)	1.9±1.0 * (-87.9)
	366 DAA1	11.0±2.2	12.3±4.5 (12.5)	3.5±2.4 * (-87.9)
Anecic	7 DBA1	51.0±8.9	54.0±21.7 (5.9)	51.4±13.9 (0.8)
	34 DAA1	35.0±19.1	38.1±17.1 (8.8)	6.7±5.4 * (-80.8)
	56 DAA1	11.3±3.6	22.5±4.9 (99.9)	1.0±1.1 * (-91.4)
	204 DAA1	70.7±20.0	102.6±8.1 (45.1)	9.6±2.1 * (-86.4)
	366 DAA1	52.1±7.3	45.8±26.2 (-12.1)	11.6±8.4 * (-77.8)

^{a)} negative values (-) indicate lower earthworm numbers compared to the control; positive values (+) indicate higher earthworm numbers compared to the control

DBA1= days before application 1, DAA1= days after application 1

*significantly different from control (p ≤ 0.05)

Discussion

The study meets the guideline requirement for having at least 100 earthworms/m² (grassland) on initial sampling. The mean abundance of earthworms across the site was 232.9 /m² and the proportion of endogeic: anecic earthworms was approximate 8: 2, with 81.9 % endogeic and 18.1 % anecic. Again, this meets the recommendations of guidance for having at least 10 % of each group. The numbers of earthworms were reduced considerably during the warmer months of the study (third sampling occasion) recovering over the cooler months, with the exception of the reference item treated plot, which caused statistically significant effects on abundance and biomass for all subgroups as well as total earthworms.

The climatic conditions were, over the course of the whole study, dry compared to the long-term average, with an overall precipitation deficit of 28.5 %. During the period of test item application, artificial irrigation was used to increase precipitation to reflect the long-term average. The deviation from long term (total) precipitation was –

48.1 % in April 18 and +15.5 % in May 18. This indicates that total precipitation was not ideal within the earlier half of the application period. As seen in table 22, the total precipitation (mean irrigation + precipitation) over the course of 3 days post application exceeded 10 mm (in line with guidance) on the first, second and third applications. Therefore, while total precipitation was lower than long term average, the total precipitation immediately after applications 1, 2 and 3 was in line with guideline recommendations. It is not clear if sufficient precipitation occurred during applications 4, 5 and 6 as data for artificial and natural precipitation is not recorded between the 9th and 29th May (encompassing all three of these later applications). The data from table 32 suggests that natural precipitation may have been sufficient to ensure the guideline recommendation was met.

The application of the test item was confirmed by both measuring the weight of applied pellets and photography of random 1 m² plots which indicated that the desired rate of 8 kg product/ha was achieved at each application. The test site soil was not analysed in any way other than this visual observation. This, especially considering the dry climatic conditions (see above) leads to uncertainty regarding the representativeness of the study for wetter conditions, as it is not possible to determine the amount of degradation (creating ‘hotspots’ of high formulation concentration around the pellet) that occurred during the study. However, as discussed above, it is confirmed that at least during the first three applications that at least 10 mm total precipitation occurred in the three days after each application. There is uncertainty regarding the final three applications.

The biological results are supportive overall of there being no effects of the test item on total earthworm abundance under the conditions of the study. No statistically significant effects were noted on numbers or biomass of adult earthworms, juveniles and total earthworms between the first application and termination of the test. The reference item (Carbendazim, 10 kg/ha) caused statistically significant reductions in both abundance and biomass on every sampling occasion after the first, pre-treatment sample. MDD values were calculated after the study was completed and indicated that the numbers of earthworms were sufficient for the study to be able to detect effects of the test item in all three treatment groups (Class IV). The same conclusions can be made for endogeic earthworms, as represented by *A. calliginosa* and *A. rosea*, which made up the majority of earthworms in the site.

While the same results and overall trends were observed for anecic earthworms (represented in the study by *L. terrestris* and *A. longa*) the numbers of this group were particularly low on the second and third sampling occasions, with MDD classes of I and 0 (effects lower than 90 % cannot be detected, and no effects can be detected, respectively) for *L. terrestris*. The MDD values indicate the study is more reliable for *A. longa* (MDD class III throughout the same period), but the numbers overall are low (8.5-21.0 anecic earthworms in the control and test-item treated plots over the course of the study). As anecic earthworms come to the surface to feed, these are potentially more likely to be exposed to the intact test item than endogeic earthworms. The inability of the test to detect effects of the test item on the second and third sampling occasions for the ecologically important species *L. terrestris* means that there is high uncertainty with drawing conclusions regarding the effects of the test item on anecic species.

HSE Comments

It is noted that no batch number is provided for the iron used in the formulation, however, in light of updated consideration in the volume 4, it is concluded that the tested material is representative of the technical specification for elemental iron.

The study was conducted to GLP and follows the latest available guidance for Earthworm field studies, with modifications to allow for application of granules (instead of a standard chemical spray). The study was well reported and meets the guideline validity criteria for the numbers of earthworms (>100/m²) and overall proportions of endogeic and anecic earthworms (81.9 and 18.1 %, respectively). The toxic reference item, applied as a chemical spray (once at the same time as the first application of the test item), proved that the test organisms were sufficiently sensitive (in the context of a chemical spray), causing statistically significant effects on abundance and biomass in all groups analysed.

Six applications of the test item with a target rate of 8.0 kg/ha and an interval of 5 days were made over the period from 24 April 2018 to 19 May 2018. All application rates were applied within a limit of ±10%. Photographs taken before and after each application of the test item confirmed the correct application of the test item granules, however no chemical analysis of the soil was conducted.

No statistically significant reductions of total earthworm numbers or biomass, nor groupings (endogeic or anecic earthworms) or single species in test item treated plots compared to the respective earthworm data of the control plots occurred at any of the samplings after application of the test item.

The climatic conditions during the period of the study were dry compared to the long term average with a total precipitation deficit of 28.5 %. The ramifications of this are discussed further in the risk assessment for earthworms, but for the purposes of evaluating the study, the main effect seems to have been a generally low abundance of earthworms. Key species that could be exposed to the intact test item (*L. terrestris* and *A. longa*, i.e. Anecic species) were (possibly as a result of the dry conditions) low in abundance throughout the study, and their numbers were insufficient to be able to determine effects of the test item during the period immediately after the last application. This also lends uncertainty to the results for the toxic reference item. Therefore, while statistically significant effects of the test item were not detected for this group, the results are not considered reliable for the purposes of risk assessment.

Overall, the results indicate that under the conditions of the study, no statistically significant adverse effects of the test item (applied at 6x 8 kg product/ha, 5 day application interval) would be expected on endogeic earthworms. However the numbers of anecic earthworms were too low to be able to detect effects of the test item. The dry conditions and their effects on the usefulness of the study are discussed further in the risk assessment.

B.9.7.1.3. Other Earthworm toxicity studies

Report:	CP 10.7/02. [REDACTED] 2008
Title:	Slug & Snail Killer acute toxicity to earthworms (<i>Eisenia fetida</i> Sav.)
Report no.:	G/54/08 (R-38456)
Guidelines:	OECD 207 (1984) corresponding to method C.8
Deviations:	None
GLP:	Yes, certified
Published:	No

I. MATERIALS AND METHODS

A. MATERIALS:

1. **Test material:** Slug & Snail Killer (0.98% total iron)
Batch no.: not included in the report
Purity: 0.98% total iron
Description: grey-blue granules with characteristic odour
2. **Test organism:** *Eisenia fetida* Sav.
Age: adults
Source: Institute of Industrial Organic Chemistry, Branch Pszczyna
3. **Treatment:** 0 (control) 100, 180, 320, 560 and 1000 mg/kg (dry weight of artificial soil). (nominal)
4. **Test vessels:** glass containers covered with transparent, perforated plastic (to prevent the test medium from drying)
Soil: 10 % sphagnum peat, 20% clay, 70% industrial sand, mixed thoroughly; moisture of soil was determined to be 15% and pH 5.96
5. **Environmental conditions:**
Temperature: 21-23 °C
pH: 5.97-6.01
Moisture content: 35.0 (start) – 34.2 (end of the test) (%)
Photoperiod: continuous light (400 lux)

B. STUDY DESIGN AND METHODS

1. **In-life phase:** 27 Oct - 10 Nov 2008
2. **Test organism assignment and treatment**

Before the experiment, earthworms were acclimatised for 24 hours in artificial soil. Earthworms were washed and dried on lignin before use and then weighed (10 earthworms per replicate) and placed onto the test medium. Four replicates were used for each test group.

3. Dose preparation

A portion of the test item (granules) were milled, then the test item was introduced into the artificial soil. To 2360 g (i.e. 2000 g dry weight) of artificial soil, 720 ml of distilled water was used for each concentration in order to obtain appropriate moisture content (35%) at the beginning of the test. At the same manner control group with solvent was prepared but without test substance. The soil was placed into 1.3L glass containers by 750 g per replicate (i.e. 500 g dry weight of artificial soil).

4. Measurements and observations

The number of dead animals were assessed after 7 and 14 days from the beginning of the test. The appearance and the behaviour of the animals in each container was also recorded, as well as their weight after 14 days. Observation of the earthworms was achieved by emptying test medium onto a plastic plate, sorting worms from the medium and testing their reaction to a mechanical stimulus at the front end.

The temperature and light intensity of the test area were recorded as well as the pH values of the soil and the moisture content and the beginning and the end of the test.

Analysis on the test item was not performed.

A reference test on Chloroacetamide was conducted giving a LC₅₀ value of 73 mg/kg dry soil after 7 and 14 days.

5. Statistics

In order to determine the LC₅₀ value, statistical analysis of the obtained results was performed by using ToxRat Profesional (version 2.09). The probit method was used.

II. RESULTS AND DISCUSSION

A. Mortality and abnormal responses of earthworms

The number of dead animals and the percentage of mortality at 7 and 14 days of exposure are presented in Table B.9.7.1.3-1.

Table B.9.7.1.3-1: Mortality and behaviour of earthworms after 7 and 14 days of exposure

Nominal concentration (mg/kg dw soil)	Number of exposed earthworms	Mortality after 7 Days		Mortality after 14 Days	
		Dead/ immobilized animals (observations)	Total mortality (%)	Dead/ immobilized animals (observations)	Total mortality (%)
Test item					
0 (control)	40	0	0	0	0
100	40	0	0	0	0
180	40	0	0	0	0
320	40	1	2.5	2	5.0
560	40	7 (33 lm)	17.5	8 (32 lm)	20.0
1000	40	19 (21 lm)	47.5	20 (20 lm)	50.0

lm – less mobile animals

Table B.9.7.1.3-2: Bodyweight of earthworms after 14 days of exposure

Nominal concentration (mg/kg dw soil)	Mean weight of alive earthworms (<i>Eisenia fetida</i> Sav.)				
	Number of animals	Mean weight (mg) ± SD	Number of animals	Mean weight (mg) ± SD	Weight increase (mg)
0 (control)	40	313 ± 9	40	329 ± 5	16 (5.1%)
100	40	307 ± 3	40	332 ± 15	25 (8.1%)
180	40	307 ± 6	40	329 ± 15	22 (7.2%)

320	40	313 ± 17	38	330 ± 15	17 (5.4%)
560	40	305 ± 4	32	294 ± 30	-11 (-3.6%)
1000	40	301 ± 2	20	322 ± 30	21 (7.0%)

B. Analytical verification

No analytical verification was conducted during the test.

C. Validity criteria

The validity criterion was met: mortality in the control group was 0 % at the end of the test.

III. CONCLUSION

The NOEC after 7 and 14 days for Slug & Snail Killer to earthworm (*Eisenia fetida* Sav.) was determined to be 180 mg/kg of dry artificial soil.

The LC₅₀ was not calculated in the report.

HSE Comments:

No LC₅₀ was calculated; however, as this study is not required according to the current data requirements (284/2013) the endpoints from this study would not be used in risk assessment anyway.

This study indicates that products containing approximately 1% elemental iron have an effect on earthworm behaviour and mortality at concentrations of 320 mg/kg soil d.w. and above.

The NOEC based on mortality = 180 mg/kg soil d.w..

Study from the Literature Review search:

Report:	CA 8.4.1/01. Edwards, C.A. et.al. 2008
Title:	The relative toxicity of metaldehyde and iron phosphate-based molluscicides to earthworms
Report no.:	Not applicable
Guidelines:	Not applicable
Deviations:	Not applicable
GLP:	No
Published	Yes

I. MATERIALS AND METHODS**A. MATERIALS**

1. **Test material:** Iron phosphate (Fe(PO₄)₃), EDTA, 100% Fe(PO₄)₃ + 3% EDTA and EDTA
Batch: unknown
Purity: unknown
4. **Test substrate:** Artificial soil: 10% finely ground sphagnum peat (pH 5.5–6.0), 20% kaolinite clay (containing >30% kaolinite), 70% industrial quartz sand (dominant fine sand with more than 50% of particle size of 0.05–2.0 mm), 1% calcium carbonate (CaCO₃ – pulverized to bring the pH of mixture to 6.0 + 0.5) - 35% dry weight moisture content.
Test units: Glass jars for OECD type test, PVC pipes (15 cm diameter x 30 cm deep) with a surface area of 0.0177m² covered with fine mesh gauze (1.0 mm aperture) at top and bottom to keep the earthworms from leaving the microcosms.
5. **Test organisms:**
Species: Earthworm *Eisenia fetida* (OECD type test) and *Lumbricus terrestris* (Microcosm)
Age: not reported for *E. fetida*, large adult, clitellate *L. terrestris* at test initiation
Source: *E. fetida* bred in laboratory cultures
Weight: ca. 1 mg of each *E. fetida* and 3 ± 0.5 g (*L. terrestris* - at test initiation)
Acclimation: 7 days, under test conditions for *L. terrestris*
Diet: unreported
6. **Treatment groups:** 0 (control), 0.1, 1.0, 10.0, 100.0, 1000.0, and 10,000.0 mg a.s./kg soil d.w. soil. It was not reported for the OECD type test if the pellets were applied to the soil whole or ground. In the microcosm, the pellets were applied whole to the soil surface after the worms had burrowed.
Replicates: OECD type test: Four replicates of each test item and dose. Microcosm: six replicates of each test item and dose.

7. Environmental conditions:

Temperature: ca. 20°C (OECD type test), 4°C in microcosm
pH of soil: unreported
Water content of soil: approximately 35% of the dry weight

B. STUDY DESIGN AND METHODS**1. Dose preparation****(OECD type test) artificial soil earthworm toxicity test:**

Iron phosphate was ‘applied, mixed with 10 g finely ground quartz sand’, and EDDS and EDTA were applied in deionized water solution. The control and EDDS and EDTA treatments also received 10 g finely ground quartz sand.

Microcosm:

The following pellet treatments based on these calculations were applied to the surface of the soils in the microcosm and all treatments were replicated six times:

- Control – no chemical treatment (1 pellet per microcosm), Control – no chemical treatment (5 pellets per microcosm)
- Chelated iron phosphate (1%) (Sluggo®) recommended application rate (6 pellets per microcosm), Chelated iron phosphate (1%), Sluggo® 5x recommended application rate (30 pellets per microcosm)
- Iron phosphate only (3%), recommended application rate (6 pellets per microcosm), Iron phosphate only (3%), 5x recommended application rate (30 pellets per microcosm)
- Chelating agent only (3%), EDTA pellets recommended application rate (6 pellets per microcosm) Chelating agent only (3%), EDTA pellets recommended application rate (6 pellets per microcosm)

2. Measurements and observations**(OECD type test) artificial soil earthworm toxicity test:**

The weight of *E. fetida* was measured before addition and mortality was recorded.

Microcosm:

The numbers of pellets removed by *L. terrestris* from each microcosm were recorded daily by counting those remaining on the surface for 14 days. After 14 days, all of the remaining pellets (if any) were removed from soil surfaces. At the end of the experiment after 21 days, the earthworms were collected from each microcosm and their condition, numbers, and wet weights recorded.

3. Statistics**(OECD type test) artificial soil earthworm toxicity test:**

Data were analysed statistically by analysis of variance using S.A.S. software. The LD₅₀ values were estimated using Trimmed Spearman–Kärber Version 1.5 (S.E.P.A. Cincinnati, Ohio) designed for analysis of mortality data from acute chronic toxicity tests.

Microcosm:

The data were subjected to an analysis of variance and Fisher’s protected LSD ($P \leq 0.05$) was used to determine significant differences between treatment means. Appropriate *a priori* paired treatment means were subjected to Contrast analyses using SAS statistical software. Where possible, LC₅₀ values were estimated for treatment.

II. RESULTS AND DISCUSSION**(OECD type test) artificial soil earthworm toxicity test:**

Iron phosphate had no significant effects on the earthworms at any of the concentrations tested. The earthworms. However, EDTA and EDDS had significant effects ($P \leq 0.05$) on earthworm numbers at concentrations between 100 and 1000 mg kg⁻¹ (ppm). The combination of iron phosphate with EDTA had even greater effects on the earthworm numbers. This conclusion is emphasized by the LD₅₀ calculations for metaldehyde, iron phosphate, EDTA and EDDS and their mixtures.

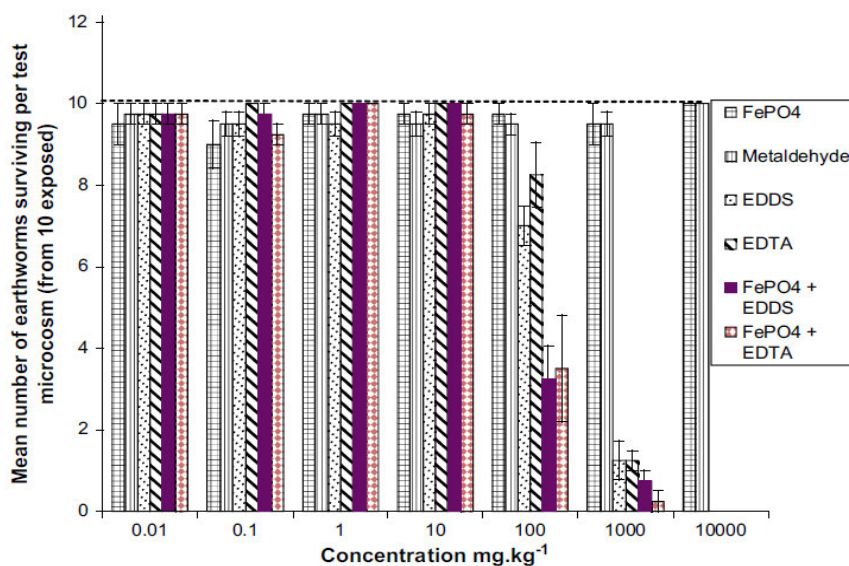


Figure B.9.7.1.3-1: Effects of molluscicides on earthworm (*Eisenia fetida*) activity in artificial soil (OECD test). Number surviving (\pm SE).

The data from the OECD artificial soil test showed that when *E. fetida* were exposed to iron phosphate on artificial soil, they were not killed by concentrations even as high as 10,000 ppm. By contrast, when they were exposed to EDTA they were affected by concentrations as low as 100 ppm with LD₅₀ values of 156.46 mg/kg. When they were exposed to iron phosphate chelated with EDTA the toxicity was even greater (LD₅₀ = 78.16 mg/kg).

Microcosm:

There was virtually no earthworm (*L. terrestris*) mortality over the 14 days of the experiment, but there were considerable differences in earthworm weights, although none of them differed significantly ($P \leq 0.05$), from the control earthworm mean weights. The earthworms that were exposed to Chelated iron phosphate (Sluggo®) at the recommended application rate gained significantly less weight ($P \leq 0.05$) than those exposed to iron phosphate only, as did those exposed to five times the recommended application rate of Chelated iron phosphate (Sluggo®).

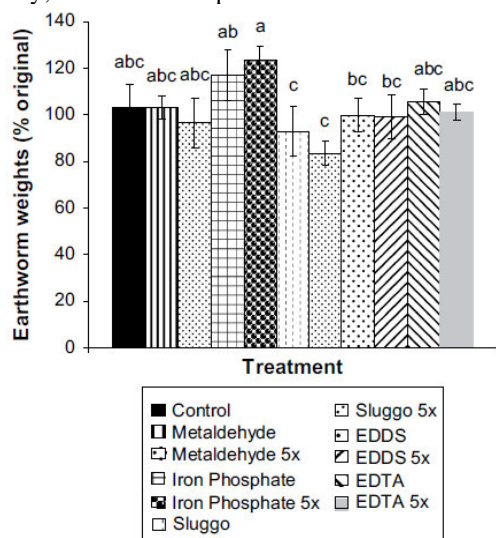


Figure B.9.7.1.3-2: *L. terrestris* bodyweight as percentage of starting weight after exposure to control, molluscicide pellet or chelating agent in microcosm conditions. (Columns with same letters a, b, or c are not significantly different ($P \leq 0.05$).)

III. CONCLUSION

EDTA and its complex with iron phosphate increased the toxicity of earthworms from this form of exposure. These data together provide clear evidence that molluscicides containing iron phosphate combined with either EDTA can have adverse effects on earthworm activity or growth and may possibly be toxic to them.

HSE Comments:

Some basic information was missing from the report, such as batch number, details of application method (OECD type test) and worm age (OECD type test). The application methods will need to be considered further in the context of the risk assessment. Confidence intervals for the LC₅₀ endpoints were not reported.

However, the study does indicate some basic conclusions, i.e. Ferrous phosphate is not toxic to earthworms, EDTA is toxic to earthworms and Ferrous phosphate with EDTA is very toxic to earthworms. The conclusions of this study should be considered for relevance and reliability in the context of the risk assessment.

B.9.7.2. Effects on non-target soil meso- and macrofauna (other than earthworms)

Report:	CP 10.4.2/01. [REDACTED] 2018c
Title:	Final Bite - 0402206: Effects on Reproduction of the Collembola <i>Folsomia candida</i> in Artificial Soil
Report no.:	127511016 (R-37823)
Guidelines:	OECD 232 and ISO 11267
GLP:	Yes, certified
Published:	No

I. MATERIALS AND METHODS

A. MATERIALS:

1. **Test material:** Final Bite - 0402206
Batch no.: KM80174200H
Purity: 10 g/kg elemental iron (nominal); 1.21 % w/w (analysed)
Description: Solid blue pellets
2. **Reference material:** Boric acid
Ref. concentration: 100.3%
3. **Vehicle:** None
4. **Test substrate:** Artificial soil: 5% Sphagnum peat, 20% Kaolin clay, 74.8% fine quartz sand, 0.2% calcium carbonate
Test units: Glass containers (volume: 100 mL; diameter: 5 cm), closed tightly to avoid water evaporation, filled with 30 g ± 1.0 g artificial soil fresh weight
5. **Test organisms:** Collembola; Isotomidae (commonly known as springtails)
Species: *Folsomia candida* (Willem 1902)
Age: Juveniles (at experimental start), adults, 9 - 11 days
Source: The synchronised individuals were bred at ibacon and were fed with granulated dry yeast and kept under breeding conditions until test start
Diet: After the introduction of the test organisms (day 0), and after 14 days, approximately 2 mg (one spoon spatula) of granulated dried yeast
Ventilation: All vessels including the additional containers were ventilated on day 3, 5, 7, 10, 12, 14, 17, 19, 21, 24 and 26 by opening the lids for a short period.
6. **Treatment:** Control, 334, 484, 702, 1018, 1476, 2140, 3103 and 4500 mg Final Bite - 0402206/kg soil (nominal)
7. **Environmental conditions:**
Temperature: 18.0 - 22.0°C
pH of soil: at experimental start 5.3 to 5.6, pH at experimental end 5.4 to 5.5
Water content of soil: at experimental start 22.1% to 23.1% (50.2% to 52.5% of the maximum water holding capacity); at experimental end 18.7% to 20.9% (42.5% to 47.4% of the maximum water holding capacity)
Photoperiod: 16 h light : 8 h dark, light intensity within the range of 400 to 800 lux

B. STUDY DESIGN AND METHODS

1. **In-life phase:** 10 Nov. – 11 Dec 2017
2. **Test organism assignment and treatment**

Individuals were collected with an aspirator, put into a small glass tube, counted to ensure that 10 Collembolans were assigned in each unit and placed onto the surface of the treated artificial soil. In total, 4 replicates were set per treatment group and 8 for the control, 1 additional container per treatment to check the pH and water content of the test substrate after 28 days.

3. Dose preparation

Amounts of Final Bite - 0402206 were weighed separately for each concentration using an analytical balance and 75 g fine quartz sand was added. The test item was pulverized using mortar and pestle before mixing it with fine quartz sand to reach a homogeneous distribution of the test item within the sand. The mixture was added to artificial soil with reduced sand fraction equivalent to 300 g final dry weight (225 g soil dry weight plus 75 g sand).

4. Measurements and observations

To measure the mortality, the numbers of living adult Collembola at day 28 after application were recorded. Missing adult Collembola were recorded as dead as it is assumed that missing adult Collembola had died and degraded during the test period. Surviving Collembola were observed for any abnormal behaviour or conditions at day 28 after application. The number of juvenile Collembola were also measured to calculate the reproduction effects at day 28 after application.

The contents of the test containers were suspended in water; the suspensions were tinted with dark ink and stirred with a fine brush. The Collembola drifted to the surface. Adult animals were counted once visually; juvenile animals were counted at least twice under binocular microscopes. 3 of the replicates were counted three times because the first two counts deviated more than 10% from their mean value. (The extraction efficiency was checked separately in September 2017, the extraction efficiency was 99.2%.)

5. Statistics

Mortality data were statistically analysed using Fisher's Exact Binomial Test (multiple comparison, with Bonferroni Correction, $\alpha = 0.05$, one-sided greater).

Reproduction data were tested for normal distribution and homogeneity of variance using Shapiro-Wilk's test and Levene's test ($\alpha = 0.05$). Since the reproduction data were normally distributed and homogeneous and did follow a monotonicity trend (contrast trend) the Williams t-test (multiple comparison, $\alpha = 0.05$, one-sided smaller) was used to compare treatment and control values.

The determination of the NOEC and LOEC values was based on the results of the statistical evaluation. The EC values for reproduction were calculated by Probit Analysis. The software used to perform the statistical analysis was ToxRat Professional, Version 3.2.1, ToxRat® Solutions GmbH.

II. RESULTS AND DISCUSSION

A. Mortality and abnormal responses of collembolans

Mortality of *Folsomia candida* was not statistically significantly different compared to the control up to and including the highest test concentration of 4500 mg test item/kg soil (Fisher's Exact Test, $\alpha = 0.05$, one-sided greater). There was no biologically significant effect on mortality in any of the tested groups. The No Observed Effect Concentration (NOEC) for mortality was determined to be 4500 mg test item/kg soil and the Lowest Observed Effect Concentration (LOEC) was estimated to be >4500 mg test item/kg soil.

The LC₅₀ of Final Bite - 0402206 for *Folsomia candida* in artificial soil was estimated to be >4500 mg test item/kg soil. No abnormal behaviour was observed with the surviving Collembola.

There were no statistically or biologically significant effects on reproduction of *Folsomia candida* up to and including the concentration of 1476 mg/kg soil. At the test concentration of 2140 mg/kg soil and above reproduction was statistically significantly reduced when compared to the control (Williams t-test, $\alpha = 0.05$, one-sided smaller).

Therefore, the NOEC for reproduction was determined to be 1476 mg test item/kg soil and the LOEC was determined to be 2140 mg test item/kg soil. The EC₁₀ was determined to be 1154.8 mg test item/kg soil (95% confidence levels: 538.8 – 1611.3 mg test item/kg soil), the EC₂₀ was determined to be 2143.4 mg test item/kg soil

(95% confidence levels: 1502.2 – 2735.6 mg test item/kg soil) and the EC₅₀ was estimated to be >4500 mg test item/kg soil.

Table B.9.7.2-1: Mortality and sub-lethal effects on collembola and resulting endpoints

Final Bite - 0402206 [mg/kg soil dry weight]	Control	334	484	702	1018	1476	2140	3103	4500
Mortality (day 28) [%]	13	20	8	5	10	13	18	28	23
Significance ¹⁾	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
No. of juveniles (day 28)	548	564	510	548	519	517	434	377	349
Significance ²⁾	-	n.s.	n.s.	n.s.	n.s.	n.s.	*	*	*
Reproduction in [%] of control (day 28)	-	103	93	100	95	94	79	69	64
Endpoints [mg test item/kg soil dry weight]									
NOEC (mortality)	4500								
LOEC (mortality) ³⁾	>4500								
LC ₅₀ (mortality) ³⁾	>4500								
NOEC (reproduction)	1476								
LOEC (reproduction)	2140								
EC Values (reproduction)	EC ₁₀ ⁴⁾			EC ₂₀ ⁴⁾			EC ₅₀ ³⁾		
	1154.8			2143.4			>4500		
95% confidence limits	538.8 – 1611.3			1502.2 – 2735.6			-		

n.s. = not significantly different compared to the control * = significantly different compared to the control

¹⁾ Fisher's Exact Test, $\alpha = 0.05$, one-sided greater

²⁾ Williams t-test, $\alpha = 0.05$, one-sided smaller

³⁾ estimated value ⁴⁾ Probit analysis

- not applicable

B. Analytical verification

No analytical verification was conducted.

C. Validity criteria

Mean mortality in the controls was 13% ($\leq 20\%$), the mean number of juvenile Collembola per replicate was 430 to 625 (≥ 100 juveniles per container) and the coefficient of variation of the control reproduction was 10.9% ($< 30\%$) and therefore, all validity criteria were met.

III. CONCLUSION

Final Bite - 0402206 caused no statistically significant effects on mortality of *Folsomia candida* up to and including the concentration of 4500 mg test item/kg soil and no effects on reproduction up to and including the concentration of 1476 mg test item/kg soil.

The No Observed Effect Concentration (NOEC) for mortality was determined to be 4500 mg test item/kg soil and the Lowest Observed Effect Concentration (LOEC) was estimated to be >4500 mg test item/kg soil. The NOEC for reproduction was determined to be 1476 mg test item/kg soil and the LOEC was determined to be 2140 mg test item/kg soil. The EC₁₀ was determined to be 1154.8 mg test item/kg soil (95% confidence levels: 538.8 – 1611.3 mg test item/kg soil), the EC₂₀ was determined to be 2143.4 mg test item/kg soil (95% confidence levels: 1502.2 – 2735.6 mg test item/kg soil) and the EC₅₀ was estimated to be >4500 mg test item/kg soil.

HSE Comments:

It is noted that no batch number is provided for the iron used in the formulation, however, in light of updated consideration in the volume 4, it is concluded that the tested material is representative of the technical specification for elemental iron.

The study was conducted according to the agreed guideline and in compliance with GLP. It is noted that the pellets were ground up and mixed through the test soil, i.e. exposure to whole pellets was not assessed. The endpoints are suitable for use in regulatory risk assessment. **The NOEC, EC₁₀ and EC₂₀ values for reproduction are 1476,**

1155 and 2143 mg test item/kg dry soil. It is however noted that the confidence intervals for the EC₁₀ and EC₂₀ values are rather wide; this should be considered in the context of the risk assessment.

Report:	CP 10.4.2/02. [REDACTED] 2018d
Title:	Final Bite - 0402206: Effects on Reproduction of the Predatory Mite <i>Hypoaspis aculeifer</i> in Artificial Soil
Report no.:	127511089 (R-37824)
Guidelines:	Guidelines for the testing of chemicals No. 226 Predatory Mite (<i>Hypoaspis (aculeifer)</i>) reproduction test in soil, adopted July 29, 2016
GLP:	Yes, certified
Published:	No

I. MATERIALS AND METHODS

A. MATERIALS:

1. **Test material:** Final Bite - 0402206
Batch no.: KM8017420OH
Purity: 10 g/kg elemental iron (nominal); 1.21 % w/w (analysed)
Description: Solid blue pellets
2. **Reference material:** Perfekthion
Ref. concentration: a.s. dimethoate, 400.0 g/L, nominal
3. **Vehicle:** None
4. **Test substrate:** Artificial soil: 5% Sphagnum peat, 20% Kaolin clay, 74.8% fine quartz sand, 0.2% calcium carbonate
Test units: Glass containers (volume: 100 mL; diameter: 5 cm), tight screw top closure to avoid water evaporation, filled with approximately 20 g ± 1.0 g artificial soil dry weight, the height of the soil layer in the containers was 1.5 – 2.0 cm.
5. **Test organisms:** Predatory mite
Species: *Hypoaspis aculeifer*
Age: adult females, approximately 11 days after reaching the adult stage (32 days after placing adult females in clean rearing vessels)
Source: cultured by ibacon
Diet: One spatula of cheese mites (*Tyrophagus putrescentiae* cultured by ibacon) on days 3, 5, 7, 10 and 12.
Ventilation: All vessels including the additional containers were ventilated on days 3, 5, 7, 10 and 12 by opening the lids for a short period.
6. **Treatment:** Control, 334, 484, 702, 1018, 1476, 2140, 3103 and 4500 mg Final Bite - 0402206/kg soil.
7. **Environmental conditions:**
Temperature: 18 – 22 °C
pH of soil: 5.3 to 5.6 (initial) 5.7 to 5.8 (at exp. end)
Water content of soil: at experimental start 22.1% to 23.1% (50.2% to 52.5% of the maximum water holding capacity); at experimental end 20.6% to 21.9% (46.8% to 49.7% of the maximum water holding capacity)
Photoperiod: 16 h light : 8 h dark (within the range of 400 to 800 lux).

B. STUDY DESIGN AND METHODS

1. **In-life phase:** 10 Nov. – 28 Nov. 2018
2. **Test organism assignment and treatment**

Adult female mites were collected with a fine brush, put into a small glass tube, counted to ensure that 10 females were assigned in each unit and placed onto the surface of the treated artificial soil. In total, 4 replicates were set per treatment group and 8 for the control, 1 additional container per treatment to check the pH and water content of the test substrate after 14 days.

3. Dose preparation

Amounts of Final Bite - 0402206 were weighed separately for each concentration using an analytical balance and 75 g fine quartz sand was added. The test item was pulverized using mortar and pestle before mixing it with fine

quartz sand to reach a homogeneous distribution of the test item within the sand. The mixture was added to artificial soil with reduced sand fraction equivalent to 300 g final dry weight (225 g soil dry weight plus 75 g sand).

4. Measurements and observations

After 14 days exposure the soil was filled into Millipore pots with attached plastic containers for collecting the escaping mites. These extraction units were placed in a Kempson extractor. The soil including the mites was exposed to a temperature of approximately 25°C and 30°C for approximately 3 days. Escaping mites were collected in a fixing liquid, cooled at a temperature of approximately 16°C. The fixing liquid contained glycol and a detergent. Adult animals were counted once visually, juvenile animals were counted twice under binocular microscopes. One of the replicates was counted three times because the first two counts deviated more than 10% from their mean value. The extraction efficiency was checked separately in June 2017; it was determined to be 96.8%.

Number of surviving adult female predatory mites 14 days after test initiation was recorded (counted after extraction). Missing adult predatory mites were recorded as dead as it was assumed they would have died and degraded during the test period. The living predatory mites were observed for differences in morphology or any abnormalities at experimental end. Number of juvenile mites at day 14 after application, counted after extraction.

5. Statistics

Mortality data were statistically analysed using Fisher's Exact Binomial Test (multiple comparison, with Bonferroni Correction, $\alpha = 0.05$, one-sided greater). The LC_{50} at day 14 was not determined by statistical analysis as no mortality above 50% was observed.

Reproduction data were tested for normal distribution and homogeneity of variance using Shapiro-Wilk's test and Levene's test ($\alpha = 0.05$). Since the reproduction data were normally distributed and homogeneous and did follow a monotonicity trend (contrast trend) the Williams t-test (multiple comparison, $\alpha = 0.05$, one-sided smaller) was used to compare treatment and control values.

The determination of the NOEC and LOEC values was based on the results of the statistical evaluation. The EC values could not be determined by statistical analysis since there was no adequate concentration response.

The software used to perform the statistical analysis was ToxRat Professional, Version 3.2.1, ToxRat® Solutions GmbH.

II. RESULTS AND DISCUSSION

A. Mortality and abnormal responses of Predatory mite

One mite died in the control group and one in the lowest tested concentration group, corresponding to 1 and 3% mortality, respectively. No mortality was observed in any other test item groups. The mortality was not considered to be biologically relevant and was lower than the validity criterion threshold of 20%. Therefore, the No Observed Effect Concentration (NOEC) for mortality was determined to be 4500 mg test item/kg soil and the Lowest Observed Effect Concentration (LOEC) was estimated to be >4500 mg test item/kg soil.

No differences in morphology of the mites between the test item treated groups and the control were observed.

There were no biologically or statistically significant effects on reproduction of *Hypoaspis aculeifer* up to and including the concentration of 3103 mg/kg soil. At the concentration of 4500 mg test item/kg soil a statistically significant decrease of reproduction was observed (Williams t-test, $\alpha = 0.05$, one-sided smaller). Therefore, the No Observed Effect Concentration (NOEC) for reproduction was determined to be 3103 mg test item/kg soil and the Lowest Observed Effect Concentration (LOEC) was determined to be 4500 mg test item/kg soil. The EC_{10} and EC_{20} values for reproduction were not determined by statistical analysis since there was no adequate concentration response. Results are summarised in the table below.

Table B.9.7.2-2: Mortality and sublethal effects on *Hypoaspis* and resulting endpoints

Final Bite - 0402206 [mg/kg soil dry weight]	Control	334	484	702	1018	1476	2140	3103	4500
Mortality (day 14) [%]	1	3	0	0	0	0	0	0	0
Statistical significance ¹⁾	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
No. of juveniles (day 14)	203	203	203	176	185	173	198	186	166
Reproduction in [%] of control (day 14)	-	100	100	86	91	85	97	91	82
Statistical significance ²⁾	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
Endpoints [mg/kg soil dry weight]									
NOEC (mortality)	4500								
LOEC (mortality) ³⁾	>4500								
LC_{50} (mortality) ³⁾	>4500								
NOEC (reproduction)	3103								
LOEC (reproduction)	4500								
EC_{50} (reproduction) ³⁾	>4500								

n.s. = not statistically significantly different compared to the control

* = statistically significantly different compared to the control

¹⁾ Fisher's Exact Test, $\alpha = 0.05$, one-sided greater

²⁾ Williams t-test, $\alpha = 0.05$, one-sided smaller

³⁾ estimated value

- not applicable

B. Validity criteria

Mean mortality of the controls was 1% ($\leq 20\%$ of the introduced adult female animals), the number of juvenile mites per replicate was 159 to 223 (≥ 50 juveniles per test unit) and the coefficient of variation (CoV) was calculated to be 9.9% ($\leq 30\%$) and therefore the study met all the validity criteria.

III. CONCLUSION

Final Bite - 0402206 caused no statistically significant effects on mortality of *Hypoaspis aculeifer* up to and including the concentration of 4500 mg test item/kg soil and no effects on reproduction up to and including the concentration of 3103 mg test item/kg soil.

The No Observed Effect Concentration (NOEC) for mortality was determined to be 4500 mg test item/kg soil and the Lowest Observed Effect Concentration (LOEC) was estimated to be >4500 mg test item/kg soil. The NOEC for reproduction was determined to be 3103 mg test item/kg soil and the LOEC was determined to be 4500 mg test item/kg soil. The EC_{10} and EC_{20} values for reproduction were not determined by statistical analysis since there was no adequate concentration response.

HSE Comments:

It is noted that no batch number is provided for the iron used in the formulation, however, in light of updated consideration in the volume 4, it is concluded that the tested material is representative of the technical specification for elemental iron.

The study was conducted according to the agreed guideline and in compliance with GLP. It is noted that the pellets were ground up and mixed through the test soil, i.e. exposure to whole pellets was not assessed. The endpoints are suitable for use in regulatory risk assessment. EC_{10} and EC_{20} values could not be determined. **The NOEC for reproduction was 3103 mg test item/kg dry soil.**

B.9.8. RISK ASSESSMENT FOR NON-TARGET SOIL MESO- AND MACROFAUNA

The toxicity endpoints have been determined in terms of the test item, i.e. the product per kg soil. Two sub-lethal toxicity studies were submitted for earthworms and two sub-lethal toxicity studies were submitted for other soil macro-organisms on *Folsomia candida* and *Hypoaspis aculeifer*.

The derived toxicity endpoints for soil meso- and macrofauna are summarised in Table B.9.8-1.

Table B.9.8-1: Summary of toxicity data to Earthworms and soil macro-organisms

Test species	Time scale	Test material	Endpoint *	Data point Author, year
<i>Eisenia fetida</i>	Long-term 8 w	Final Bite – 0402206 ¹⁾	NOEC _{weight} = 1050 mg product/kg dw soil NOEC_{repro} = 100 mg product/kg dw soil LOEC _{repro} = 180 mg product/kg dw soil EC ₁₀ = 147.1 mg product/kg dw soil EC ₂₀ = 188.6 mg product/kg dw soil EC ₅₀ = 303.7 mg product/kg dw soil	CP 10.4.1.1/01. [REDACTED] 2018a
<i>Eisenia andrei</i>	Long-term 8 w	Final Bite – 0402206 ²⁾	NOEC _{mortality} = 95.9 pellets/m ² LOEC _{mortality} = 1391 pellets/m ² LC ₁₀ = 1109 pellets/m ² LC ₂₀ = 1667 pellets/m ² LC ₅₀ = 3633 pellets/m ² NOEC _{weight} = 662 pellets/m ² LOEC _{weight} = 959 pellets/m ² NOEC _{repro} = <662 pellets/m ² LOEC _{repro} = 662 pellets/m ² dw soil EC ₁₀ = n.d. EC₂₀ = 460 pellets/m² EC ₅₀ = 743 pellets/m ²	CP 10.4.1.1/02. [REDACTED] 2018b
<i>Folsomia candida</i>	Long-term 28 d	Final Bite – 0402206 ¹⁾	NOEC _{mortality} = 4500 mg product /kg soil LOEC _{mortality} > 4500 mg product /kg soil NOEC _{reproduction} = 1476 mg product /kg soil LOEC _{reproduction} = 2140 mg product /kg soil EC_{10 repro} = 1154.8 mg product /kg dw soil EC _{20 repro} = 2143.4 mg product/kg dw soil EC _{50 repro} > 4500 mg product/kg dw soil	CP 10.4.2/01. [REDACTED] 2018c
<i>Hypoaspis aculeifer</i>	Long-term 14 d	Final Bite – 0402206 ¹⁾	NOEC _{mortality} = 4500 mg product /kg soil LOEC _{mortality} > 4500 mg product /kg soil NOEC_{reproduction} = 3103 mg product /kg soil LOEC _{reproduction} = 4500 mg product /kg soil EC ₁₀ = n.d.	CP 10.4.2/02. [REDACTED] 2018d

n.d. = not determined

¹⁾ test item (granules) mixed into the artificial soil - homogeneous distribution²⁾ test item (granules) applied onto the soil surface*no correction for the study with the formulation was performed since the logK_{ow} of elemental iron cannot be defined due to the insolubility of iron in waterNote: values in **bold** were used for the risk assessment

Elemental iron is a naturally occurring element in soil (UK Fate and Behaviour specialists estimate background levels of 2000-50000 mg/kg soil), indicating that the element is present in all soils. The calculated PEC_{soil,accumulation} is 12.8 mg a.s./kg based on the proposed use of the product accumulating over a 20 year period. This PEC, resulting from the worst case use of the product is significantly lower than the background levels; therefore, no quantitative risk assessment for exposure to the active substance has been carried out.

The published literature study (Edwards *et al.*, 2008) indicates that Ferrous phosphate is not toxic to earthworms, EDTA is toxic to earthworms and Ferrous phosphate with EDTA is very toxic to earthworms. This study did not investigate the effects of elemental iron alone, or in combination with EDTA.

In the toxicity tests with *Folsomia* and *Hypoaspis* the 'Final Bite' pellets were ground up and mixed through the test soil, i.e. exposure to intact pellets was not assessed. Soil-dwelling invertebrates will not directly consume whole pellets as they are too large; however, they might consume parts of pellets, either directly after the pellets have begun to degrade, or indirectly if their food becomes contaminated. The toxicity tests performed are considered to have covered the exposure route of direct and indirect consumption of degraded pellets in the most appropriate way possible in the laboratory situation, i.e. using a simulated degradation process. However, there is an inherent uncertainty with the method as its efficacy was not confirmed using a granular reference item. This

approach was discussed with the Expert Committee on Pesticides (ECP) in January 2022, who agreed it was sensible although considered the species tested to not be the key detritivores that might be at risk. That said, they recognised that the species *F. candida* and *H. aculeifer* were model organisms and form part of the data requirements for active substances, on that basis they agreed that a risk assessment based on these organisms was (at least formalistically) appropriate.

In the chronic earthworm toxicity study of [REDACTED] 2018b (*Eisenia andrei*) there were large, statistically significant effects on reproduction at the lowest tested concentration, therefore no NOEC or EC₁₀ could be derived. A EC₂₀ was derived, however. The method of application in this study was the placing of whole pellets on the test soil surface. In the chronic earthworm study of [REDACTED] 2018a (*Eisenia fetida*), the test item was ground up and homogenously distributed into the soil, as in the case of the soil macro-organisms also tested (see paragraph above). The effects were not as severe as in [REDACTED] 2018b (*Eisenia andrei*), with an overall NOEC of 100 mg product/kg. It is possible that this is due to the different species used, or could be due to the exposure method, which creates a homogenous distribution of (relatively) manageable concentration instead of limited areas of highly toxic concentration (i.e. the pellet and immediate surrounding area). Considering the high background levels of iron in soil and the high toxicity of complexing agents (both alone and in combination with a ferrous active substance - as shown in Edwards *et al.*, 2008) it is likely that the toxicity of the product to earthworms is being driven by the complexing agent in the formulation.

In VOL 3CP Section 8, Fate and Behaviour calculated a PEC_{SOIL} formulation based on the product application rate 48 kg product/ha. The PEC_{SOIL} of 'Final Bite ®' is 64 mg/kg.

The exposure estimate for the 'application rate' risk assessment will be based on the worst case GAP and will not take into account any dilution by mixing in. The exposure estimate calculation is summarised in the table below.

Table B.9.8-3: Soil exposure for use in the 'application rate' risk assessment

Test substance	Max application rate single	No. of applications	MAF	In-field PER
'Final Bite'	60 pellets/m ²	6	6.0	360 pellets/m ²

However, the earthworm field study conducted by Axmann (2019) in support of this application can be used as surrogate 'field data' regarding the accumulation of pellets in a worst-case application scenario (6x 8 kg product/ha, 5 day application interval, no interception from foliage). In this study, the amount of pellets before and after application were analysed by photographing 8x 1 m² plots. The maximum accumulated number of pellets per m² was 156. This is much lower than the worst-case approach of assuming a MAF of 6, and lower than the EC₂₀ measured for earthworms (460 pellets/m²: see the table below). In the aforementioned field study, the average number of pellets after one application was 66.3/m² (range: 46-99).

The risk assessment has been carried out in accordance with SANCO/10329/2002 rev 2 final (17 October 2002). The following formula has been used to calculate toxicity:exposure ratios:

$$TER_{LT} = \frac{\text{Toxicity endpoint}}{\text{Exposure estimate}}$$

The resulting TERs have been compared to the relevant trigger value, which is 5 for chronic effects on soil meso and macro-fauna. The risk assessment is summarised in Table B.9.8-4.

Table B.9.8-4: Long term risk assessment for earthworms exposed to 'Final Bite'

Organism	Species	Toxicity endpoint	Exposure estimate	Endpoint	TER _{LT}	Trigger value
Earthworm	<i>Eisenia fetida</i>	NOEC _{repro} ¹⁾	64 mg product/kg	100 mg product/kg	1.56	5
Earthworm	<i>Eisenia fetida</i>	Reproduction EC ₂₀ ²⁾	156* pellets/m ²	460 pellets/m ²	2.9	5
Collembola	<i>Folsomia candida</i>	EC _{10 repro}	64 mg product/kg	1154.8 mg product/kg dw soil	18.04	5

Predatory mites	<i>Hypoaspis aculeifer</i>	NOEC _{reproduction}	64 mg product/kg	3103 mg product/kg dw soil	48.48	5
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¹⁾ NOEC_{repro} derived from [REDACTED] 2018a

²⁾ Reproduction EC₂₀ derived from [REDACTED] 2018b

*156 pellets m² worst case accumulated number of pellets in Axmann (2019).

For *Folsomia candida* and *Hypoaspis aculeifer* the TER_{LT} values for ‘Final Bite’ are above the trigger value of 5, demonstrating an acceptable chronic risk following application in accordance with the proposed uses. The exposure method used in the toxicity studies is a reasonable worst case for these soil dwelling-organisms. No further consideration is necessary.

For *Eisenia fetida* the TER_{LT} value for ‘Final Bite’ is below the trigger value of 5 for both means of exposure, indicating an unacceptable risk at first tier. Refinement of the risk is required.

Further consideration of risk of representative product ‘Final Bite’ to Earthworms

Field Study

The applicant has submitted a field study (Axmann, 2019) which investigated the effects of ‘Final Bite’ applied six times to grassland plots, with a five day application interval, at an individual application rate of 8 kg product/ha. The application used could therefore be considered to be in excess of what would normally be applied (the requested GAP is for 6 such applications but over the course of a single growing season/year). The application period was April-May 2018 and the study continued until April 2019. This study was considered in section B.9.7.1.2 (this document) and found to be valid. No statistically significant adverse effects of the test item to earthworms were detected over the course of the year. Indeed, there were no observable differences between the control and test item-treated plots in abundance or biomass for any of the earthworm populations sampled during the study. However, there were some shortcomings identified.

- For anecic species (represented by *L. terrestris* and *A. longa*) the abundance was low and in the case of *L. terrestris*, corresponding MDD values were not low enough to detect even large effects of the test item on the second and third sampling occasions (MDD class 1 and 0 respectively). The low abundance of this species, and anecic species in general, was thought to be due to the high temperatures and dry environment. Anecic species, which live in deep vertical burrows and come to the soil surface to feed, can be exposed directly to the test item, so for the purposes of this risk assessment are arguably the key species group. The lack of effects on endogeic earthworms (sufficiently abundant in the study) is somewhat negated by the fact that these species, living beneath the soil surface, are unlikely to be exposed to the test item except when it degrades into the soil.
- The dry environment also means that the study is not necessarily representative of wetter environments frequently encountered in the UK, particularly during autumnal applications (application in the study was in the Spring). The representative product is designed to be water-resistant (pellets after rainfastness trials were still mostly intact after 14 days of 10 mm daily rainfall), however this does not necessarily reduce the risk of ‘hotspots’ of the formulation in the immediate vicinity of the pellets even if they are consumed before they breakdown (as the iron and other cofomulants may remain after the target organism has perished). The concentration of iron and cofomulants in the soil was not investigated by chemical analysis in the study. Wetter, milder environments might produce a greater quantity of earthworms potentially exposed to the test item, and also increase the activity of the earthworms, meaning they would be more likely to be exposed to the test item. It would also result in greater breakdown of the product meaning it would be more bioavailable to the earthworms.
- Furthermore, the reference item tested (carbendazim) is applied as a spray so while the results show that the earthworms were sensitive, it does not necessarily show that the test system itself was sensitive to the effects of granule-based test items.

Consultation with the Applicant and ECP

The applicant did not agree with CRD’s position on the Axmann (2019) study and sought advice from a specialist in earthworm field studies who considered the study and concluded that it was in fact reliable and relevant for the GAP (Brown, 2021 – see Appendix 4). CRD examined this position paper but concluded that it did not sufficiently address the concerns previously raised – in particular, the low number of anecic earthworms and the extrapolation

from a spray reference item to a granular test item. The paper did, however, address concerns that the dry conditions observed in the study during April-August were representative of key UK agricultural locations (East Anglia, the Southeast) during the same period. That said, this did not allay concerns regarding the representativeness of the study for climatic conditions during the autumn (this is discussed in detail under ‘Literature Review Report’ below). The applicant, following a meeting with CRD in December 2021, agreed that a literature review focusing on earthworms and granular applications would be the best next step in terms of addressing CRD’s concerns. In addition to this, CRD’s conclusions were taken before the Expert Committee on Pesticides (ECP) in January 2022. Their written conclusion was as follows:

“The ECP noted that the proposed product use was for spring and autumn applications and concluded that the study was only directly relevant to the former. However, the Committee also noted that there are seasonal peak periods of earthworm activity that coincide with the two proposed application periods, and so the study was representative of earthworm behaviour at both proposed times of product application. Non-anecic earthworm species are usually more numerous in agricultural soils and the number of anecic worms reported in this study compares favourably with population densities typical of UK and European farm habitats (Rutgers et. al. (2016) Mapping Earthworm Communities in Europe. Applied Soil Ecology 97, 98-111.).

The ECP concluded that sufficient numbers of anecic earthworms were recovered for the study to be considered representative of field populations, and that they were susceptible to the toxic standard treatment.”

Literature Review Report

In response to these concerns the applicant submitted an additional literature review report (Brown, 2022 – see Appendix 4) which considered the following questions:

- 1) What are typical UK numbers of the anecic species *L. terrestris* and *A. longa*, and how do these compare with the field study by Axmann (2019)?
- 2) What are the ideal growth conditions for the anecic species *L. terrestris* and *A. longa*, and how do these compare with the field study by Axmann (2019)?
- 3) Do foraging anecic earthworms encounter and consume slug pellets and are they affected by iron-containing slug pellets? Do the results of Axmann (2019) indicate any such effects?

The literature review was not limited to a particular time period. Three databases were searched – Web of Science (interdisciplinary citation database); PubMed (biomedical science and toxicology citation database); Science direct (interdisciplinary citation database). The methodology of the literature review is in line with EFSA (2011) and the conclusions of the CRD/ADAMA meeting in December 2021, by not imposing a time window restriction (EFSA 2011 standard literature searches are normally limited to the ten years prior to submission). It is noted that while the returned publications cover a timespan of 1964-2022, the majority seem to have been published within the last 20 years. It was noted that none of the search terms were iron-specific.

Overall, 274 articles were returned not including duplicates. These were sifted, based on their abstracts, for relevance to the questions above. Those considered relevant based on the abstract were ordered and reviewed in full for reliability and relevance to the questions. Of the 274 from the initial search, 22 (plus an additional 2) were considered relevant and reliable for question 1, 39 were considered so for question 2 and 8 were considered so for question 3, a total of 71 publications.

Each question addressed by the literature review is considered below.

1) What are typical UK numbers of the anecic species *L. terrestris* and *A. longa*, and how do these compare with the field study by Axmann (2019)?

Brown (2022) collated information from several UK-specific sources derived from the literature review and found that anecic earthworm species *A. longa* and *L. terrestris* typically varied in number from 1 to 20 individuals/m², with as few as 0/m² or as many as 50/m² reported, though sources varied in their sampling methods in that some counted adults only and others included juveniles. Populations of both species were consistently higher in grassland than in arable soils, where tillage is the main factor affecting earthworm abundance. It was noted that sampling methods used in the cited sources may have been biased against sampling adult anecic earthworms, with shallow surface digging and limited observation periods meaning that mostly juveniles (which cannot burrow as quickly as adults) were observed. Similar sampling methods were used in the Axmann (2019) study, where juveniles were not identified in terms of being anecic but rather just tanylobous or epilobous.

Numbers of adult *L. terrestris* ranged from 1.0 to 13.3/m² in the control plots and 3.5 to 12.3/m² in the Final Bite treated plots of the Axmann (2019) study. Similarly, numbers of adult *A. longa* ranged from 3.3 to 13.8/m² in the control plots and 3.8 to 21.0/m² in the Final Bite treated plots of the Axmann (2019) study. These figures then are broadly average, at least compared with the information from the available literature. They are not as high as observed in Boag (1997), where sampling was conducted in the Autumn (September, October, November) across 100 farms in Scotland and found that *L. terrestris* had a mean abundance in grass fields of 44.8 individuals/m² and in arable 31.2/m², and *A. longa* had a mean abundance in grass fields of 56.8 individuals/m² and 38.4/m² in arable fields. However, it was noted by Brown (2022) that in this study, no differentiation was made between adult or juvenile earthworms and the results for the two were combined – this means it is difficult to compare these results with those of Axmann (2019).

In addition to the open literature, Brown (2022) also compared data from ADAMA-commissioned field studies. The UK study (Forster and Salaun, 2003) in a long-term grass field in South West England was sampled using the formalin method only. In 2003 this was accepted practice with digging being introduced later, initially as an efficiency check for the formalin sampling and then as an additional approach to complement it. *A. longa* was not present in this study in large enough numbers to draw any conclusions. *L. terrestris* numbers ranged from 1.3 adults and 8.5 juveniles/m² in May 2001 to 4 adults and 15 juveniles/m² in July 2002. Of the 15 studies performed in Germany all recorded numbers of *L. terrestris* but seven recorded no *A. longa* adults or juveniles. All of these studies except one were sampled using digging to a 20 cm depth followed by either formalin or more recently AITC to sample anecic worms. These earthworm field studies were conducted in a range of crop systems from grass fields, barley fields and maize fields to bare soil sown with clover after application.

In many of the studies where sampling took place in June or July numbers of adult *L. terrestris* were very low. There was considerable variability between studies with some having relatively high numbers of anecic worms and others very low numbers. *A. longa* was completely absent from 6 of the 15 German field studies. The octet method for sampling with electrical currents used in the study of Rozencranz (2009; Study 6 in Table 6 below) appears to have been particularly effective when sampling juvenile *L. terrestris* but generated very low numbers of adults.

The table below compares mean abundance of adult *L. terrestris* and *A. longa* in all ADAMA-owned field studies. Studies that combined juvenile and adult numbers have been left out as these cannot be compared with the Axmann (2019) study. Rows in green were conducted in Spring, those in orange were conducted in Autumn.

Study	Location	System	Mean Control adult <i>L. terrestris</i> /m ²	Mean Control adult <i>A. longa</i> /m ²	Mean Control adult anecic earthworm total/m ²
Axmann 2019	Germany	Grass	7.16	9.08	16.24
1	UK	Grass	2.53	1.125	3.66
2	Germany	Spring barley	1.95	0	1.95
3	Germany	Grass	3.375	13.125	16.5
4	Germany	Grass	2	0	2
5	Germany	Grass	8	0	8
6	Germany	Maize	6	0.625	6.625
7	Germany	Grass	16.05	6.5	22.55

8	Germany	Grass	8.875	4.875	13.75
9	Germany	Bare soil (arable)	11.83	10	21.83
10	Germany	Bare soil with grass clover sown after app	12.525	0	12.525
11	Germany	Grass	11	0	11
12	Germany	Bare soil then clover	7.7	11.02	18.72
13	Germany	Bare soil then grass and clover	12.38	0	12.38
Overall means			7.95	4.025	11.975

Numbers of *L. terrestris* observed in the Axmann (2019) study are a little lower than average, while for *A. longa* the abundance is notably greater than average. Overall numbers of adult anecic earthworms are correspondingly greater than average. It is noted that in this sample of field studies, *L. terrestris* was generally more common than *A. longa*, appearing in greater numbers generally and in more studies. It was also quite prevalent in the studies that used agricultural soils, though it is not possible to comment further on this without knowledge of the farming techniques used (e.g. tillage/minimally invasive techniques etc). It is noteworthy that time of year did not noticeably affect numbers; most studies started in the spring but those that did start in autumn had equally variable numbers, suggesting this does not affect earthworm abundance.

Overall, the data from the literature review report indicates that the abundance of anecic species – *L. terrestris* in particular – are in line with what could be expected from a typical field study. Data from the UK in particular is limited but where data is available for adults, it does not suggest a significant difference from the body of evidence from field studies conducted by ADAMA.

2) What are the ideal growth conditions for the anecic species *L. terrestris* and *A. longa*, and how do these compare with the field study by Axmann (2019)?

HSE CRD's comments regarding the relatively dry conditions in the study relate to how they could be the reason for the low numbers of anecic earthworms observed. In that way, the dry conditions could have impacted the reliability of the study. The dry conditions could also have affected the breakdown of the pellet, or limited activity of the earthworms. The UK has a wide range of weather conditions depending on location.

Brown (2021) compared the conditions observed in the Axmann (2019) study with historical weather data (UK Met Office) over the months of April, May, June, July and August in order to address the concerns over the relatively dry conditions. The application period during the field study was 24th April to 19th May 2018. The area chosen to compare with the study was East Anglia, using a weather station at Cambridge, as this was considered to be 'a major UK agricultural area'. The results are tabulated below (table copied from the position paper):

Table 1: Monthly long-term average rainfall at Cambridge UK and recorded rainfall +irrigation in the earthworm field study of Axmann, (2019)

Month	41 year monthly average rainfall (mm)	20 year monthly average rainfall (mm)	Rainfall at the study site (mm)	Irrigation at the study site (mm)	Rainfall + irrigation at the study site (mm)
April	39.3	31.6	10.7	22.5	33.2
May	43.6	46.8	74.8	45.3	120.0
June	50.4	42.5	22.0	0	22.0
July	49.0	54.0	55.0	24.3	79.3
August	53.8	61.4	80.8	13.0	93.8
Total April - August	236.0	236.3	243.3	105.1	348.3

This table indicates that compared to the long-term average rainfall in Cambridge, UK, the total soil moisture (rainfall + irrigation) in the study was actually greater for the same period. Although East Anglia is a major agricultural region, in terms of arable crops the southeast is the most productive¹⁷, so the HSE evaluator used Met office data for a weather station in Eastbourne to see if the rainfall was similar to that determined for Cambridge:

HSE Table 1 – monthly long term average rainfall at Eastbourne UK and recorded rainfall + irrigation in the earthworm field study of Axmann, 2019.

Month	41 year Monthly Average (mm)	20 year Monthly Average (mm)	Rainfall at study site (mm)	Irrigation at the study site (mm)	Rainfall + irrigation at the study site (mm)
April	44.34	36.805	10.7	22.5	33.2
May	46.35	47.265	74.8	45.3	120
June	47.10	38.47	22	0	22
July	51.17	53.145	55	24.3	79.3
August	56.09	62.465	80.8	13	93.8
TOTAL	245.04	238.15	243.30	105.10	348.30

The 41- and 20 year monthly average rainfalls for Eastbourne are slightly greater than for Cambridge across the same timespan (April-August). However, the total precipitation (rainfall + irrigation) in Axmann (2019) was about 100 mm greater over the application period and just after (April-August). Indeed, even without irrigation the total rainfall in the Axmann study was comparable to the 41 and 20 year average in East Anglia and the South East regions of the UK.

The exception is the month of June where, in the field study, the total rainfall was 22 mm (compared to, 47.1 mm and 38.5 mm, 41 and 20 year average rainfalls in Eastbourne, UK respectively). No irrigation took place. It is however noted that the soil moisture in the study site in this period remained at 28.3 % at 5cm depth and 28 % at 20 cm depth which indicates that despite the dry conditions the soil moisture levels were comparable to the previous month (May, 32.1 % at 5 cm depth and 29.5 % 20 cm depth) where total precipitation was nearly 3-fold greater (120 mm) than the Eastbourne average rainfall (46.4 and 47.3 mm). Therefore, it could be argued that this deviation from the average in June would not be likely to have had a significant impact – perhaps due to the excessive precipitation in the previous month.

¹⁷ Agriculture in the English Regions 2017, 1st estimate – statistics notice (30 August 2018) – www.gov.uk/government/statistics/agriculture-in-the-english-regions

Overall, this data is indicative that while the conditions were relatively dry across the whole year of the study, the total precipitation in the study was comparable, if not greater than, major agricultural areas in the UK during the period April-August. This was corroborated by HSE CRD Environmental Fate and Behaviour specialists which confirmed below:

HSE Table 2 – comparison of precipitation in Axmann (2019) with historic weather data used by HSE CRD Environmental Fate specialists for higher tier drainflow modelling. The table compares the trial site values (highlighted orange) against the original M4 weather sites (highlighted green) and the updated MARS weather sites (highlighted blue).

Month	41 year monthly average rainfall (mm)	20 year monthly average rainfall (mm)	Rainfall at the study site (mm)	Irrigation at the study site (mm)	Rainfall + irrigation at the study site (mm)	M4 dry (Cambridge) 1959-88 average (mm)	M4 medium (Mylnefield) 1959-88 average (mm)	M4 wet (Keel) 1959-88 average (mm)	MARS dry (Cambridge) 1989-2020 average (mm)	MARS medium (Mylnefield) 1989-2020 average (mm)	MARS wet (Herefordshire) 1989-2020 average (mm)
April	39.3	31.6	10.7	22.5	33.2	40.3	41.0	55.8	39.6	45.0	55.2
May	43.6	46.8	74.8	45.3	120.0	63.8	52.4	78.2	42.2	51.2	57.4
June	50.4	42.5	22.0	0	22.0	63.5	47.3	69.7	44.4	60.8	58.8
July	49.0	54.0	55.0	24.3	79.3	45.2	60.3	54.7	43.9	62.5	56.9
August	53.8	61.4	80.8	13.0	93.8	55.2	74.8	81.1	50.5	66.8	61.7
Total April - August	236.0	236.3	243.3	105.1	348.3	268.0	275.7	339.6	220.6	286.4	289.9

In summary, despite in some cases significant differences in monthly amounts, the total Rainfall + Irrigation value was greater than that observed for any of the M4 or MARS weather sites. Overall the study is considered representative of wetter conditions for the months considered (April to August) in the UK.

An important part of the risk assessment is consideration of the relevance of field studies to the proposed use of the product in question. As discussed above, Axmann (2019) applied the product Final Bite (1% iron) in April-May, and ensured wet conditions throughout the application period up to August of that year. However, application conditions may not be representative of later or earlier periods in the year and HSE CRD is aware that the requested GAP for Elemental Iron is essentially for all crops and amenity vegetation, meaning that applications could take place throughout the year.

The PUSG data indicate that for 2016 (the last year surveyed for arable crops):

- 85539 kg molluscicides applied to cereals
- 54649 kg molluscicides applied to Oilseed Rape (OSR)
- 18991 kg molluscicides applied to potatoes
- 160624 kg molluscicides applied to all crops

This translates to 53% of the total on cereals, 34% on OSR and 12% on potatoes (i.e., 99% combined). Consultation was sought with HSE CRD Efficacy specialists who provided the following data regarding when slug treatments were conducted in the UK for OSR, cereals and potatoes.

Table HSE 3 - acceptable BBCH use timings for slug pellets in the UK.

CROP	BBCH	Timing
BRSNN Oilseed Rape	BBCH0-14	Mid-August-end of September
TRZAW Winter wheat	PRE EM	Beginning September-end October
HORVW Winter barley	PRE EM	Mid-September-Mid-October
SOLTU Potato	BBCH31-89	Mid-June-End September but can vary a lot depending on weather

The table above indicates that the majority of slug pellet use is in the Autumn months. A 3-month comparison was made between rainfall in the months of April-June in the study and typical conditions in the UK during September-November, based on the same modelling data used in HSE Table 2 above.

HSE Table 4 – comparison of precipitation in Axmann (2019) with historic weather data used by HSE CRD Environmental Fate specialists for higher tier drainflow modelling, this time comparing rainfall in the study with rainfall in Autumn in the UK. The table compares the trial site values (highlighted orange) against the original M4 weather sites (highlighted green) and the updated MARS weather sites (highlighted blue).

Month	41 year monthly average rainfall (mm)	20 year monthly average rainfall (mm)	Rainfall at the study site (mm)	Irrigation at the study site (mm)	Rainfall + irrigation at the study site (mm)	Month	M4 dry (Cambridge) 1959-88 average (mm)	M4 medium (Mylnefield) 1959-88 average (mm)	M4 wet (Keele) 1959-88 average (mm)	MARS dry (Cambridge) 1989-2020 average (mm)	MARS medium (Mylnefield) 1989-2020 average (mm)	MARS wet (Herefordshire) 1989-2020 average (mm)
April	39.3	31.6	10.7	22.5	33.2	September	45.8	70.1	69.3	43.1	55.8	62.5
May	43.6	46.8	74.8	45.3	120	October	54.9	60.7	65.5	54.7	85.6	91.1
June	50.4	42.5	22	0	22	November	45.1	62	87.9	50.7	71.6	87.5
SUM (mm)	133.3	120.9	107.5	67.8	175.2	SUM (mm)	145.8	192.8	222.7	148.5	213	241.1

The total of rainfall plus irrigation in the Axmann (2019) study exceeds rainfall for both the M4 and MARS 'dry' sites, but is less than what would be seen in both the M4 and MARS 'medium' and 'wet' sites. According to HSE Environmental Fate and Behaviour specialists, however, these summed values for the trial sites are not necessarily representative of all 'dry', 'medium' and 'wet' areas in the UK. While the 'dry' scenario could reasonably be expected to cover a large area of land devoted to agriculture, particularly in the aforementioned eastern areas of England, it should not be taken to be representative of the whole country, or indeed a worst-case trial site.

In Brown (2022) it is argued that the conditions experienced by earthworms in the Axmann (2019) study align with the ideal growing conditions for anecic earthworms (represented by *L. terrestris* and *A. longa*). The main

factors affecting growth, survival and reproduction were soil moisture and temperature, and are described in the table below. Overall, the optimum soil moisture content for culturing anecic earthworm species in the laboratory was found to be 25-30 %, with both *L. terrestris* and *A. longa* preferring soil moisture content above 14 %. The optimum temperature is considered to be 15 °C for *A. longa* and 15-20 °C for *L. terrestris*. In terms of pH, both species prefer neutral, or neutral to alkaline soil. *L. terrestris* thriving at pH 6.2-10 and *A. longa* at pH 6.7.

Table 1: Summary of optimal conditions for *Lumbricus terrestris*

Reference	Life history parameters studied	Environmental factors studied	Optimum conditions found	Limiting conditions
Berry and Jordan, 2001	Growth	Temperature Soil moisture	20 °C 30% soil moisture	Death at 30 °C after 14 days Death at 25 °C after 182 days
Butt, 1991	Growth Cocoon production Rate Survival	Temperature	20 °C optimum for cocoon development and hatchling growth	Hatchling survival: 96% at 15 °C 87% at 20 °C 11% at 25 °C
Daniel <i>et al.</i> , 1996	Weight gain of juveniles	Temperature	15 – 17.5 °C At 40% soil moisture	Time from hatching to maturity: 25.7 weeks at 7.5 °C 23.7 weeks at 9 °C 14 weeks at 15 °C 9.4 weeks at 20 °C
Daughjerg, 1988	Adult and juvenile choice over 5 days in soil columns with temperature gradients	Temperature at 20% moisture	10 °C for adults	13% preferred 5 °C 60% preferred 10 °C 13% preferred 15 °C 13% preferred 20 °C <10% soil moisture induces diapause.
Holmstrup <i>et al.</i> , 1996	Incubation time of cocoons	Temperature		Cocoon incubation: 48 days at 20 °C 152 days at 15 °C 160 days at 10 °C
Khan <i>et al.</i> , 2012	Heartbeat rate Blood oxygen level Enzyme activity	Temperature	14 °C had highest respiration and haemoglobin synthesis	20 – 23 °C respiration rate and haemoglobin synthesis decreased. Death after a few days at 25 °C
Lowe and Butt, 2005	Growth Cocoon development	Temperature Soil moisture	Growth: 15 – 20 °C 30% soil moisture. 20 °C optimum for cocoon development.	
Miles, 1963	Mortality	Temperature	Researched lethal temp.	28 °C for 6 h 40 minutes was lethal.
Moreau Valancogne <i>et al.</i> , 2013	Review of publications. Growth Cocoon development	Temperature	16.9 °C for growth 21.6 °C for cocoon development	No growth above 25.9 °C No growth below 2.5 °C No cocoon development below 2.6 °C
Perreault and Whalen, 2006	Weight gain Surface casting	Temperature Soil moisture	20 °C 30% soil moisture	Soil moisture a key driver, as greater weight gain at 20 °C and 30% moisture than lower temps and drier soil.

Table 2: Summary of optimal conditions for *Aporrectodea longa*

Reference	Life history parameters studied	Environmental factors studied	Optimum conditions found	Limiting conditions
Baker and Whitby, 2003	Hatchling growth Cocoon hatching	Temperature At fixed 25% soil moisture pH	15-20 °C	Development time from hatching to adults: 9 months at 15 °C. 6 months at 20 °C.

				80% death at 25 °C Requires pH >4.5
Bouché, 1972	Growth and survival	pH	soil pH = 6.7	
Daughjerg, 1988	Adult and juvenile choice over 5 days in soil with temperature gradients	Temperature at 20% moisture	10 – 15 °C for adults 10 °C for juveniles	Approx.: 4% preferred 5 °C 40 % preferred 10 °C 40 % preferred 15 °C 12% preferred 20 °C Approx.: 12% preferred 5 °C 64 % preferred 10 °C 14 % preferred 15 °C 4 % preferred 20 °C
Miles, 1963	Mortality as induced by temperature	Temperature	15 °C	>25.7 °C for 12 hours is lethal threshold
Holmstrup <i>et al.</i> 1996	Incubation time of cocoons	Temperature		50 days at 20°C 69 days at 15 °C 125 days at 10 °C
Holmstrup, 1999	Development time for cocoons	Temperature	22.5 °C	Cocoon development: 106 days at 9.6°C 54.1 days at 15 °C 37.3 days at 20 °C 32.4 days at 22.5 °C 42.1 days at 26 °C

The Axmann study conditions were compared with these published optimal conditions. Overall, soil moisture (% volume) was found to be at or close to 30 % in the first 10 cm of soil depth during the first 52 days of the study, ranging from approximately 29 to 36 %. The 10-20 cm depth was generally lower ranging from approximately 24 % to peaks of 34 %. For temperature, the first 5 cm of soil depth varied over the period of test item application, with averages of 12-23 °C. The daily maximum temperature regularly exceeded 25 °C approximately 30-46 days after the first application, which would have been sub-optimal for the anecic worms, but by retreating further into their vertical burrows they would have avoided these higher temperatures. The soil temperatures at 20cm did not vary as greatly as the 5 cm layer but followed the same trend.

Air temperature was also considered relevant as anecic species come out at night to feed. Brown (2022) considered that the typical temperatures that the worms would have been exposed to would have been between the mean and minimum temperatures accordingly. The mean ranged from 9 to 23 °C and the minimum from 1 to 14 °C. With the optimum temperature of the anecic species being between 15 and 20 °C, these temperatures are considered to be suitable for foraging, and this is borne out by the results for the spray reference item used in the test which caused statistically significant effects on abundance and biomass even with the low numbers of anecic individuals sampled, suggesting that the worms were coming up, foraging and being exposed to the sprayed reference item at the soil surface.

It is agreed that the conditions in the Axmann (2019) study are, based on the data gathered from the literature review, within the range considered optimal for growth of anecic species *L. terrestris* and *A. longa*. This can be corroborated by the discussion around question 1) relating to abundance of these species observed in the study, which found that the numbers of adult individuals observed per m² were typical.

3) Do foraging anecic earthworms encounter and consume slug pellets and are they affected by iron-containing slug pellets? Do the results of Axmann (2019) indicate any such effects?

Final Bite is a molluscicide product containing 1 % Iron (10,000 mg/kg). The product works by combining the elemental iron (Fe0) in the product with the [REDACTED], in the low pH (<3.0) environment of the slug gut. The [REDACTED] interferes with the haemocyanin-based oxygen transport chain specific to molluscs, similarly to how carbon monoxide interferes with oxygen uptake by mammalian haemoglobin. As a result, ingestion of iron-based products by molluscs leads to interruption of feeding and locomotion due to anoxia.

Earthworm digestive tract pH is close to neutral¹⁸, meaning the [REDACTED] might not form. In addition, the earthworm blood pigment is Haemoglobin, not Haemocyanin. Nevertheless, significant toxic effects of the product 'Final Bite' were observed in the first-tier laboratory studies by [REDACTED] (2018a and 2018b). It is likely that these effects were at least in part mediated by the [REDACTED], which has been shown to be toxic to earthworms (*E. fetida*) under laboratory conditions (LC50 = 156.56 mg/kg), particularly in combination with an iron-salt such as Ferric Phosphate (LC50 = 78.16 mg/kg; both endpoints from Edwards *et al.* 2008). Of note, this study showed almost no toxicity to earthworms from Ferric Phosphate alone, at concentrations up to and including 10,000 mg/kg. This supports the conclusion of low risk to earthworms from elemental iron as a stand-alone active substance.

Microcosm experiments with Ferric phosphate + EDTA pellets and the anecic species *L. terrestris* (Edwards *et al.*, 2008; Langan and Shaw, 2006) showed that this species does consume slug pellets while foraging, though in Langan and Shaw (2006) earthworms took more metaldehyde pellets into their burrows. It is not clear if this is due to the different chemical composition of these pellets or if the size of the pellets is a factor. Either way, Ferric phosphate + EDTA resulted in lower activity and decreased growth at rates ranging from 8x the recommended field rate, though these were not statistically significant. It is worth noting that no observable negative effects or statistically significant effects were observed on the species *L. terrestris* in two field studies conducted during the renewal of Ferric Phosphate (Luhrs, 2009/2010), which tested up to seventeen times the proposed field rate of the respective products. Therefore, under field conditions, there is no precedent for Ferric-EDTA complexes to cause adverse effect on this species.

It is difficult to compare the studies by [REDACTED] with those referenced above, as the studies are not strictly comparable, with formulations and delivery methods of EDTA differing between studies. [REDACTED]

The results of the Axmann (2019) field study for anecic species are summarised in the tables below, from the full study report.

¹⁸ Horn *et al.*, 2003. The Earthworm Gut – an ideal habitat for ingested N₂O-Producing Microorganisms

Table 11: Mean number (n/m²), standard deviation and percentage change of Anecic earthworm abundance in the test item treatment (T) and toxic reference treatment (R) relative to the control (C)

Species / group	Timing	Mean number (n/m ²) ± standard deviation and deviation from control (%) ^{a)}		
		C (untreated)	Final Bite – Blue (T)	Carbomax (R)
<i>Aporrectodea longa</i>	7 DBA1	7.5±1.0	8.8±4.6 (16.7)	6.8±1.7 (-10.0)
	34 DAA1	13.3±5.7	9.8±4.7 (-26.4)	2.3±2.1 * (-83.0)
	56 DAA1	7.5±2.6	9.5±4.8 (26.7)	1.0±1.2 * (-86.7)
	204 DAA1	13.8±4.6	21.0±5.8 (52.7)	2.0±2.2 * (-85.5)
	366 DAA1	3.3±1.9	3.8±2.1 (15.4)	0.0±0.0 * (-100.0)
<i>Lumbricus terrestris</i>	7 DBA1	13.3±2.6	12.3±4.7 (-7.5)	11.8±3.5 (-11.3)
	34 DAA1	6.5±4.5	8.3±5.1 (26.9)	2.0±2.2 (-69.2)
	56 DAA1	1.0±0.8	3.5±1.7 (250.0)	0.0±0.0 (-100.0)
	204 DAA1	8.0±3.7	12.0±4.1 (50.0)	1.5±0.6 * (-81.3)
	366 DAA1	7.0±2.2	5.8±3.3 (-17.9)	2.0±1.4 * (-71.4)
Anecic	7 DBA1	20.8±3.1	21.0±8.8 (1.2)	18.5±5.1 (-10.8)
	34 DAA1	19.8±8.7	18.0±7.1 (-8.9)	4.3±3.2 * (-78.5)
	56 DAA1	8.5±3.3	13.0±3.5 (52.9)	1.0±1.2 * (-88.2)
	204 DAA1	21.8±5.9	33.0±1.8 (51.7)	3.5±1.7 * (-83.9)
	366 DAA1	10.3±2.2	9.5±4.9 (-7.3)	2.0±1.4 * (-80.5)

^{a)} negative values (-) indicate lower earthworm numbers compared to the control; positive values (+) indicate higher earthworm numbers compared to the control

DBA1= days before application 1, DAA1= days after application 1

*significantly different from control ($p \leq 0.05$)

Table 12: MDDs calculated for abundance a posteriori for Dunnett's test (3 treatment groups: control, T, R, $\alpha = 0.05$, 4 replicates per treatment group, left sided test)

% MDD	Abundance				
Taxon	7 DBA1	34 DAA1	56 DAA1	204 DAA1	366 DAA1
<i>Aporrectodea longa</i>	59 (III)	51 (III)	66 (III)	50 (IV)	77 (II)
<i>Lumbricus terrestris</i>	43 (IV)	98 (I)	170 (0)	62 (III)	53 (III)
Anecic	46 (IV)	53 (III)	52 (III)	26 (IV)	49 (IV)

(MDD classification (EFSA PPR PANEL (2013)): 0: >100%, I: >90-100%, II: >70-90%, III: >50-70%, IV: ≤50%)

As previously discussed the figures for *L. terrestris*, particularly for the sampling time points 34 DAA1 and 56 DAA1, make the results harder to interpret, as the MDD values are high (Class 1 and 0 at each time point, respectively) meaning it is not possible for the study to statistically determine effects of the test item on these species at these time points. This is corroborated by the abundance figures for the reference item and this species, which are not considered statistically significant at these time points, despite being observably lower than in the case of both the control and the test item-treated groups.

Nevertheless, the study does not demonstrate any observable negative effects on *L. terrestris*. In addition, data for *A. longa* – based on the MDD values for this species – is reliable and shows no statistically significant or potentially relevant negative effects of the test item. The same can be said of anecic species overall.

At this point it is worth discussing the effects of the reference item. Initially the concern from HSE CRD was that the reference item (Carbendazim) was being applied as a spray, and as the test item was a granule, the study was showing it was sensitive to effects of sprays, with high uncertainty that this could be extrapolated to granular test items. The extended literature data suggests that anecic earthworms (*L. terrestris* at least) are capable of encountering and consuming granular products. The fact that the reference item is having a statistically significant, or at least observably negative, effect on both *L. terrestris* and *A. longa* in this field study suggests that these worms are coming out to forage and can be exposed to both types of test item. This provides some reassurance that the anecic earthworms are being given the opportunity to encounter the test item in the test.

Overall conclusions

The new literature data provides some reassurance that anecic worms had the potential to come into contact with granules and hence be exposed in the study, therefore helping to reduce uncertainties in one of the areas identified as problematic. Data on growth conditions and abundance suggests the anecic numbers in the study are not atypical and this helps with the read across point between times of year. The reference item data for this study, and the extended literature, indicate that the anecic earthworms are potentially being exposed to the test item. The issue remains that the statistical power of the study to detect effects in *L. terrestris* was low at the key timepoints after exposure, hence there is uncertainty that the formulation does not impact this species in the short-medium term.

Data on another anecic species (*A. Longa*) and the data on anecic species in total suggests that where it can be adequately assessed, anecic species populations aren't impacted at all by the formulation. Therefore *L. terrestris* would have to be significantly more sensitive to the formulation for there to be a concern over the impact on this species. There is limited information on this latter point, but there is nothing to indicate that *L. terrestris* would suffer statistically significant effects of ferric test items at the requested field rates.

It is also by no means certain that an additional field study would further resolve the remaining uncertainties. On balance, there is now sufficient evidence for HSE CRD to conclude an acceptable risk to earthworms for the proposed use of the representative product, in line with the ECP recommendation.

B.9.9. EFFECTS ON SOIL NITROGEN TRANSFORMATION

Report:	CP 10.5/01. ████████ 2008
Title:	Slug & Snail Killer Soil Microorganisms: Nitrogen Transformation Test
Report no.:	G/55/08 (R-38457)
Guidelines:	OECD 216 (2000) and corresponding EU method C.21
GLP:	Yes, certified
Published:	No

I. MATERIALS AND METHODS**A. MATERIALS**

- Test material:** SLUG & SNAIL KILLER
Batch no.: Not included in the report
Date of production: 26.06.2008 (stability: 3 years from production)
Purity: 0.98% total iron (analysed)
Description: blue granules with characteristic odour
- Test soil:**
Soil type: Agricultural soil
Source: 49°59', 780N and 018°55', 190E adjoining to Institute of Industrial Organic Chemistry, Branch Pszczyna
Test chamber: not described
Substrate: Lucerne: 5 g/kg dry weight of soil

3. Treatment groups: 0 (control), 66.7 and 333.5 mg /kg soil dry weight

4. Environmental conditions

Temperature: 20 – 22°C

Photoperiod: In the dark

pH-value: 6.5

Moisture content in soil: 40 – 60% of the WHC_{max}

B. STUDY DESIGN AND METHODS

- In life dates:** 04 Nov – 30 Dec 2008
- Soil preparation**

The soil was collected from a field covered by grass, which had not been used for agricultural purposes for five years. No PPPs or fertilisers had been used on the site. Samples were taken from a depth of 0 – 20 cm from different parts of the field and pooled into one sample collection.

The freshly collected soil from the field for test was used. The soil was manually cleared of large objects and then sieved to particle size equal to 2 mm.

The soil was amended with Lucerne (C - 31 .1 % d.w.; N - 2.49 % d.w.) as an organic substrate at a rate of 5 g of lucerne per kilogram of soil (dry weight). Lucerne was sieved to particle size equal to 0.3 mm.

3. Dose preparation

The soil (4500 g) with lucerne was thoroughly mixed and then divided into three portions of equal weight. The test material granules were milled before being mixed with fine grained quartz sand (10 g of sand per kg dry weight of soil). Then this sand/test material mix was mixed thoroughly with the soil. Two portions of soil were mixed with the test substance and the third was mixed with sand without the substance (control).

The test material was introduced into soil in concentrations of 66.7 and 333.5 mg/kg of soil. The control soil and soil treated with test material were incubated in three replicates.

At the beginning of the test, the moisture content of the soil was adjusted with distilled water to a value of 50 % of the maximum water holding capacity.

4. Measurement of nitrate

The test duration was 28 days. Nitrogen transformation rate was tested on samples of soil (three from each replicate of treatments and control) collected after 0, 7, 14 and 28 days of incubation. The mean nitrate formation rate in each soil sample treated with test material was compared with that in control and the percent deviation from the control was calculated.

To prepare the soil extracts, 10 grams of soil sample was weighed into the Erlenmeyer flask. Next, 100 ml of working extract solution was added. The obtained soil suspension was mixed and left for 24 hours, after which the soil suspension was filtrated. For the blank sample (without soil), it was prepared in the same way as soil extract.

The method used to analyse the nitrate formation rate was based on the spectrophotometric measurement of the nitrate ions concentration in soil extract of the 1% potassium sulphate (VI) solution. The analysis was based on the measurement of the intensity of yellow colour, generated in reaction with phenyldisulphonic acid at 410 nm in the spectrophotometer.

The obtained soil extract (20 mL) and 0.5 ml of 0.4% sodium hydroxide solution was evaporated in a water bath. After cooling, phenyldisulphonic acid solution (1.5 ml) and distilled water (20 ml) were added and then 25% sodium hydroxide solution (20 ml). The obtained solution was mixed and the extinction at 410 nm was measured. The values of the extinction and the amount of nitrate ions determined on the basis of a standard curve were calculated.

A series of standard solutions were prepared and a standard curve was produced plotting amount of nitrate against the extinction at 410 nm.

5. Statistics

The results were evaluated by analysis of variance (ANOVA). The statistical significance ($P < 0.05$) of differences was assessed by post hoc comparison of means using lowest significant differences (LSD-test) with help of programme STATISTICA v.5.0.

II. RESULTS AND DISCUSSION

A. Findings

There were no effects in either test item group of more than $\pm 25\%$. The nitrate content as determined in the soils at the different sampling times are summarised in Table B.9.9-1 below. The nitrate transformation rate in the control and test item groups and how the test item groups compare to the control are summarised in Table B.9.9-2.

Table B.9.9-1: Effects of Slug & Snail Killer on Soil Nitrate Content (Mean Values)

	NO ³ -N (mg/kg soil dry weight)		
	0 (control)	66.7 mg/kg soil dw	333.5 mg/kg soil dw
Day 0	4.92	4.86	4.84
Day 7	6.6	6.46	6.61
Day 14	9.36	9.26	9.16
Day 28	20.42	19.71	19.04

Table B.9.9-2: Effects of slug and snail killer on the nitrate transformation rate

Treatment group	Mean Nitrogen transformation rate (mg NO ₃ -N/kg soil d.w./day) (% inhibition in comparison to the control)		
	Day 0-7	Day 7-14	Day 14-28
Control	0.24	0.39	0.79
66.7 mg/kg soil	0.23 (5.15 %)	0.40 (-1.33)	0.75 (5.46)
333.5 mg/kg soil	0.25 (-4.95)	0.36 (7.84)	0.71 (10.55)

B. Validity criteria

The results follow the following validity criteria:

- The variation between replicate samples in control was less than $\pm 15\%$.

III. CONCLUSION

There were no effects in either test item group (66.7 and 333.5 mg/kg of soil dry weight) of more than $\pm 25\%$

UK Comments:

It is noted that no batch number is provided for the formulation however, in light of updated consideration in the volume 4, it is concluded that the tested material is representative of the technical specification for elemental iron.

The study was carried out according to the agreed guideline and in accordance with GLP. The endpoints are acceptable for use in regulatory risk assessment. No effects $>25\%$ were observed at 333.5 mg/kg soil d.w..

B.9.10. RISK ASSESSMENT FOR SOIL NITROGEN TRANSFORMATION

The Nitrogen transformation test was carried out with a slightly different formulation to the representative formulation. The Applicant provided a comparison of the two formulations, which has been considered by Chemistry and Ecotoxicology (see section C.1.3). There is a major difference with one of the co-formulants, which will be considered further below, in the context of the risk assessment.

A summary of the study and the endpoints from the study are found in Table B.9.10-1.

Table B.9.10-1 : Summary of endpoints on the toxicity of Final Bite to soil micro-organisms

Test type	Time scale	Test material	Endpoint	Data Author, year	point
Nitrogen transformation	28 days	Slug & Snail Killer	-6.5% effect at up and including 333.5 mg product/ kg of soil dry weight after 28 days <25% deviation from control by the study end	CP Jerzy, O., 2008	10.5/01.

Elemental iron is a naturally occurring element in soil (UK Fate and Behaviour specialists estimate background levels of 2000-50000 mg/kg soil), indicating that the element is present in all soils. The calculated $PEC_{\text{soil, accumulation}}$ is 12.8 mg a.s./kg based on the proposed use of the product accumulating over a 20 year period. This PEC, resulting from the worst case use of the product is significantly lower than the background levels; therefore, no quantitative risk assessment for exposure to the active substance has been carried out.

The PEC_{soil} of the Formulation is calculated as 64 mg product/kg soil. This is exceeded by the endpoint above (<25 % effects at 333.5 mg product/kg soil) with a high margin of safety. Overall the risk from soil micro-organisms can be considered acceptable.

B.9.11. EFFECTS ON TERRESTRIAL NON-TARGET HIGHER PLANTS

B.9.11.1. Summary of screening data

No data was submitted.

B.9.11.2. Testing on non-target plants

No data was submitted.

B.9.11.3. Extended laboratory studies on non-target plants

No data was submitted.

B.9.11.4. Semi-field and field tests on non-target plants

No data was submitted.

B.9.12. RISK ASSESSMENT FOR TERRESTRIAL NON-TARGET HIGHER PLANTS

The risk to non-target plants has been assessed in line with the SANCO Terrestrial Guidance document (2002)¹⁹, with further reference to the data requirements as stipulated under Regulation EU 283/2013²⁰. In the case of elemental iron, the representative product Final Bite is formulated as a ready-to-use bait granule, applied to the soil around the target crops by spreading. The risk of exposure to off-crop non-target plants is therefore considered to be minimal, limited to dust drift. No guidance is currently available to assess the risk from this method of exposure, though with an optical dust factor of 1.33 (See Vol 3CP 2.8.5/01 of this dossier), it is considered that the representative product produces low levels of dust.

Even allowing for exposure, elemental iron is a naturally occurring element in soil (UK Fate and Behaviour specialists estimate background levels of 2000-50000 mg/kg soil) and used as a nutrient in plants²¹. No unacceptable effects are to be expected after application of Final Bite according to the proposed GAP, which has a maximum soil PEC of 12.8 mg/kg, which will not add significantly to the background levels of iron. Hence, germination and growth is unlikely to be impacted. This is corroborated by the efficacy trials data (see Section 6 of this dossier) which indicate no phytotoxicity in cereals, oilseed rape, cabbage and lettuce treated with Final Bite®.

Overall, considering the predicted exposure is low compared to background levels, low observed phytotoxicity in field efficacy trials, and the high naturally occurring nature of the active substance, the risk to off-crop non-target terrestrial plants from the proposed uses of elemental iron is concluded to be acceptable.

¹⁹ SANCO/10329/2002 rev 2 final, Chapter 7 (pages 31 – 35)

²⁰ Commission Regulation (EU) No 283/2013, Section 8.6 (pages 63-64)

²¹ Connorton, J., J. Balk and J. Rodriguez-Celma. 2017. 'Iron homeostasis in plants – a brief overview'. *Metallomics* 9, 813-823.

B.9.13. EFFECTS ON OTHER TERRESTRIAL ORGANISMS (FLORA AND FAUNA)**B.9.13.1. Effects on Carbon Transformation**

Report:	CP 10.7/01. █████ 2008
Title:	Slug & Snail Killer Soil Microorganisms: Carbon Transformation Test
Report no.:	G/56/08
Guidelines:	OECD 217 (2000) and corresponding EU method C.22
GLP:	Yes, certified
Published:	No

I. MATERIALS AND METHODS**A. MATERIALS**

- Test material:** SLUG & SNAIL KILLER
Batch no.: Not included in the report
Date of production: 26.06.2008 (stability: 3 years from production)
Purity: 0.98% total iron
Description: grey-blue granules with characteristic odour
- Test soil:**
Soil type: Agricultural soil (unused for agricultural purposes for 5 years)
Source: 49°59', 780N and 018°55', 190E adjoining to Institute of Industrial Organic Chemistry, Branch Pszczyna
Test chamber: not described

3. Treatment groups: 0 (control), 66.7 and 333.5 mg /kg soil dry weight

4. Environmental conditions

Temperature: 20 – 22°C

Photoperiod: In the dark

pH-value: 6.5

Moisture content in soil: 40 – 60% of the WHC_{max}

B. STUDY DESIGN AND METHODS

- In life dates:** 12 Nov – 11 Dec 2008
- Soil preparation**

The freshly collected soil from the field for test was used. The soil was manually cleared of large objects and then sieved to a particle size equal to 2 mm. The soil (4500 g) was thoroughly mixed and then divided into three portions of equal weight.

3. Dose preparation

The test material (granules) was milled and homogenously mixed with the soil. Appropriately weighed amounts of the test substance were mixed with fine-grained quartz sand (10 g of sand per kg dry weight of soil). Thereafter, sand containing test material was mixed thoroughly with soil.

Two portions were mixed with sand containing the test material and the third was mixed with sand without the substance (control). At the beginning of the test, the moisture content of the soil was adjusted with distilled water to a value of 50 % of the maximum water holding capacity. Concentrations of 66.7 and 333.5 mg/kg of soil were prepared.

4. Measurement of respiration rates

The Substrate-Induced Respiration (SIR) method was used for the determination of the rate of soil respiration. The principle of operation was based on the measurements of the pressure difference in a closed system. During respiration, the carbon dioxide generated was bound to an absorber (45% KOH), that resulted in pressure -drop, which was proportional to the soil respiration.

To assess the glucose-induced respiration rates three samples from treated and untreated soils were collected. Samples of 100 g each were thoroughly mixed with 2000 mg glucose/kg soil (dry weight). Prepared soil samples were

incubated for 12 hours (at $20 \pm 2^\circ\text{C}$) in an apparatus for continuous measurements of respiration rates. Measurements were started one hour after glucose was added. Over the course of 12 hours the total quantity of oxygen consumption and mean respiration rates were determined.

The test duration was 28 days. During the carbon transformation test samples of soil (three from each replicate of treatments and control) were collected after 0, 7, 14 and 28 days of incubation. The mean oxygen consumption in each soil sample treated with test material was compared with that in control, and the percent deviation from the control was calculated.

5. Statistics

The results were evaluated by analysis of variance (ANOVA). The statistical significance ($P < 0.05$) of differences was assessed by post hoc comparison of means using lowest significant differences (LSD-test) with the help of the programme STATISTICA v.5.0.

II. RESULTS AND DISCUSSION

A. Findings

During the carbon transformation test in soil, there was no significant difference of mean oxygen consumption between control and treated soils (66.7 and 333.5 mg/kg of soil).

The similar respiration rates were observed during the sampling times. No differences between treatments and control soil were obtained. The obtained percent deviations from the control in both concentrations are less than $\pm 25\%$. The mean oxygen consumption rates and pressure measured during the study are summarised in Table B.9.13.1 -1. The effects of the test item compared to the control are summarised in Table B.9.13.1 -2.

Table B.9.13.1-1: Effects of Slug & Snail Killer on Carbon transformation (Mean Values)

	Mean oxygen (O_2) consumption rate [mg/kg dry weight of soil/hour] \pm SD			Pressure determined during respiration measurements [hPa/12 hours]		
	0 (control)	66.7 mg/kg soil dw	333.5 mg/kg soil dw	0 (control)	66.7 mg/kg soil dw	333.5 mg/kg soil dw
Day 0	16.3 \pm 0.4	16.3 \pm 0.4	16.5 \pm 0.7	23.6	23.6	24.0
Day 7	14.6 \pm 0.4	14.4 \pm 0.7	14.7 \pm 0.4	21.3	21.0	21.3
Day 14	12.0 \pm 0.4	12.3 \pm 0.7	12.3 \pm 0.0	17.6	18.0	18.0
Day 28	9.8 \pm 0.4	10.1 \pm 0.4	10.3 \pm 0.0	14.3	14.6	15.0

Table B.9.13.1-2: Effects of Slug & Snail Killer on Carbon transformation – Deviations from control

Interval	% Deviation	
	66.7 mg/kg soil dw	333.5 mg/kg soil dw
Day 0	0.0	1.5
Day 7	-1.4	0.7
Day 14	1.8	2.1
Day 28	2.4	4.7

B. Validity criteria

The results follow the following validity criteria:

- The variation between replicate samples in control was less than $\pm 15\%$.

III. CONCLUSION

Taking into account the obtained results it was assessed that Slug & Snail Killer in concentrations of 66.7 and 333.5 mg/kg of soil dry weight, can be evaluated as having no long-term influence on carbon transformation in soil. No effects >25% were observed at 66.7 and 333.5 mg/kg of soil dry weight.

RMS Comments:

The study was carried out in accordance with the agreed guideline and in compliance with GLP.

It is noted that no batch number is provided for the formulation, however, in light of updated consideration in the volume 4, it is concluded that the tested material is representative of the technical specification for elemental iron.

No effects >25% were observed at 66.7 and 333.5 mg/kg of soil dry weight.

B.9.13.2 – Studies with Slug and Snail Killer on Aquatic Organisms

Studies on fish, aquatic invertebrates and algae with the product 'Slug and Snail Killer' (1 % iron) were presented by the applicant. Analysis for the concentrations of active substance in the test media was not conducted in any of the studies, and in the case of the study with algae, the validity criterion for coefficient of variation was not met. This means that the derived endpoints are not reliable for use in the risk assessment. As they cannot be used in the risk assessment, no further evaluation has been conducted and the studies have not been summarised by the UK evaluator. The studies have not been used in the risk assessment or for the purposes of classification. The Applicant text has been presented *verbatim* for informative purposes only.

Report:	CP 10.2.1/01. [REDACTED] 2008
Title:	Slug & Snail Killer Acute Toxicity for Rainbow Trout
Report no.:	W/89/08
Guidelines:	OECD 203 (1992), EU test method C.1
Deviations:	The length of the fish has exceeded the range of the recommended one in the OECD 203. Fish loading ratio exceeded the value recommended for semi-static experiments.
GLP:	Yes, certified
Published:	No
Comments:	During the preliminary test, 100% mortality was observed but was not observed during the definitive test. No analytical confirmation of exposure was conducted

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: SLUG & SNAIL KILLER (0.98% total iron)

Batch no.: Not included in the report

Purity: 0.98% total iron

Description: grey-blue granules with characteristic odour

2. Test organism: Rainbow trout (*Oncorhynchus mykiss*, Walb.)

Mean length: 5.85 ± 0.43 cm (max length 6.5 cm)

Mean weight: 3.07 g

Source: [REDACTED]

3. Treatment: 0 (control) and 10, 18, 32, 56 and 100 mg test item/L (nominal)

4. Test vessels: glass tanks (approx. 10 L volume)

Test water: Dechlorinated tap water

Loading rate: 2.15 g fish/L

5. Environmental conditions:

Temperature: 13.2 – 14.3°C

pH: 6.80 – 7.65

Dissolved oxygen: 84.3 – 102.4 %

Water hardness: 61.5 mg/L CaCO₃ (measured)

Photoperiod: 16 h light : 8 h darkness

B. STUDY DESIGN AND METHODS

1. In-life phase: 25 Nov – 29 Nov 2008

2. Test organism assignment and treatment

Fish were in good health and were acclimatised for 2 weeks in test medium of the same quality of test medium used in the test and were fed until 24 hours before the test was started. The test started when fish were placed in test vessels (7 fish/vessel) containing 10 L of defined water to which the test item had been added (mean loading was 2.15 g fish/L). During the experiment course, there was no possibility to obtain fish of smaller size and therefore the loading was calculated to be slightly higher than usual. There were totally 6 vessels which contained treated test medium of 10, 18, 32, 56 and 100 mg test item/L (nominal) and untreated dilution water (control), each.

The trout's sensitivity was checked in the test with reference substance – potassium dichromate (K₂Cr₂O₇). The LC₅₀ is 115.35 mg/L.

3. Dose preparation

In the test following range of concentration was used: 10, 18, 32, 56 and 100 mg/L (nominal). Approximately 10 g of test item was thoroughly grinded and amounts of test item was weighed out: 100, 180, 320, 560 and 1000 mg to make up each of the concentrations into 2L volume flasks. The flasks were filled up with water, up to 2 litres. Contents were vigorously mixed for 2 hours on magnetic stirrer. After mixing, insoluble fractions were removed by filtration. Obtained filtrates were added into tanks and then filled up to 10 litres resulting eventually in 10, 18, 32, 56 and 100 mg/L concentration medium (nominal, based on weighed amount). This procedure was not repeated during the test.

4. Measurements and observations

Observations of intoxication (loss of equilibrium, swimming behaviour, respiratory function and pigmentation) and mortality were made at 3, 24, 48, 72 and 96 hours from beginning of the test. Fish were considered dead if there was no reaction to external stimuli.

At the test start and 24, 48, 72 and 96 hours after measurements of pH and oxygen concentration were performed. Temperature of the water was measured continuously. Analysis on the test item was not performed.

5. Statistics

The data were not conducive to calculation of the LC_x values as there were no mortalities present.

II. RESULTS AND DISCUSSION

A. Mortality and abnormal responses of the fish

Cumulative mortality at 3, 24, 48, 72 and 96 hours is presented in the following table.

The toxicity of test item for fish is expressed as its median concentration causing 50% mortality of the population during 96 hours exposure time (LC_{50/96h} [mg/L]). The highest concentration causing no mortality (LC_{0/96h} [mg/L]) and the lowest causing 100% mortality (LC_{100/96h} [mg/L]) were determined after full test exposure. Moreover, the LC_x values were estimated after 24, 48 and 72 h based on nominal concentration of the test item.

Table: Cumulative mortality

Nominal concentration (mg/L)			Mortality (%)			
3 h	6 h	24 h	48 h	72 h	96 h	
Test item						
0	0	-	0	0	0	0
(control)						
10	0	-	0	0	0	0

18	0	-	0	0	0	0
32	0	-	0	0	0	0
56	0	-	0	0	0	0
100	0	-	0	0	0	0

B. Analytical verification

No analytical verification was conducted during the test.

C. Validity criteria

Validity criteria for the experiment were met: Mortality in the control during test was 0 % and the concentration of dissolved oxygen was kept within the range of 78.1 - 102.4 %, however, there was no analytical confirmation after the addition of the test item.

III. CONCLUSION

The 24-hour, 48-hour, 72-hour and 96-hour toxicity endpoints for Slug & Snail Killer to rainbow trout were estimated to be greater than or equal to 100 mg/L (NOEC) (nominal) and the LC50 would also be greater than 100 mg/L(nominal).

Report:**Title:****Report no.:****Guidelines:****Deviations:****GLP:****Published:****Comments:****CP 10.2.1/03. Jerzy, O., 2008**

Slug & Snail Killer *Daphnia magna* acute immobilization test

W/90/08 (R-38460)

OECD 202

None

Yes, certified

No

Validity criteria for the experiment were met and therefore the study could be considered acceptable, however no analytical confirmation of exposure was conducted. This study has been conducted with an equivalent test item to Final Bite®. The report is presented as supplementary information and the results have not been used for the risk assessment, since there is an existing test on the product supported by this dossier (Final Bite – 0402206).

I. MATERIALS AND METHODS**A. MATERIALS**

1. Test material: SLUG & SNAIL KILLER (0.98% total iron)

Batch no.: Not included in the report

Purity: 0.98% total iron

Description: grey-blue granules with characteristic odour

2. Test organism: *Daphnia magna* Straus

Age: < 24 hours old

Source: Institute of Industrial Organic Chemistry, Branch Pszczyna

Feeding: During housing the animals were fed with algae (*P. subcapitata*)

3. Treatment: 0 (control) and 10, 18, 32, 56 and 100 mg test item/L (nominal)

4. Test vessels: Glass vessels (150 mL)

Test water: Elendt water M7

5. Environmental conditions:

Temperature: 19.4 – 21.4°C

pH: 7.19 – 7.55

Dissolved oxygen: 7.39 – 9.40 (mg/L)

Photoperiod: 16 h light : 8 h darkness

B. STUDY DESIGN AND METHODS

1. In-life phase: 19 – 21 Nov 2008

2. Test organism assignment and treatment

Daphnids aged less than 24 hours at the start of the test were exposed at five concentrations of the test item for a period of 48 hours under semi-static conditions. 20 daphnids were used for each treated (10, 18, 32, 56 and 100 mg) and untreated (control) group, divided in four replicates of five animals each. No feeding occurred during the test.

3. Dose preparation

Approximately 5 g of test item was thoroughly grinded, then the following amounts of test item were weighed out: 10, 18, 32, 56 and 100 mg. Each of them were carried into separate 1L volumetric flasks and were filled up to 1L with dilution media. Contents of the flask were vigorously mixed for 2 hours on a magnetic stirrer. After mixing, insoluble fractions were removed by filtration. The size of the filter was not mentioned in the report. The filtrate was clear and colourless indicating that insoluble fractions were separated completely. Obtained concentrations of 10, 18, 32, 56 and 100 mg/L (nominal, based on weighed amount) were used for the definitive test. Elendt media was added in the control vessels.

4. Measurements and observations

The number of immobilised daphnids was assessed after 24 and 48 hours from the beginning of the test (the daphnids were considered dead or immobilised if they were not able to swim within 15 seconds after gentle agitation of the vessel). Dissolved oxygen concentrations and pH values were measured. The temperature and light intensity of the test area were recorded.

Analysis on the test item was not performed.

5. Statistics

The determination of the EC50 with the confidence limits were calculated by probit analysis using linear max. likelihood regression. For calculation ToxRat Professional ver. 2.09 computer software was used.

A. Mortality of *Daphnia*

The number of immobilized daphnids and the percentage of immobilization at 24 and 48 hours of exposure are presented in the following table.

Table: Percentage of immobilization after 24 and 48 hours of exposure

Nominal Concentration (mg/L)	No. exposed <i>Daphnia</i>	Response at 24 h		Response at 48 h	
		Dead/immobilised <i>Daphnia</i>	Total mortality (%)	Dead/immobilised <i>Daphnia</i>	Total mortality (%)
0 (control)	20	0	0	0	0
10	20	0	0	0	0
18	20	0	0	2	10
32	20	2	10	14	70*
56	20	10	50*	20	100*
100	20	14	70*	20	100*

B. Analytical verification

No analytical verification was conducted during the test.

C. Validity criteria

Validity criteria for the experiment were met: Mortality in the control during test was 0 % and the concentration of dissolved oxygen was kept within the range of 7.39-8.61 mg/L (>3 mg/L), however, no analytical confirmation of the test item was performed.

III. CONCLUSION

The 24 and 48-hour toxicity endpoints for Slug & Snail Killer to *Daphnia magna* are presented in the following table.

Table : Toxicity endpoints of the test item Slug & Snail Killer

Effect concentration	24 h	48 h
EC50 (95% confidence intervals)		
Test item (mg/L) nominal	65.587 (52.789 – 81.487)	26.873 (23.444 – 30.804)
NOEC (EC0)		
Test item (mg/L) nominal	18	10

LOEC		
Test item (mg/L) nominal	32	18

Based on the nominal test concentrations the NOEC (no observed effect concentration) of Slug & Snail Killer 48 hours after application to *Daphnia magna* was determined to be 10 mg test item/L. Based on the nominal test concentrations the LOEC (lowest observed effect concentration) 48 hours after application was determined to be 18 mg test item/L. The EC50 of Slug & Snail Killer was calculated to be 26.873 mg test item/L after 48 hours of exposure .

Report:	CP 10.2.1/05 Jerzy, O., 2008
Title:	Slug & Snail Killer Growth Inhibition Test <i>Pseudokirchneriella subcapitata</i> SAG 61.81 according to OECD Guideline No 201/ method C.3 W/91/08 (R-38461)
Report no.:	OECD 201/ method C.3.
Guidelines:	The mean coefficient of variation for section-by-section specific growth rates in the control cultures was exceeded (43.7% instead 35%), also the temperature was slightly exceeded during the test (20.5-23°C). The guideline recommends the range of 21-24°C maintained at $\pm 2^\circ\text{C}$
Deviations:	Yes,
GLP:	No
Published:	Validity criteria for the experiment were not met for the coefficient of variation in the control cultures also, no analytical confirmation of exposure was conducted. This study has been conducted with an equivalent test item to Final Bite®. The report is presented as supplementary information and the results have not been used for the risk assessment, since there is an existing valid test on the product Final Bite – 0402206
Comments:	

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: SLUG & SNAIL KILLER (0.98% total iron,

Batch no.: Not included in the report

Purity: 0.98% total iron

Description: grey-blue granules with characteristic odour

2. Test organism: Fresh water green algae

Source: *Pseudokirchneriella subcapitata*

Culture medium: AAP growth medium recommended by Guideline OECD No 201

Initial density: 1×10^4 cells/mL

3. Treatment: 10; 18; 32; 56; 100 and 180 mg/L (nominal concentration)

Replicates: 6 replicates for the control 3 replicates for each treatment, plus no algae replicate used as a background

4. Test vessels: 250 ml Erlenmeyer flask

Test water: OECD TG 201 APP medium

Shaking: on mechanical shaker

5. Environmental conditions:

Temperature: 20.5-23.0 °C

pH: 6.78 – 8.97

Photoperiod: continuous lighting 6940-7680 lux

B. STUDY DESIGN AND METHODS

1. In-life phase: 10-13 Dec 2008

2. Test organism assignment and treatment

Before the experiments, set up of the initial cultures were started (inoculated from basic cultures) in the same conditions as during the experiments. After attaining the exponential growth phase in initial cultures (about three days of incubation) algae were used for inoculation of the test cultures provided that the cells showed no abnormalities or any deformations (microscopic observation). The test started (0 hours) by inoculation of a biomass of 1.0×10^4 cells/mL test medium in each flask. Algal cells were sourced from a liquid growing stock culture (4.1 mL of algae inoculum of 0.975 millions cells per 1 mL). The test was performed with three replicates per test concentration and six replicates for the untreated control.

3. Dose preparation

The test item was used in series of 6 concentrations: 10; 18; 32; 56; 100 and 180 mg/L (nominal concentrations). 4.285 g of the test item was thoroughly mixed in mortar and then divided on: 180.24; 100.18 and 56.20 mg weighted amounts. Each of them was transferred quantitatively into mensural flask of 1 L volume. The adequate volume of AAP Medium was added. It was intensively mixed on multi-position magnetic stirrer for 2 h and insoluble fraction was separated by filtration. The obtained filtrates were uncolored and clear, what indicates that

insoluble and soluble fractions were completely separated. The filtrate was recognized as treatment based on nominal concentrations from weighed amount.

4. Measurements and observations

The absorbance was determined in every replicate of concentration and control after 24, 48 and 72 hours following the beginning of the experiment. The number of cells was established from spectrophotometric absorbance.

The test conditions of the test were monitored, temperature, illumination and pH-values were measured during the test.

5. Statistics

Statistical calculations and figures were made using the ToxRat Professional 2.09 computer software. The endpoints for growth and yield were calculated using linear regression method. The NOEC and LOEC values were calculated using statistical analysis: Range-to-standard-deviation-ratio Test on Normal Distribution, Cochran's Test Procedure on Variance Homogeneity, Williams Multiple Sequential t-test Procedure or Welch-t test for Inhomogeneous Variances with Bonferroni t-Test or Bonferroni Adjustment.

II. RESULTS AND DISCUSSION

A. Growth inhibition

The absorbance was determined in every replicate of concentration and control after 24, 48 and 72 hours following the beginning of the experiment. The number of cells was established from spectrophotometric absorbance ($A=670$ nm) and then records were recalculated with using nomogram. The number of algae cells was determined from equation of the regression curve $y=0.6003x-0.0003$ ($r^2=0.9953$).

Table: Mean number of cells

Nominal Concentration in mg/L	Parameter	Algae cells number (10^6 Cells /mL)		
		24 h	48 h	72 h
Control	Mean	0.0983	0.3610	0.9953
	SD	0.0057	0.0219	0.0649
10	Mean	0.1063	0.3557	1.0640
	SD	0.0081	0.0260	0.0203
18	Mean	0.1037	0.3403	0.9827
	SD	0.0047	0.0035	0.0108
32	Mean	0.1020	0.3137	0.9927
	SD	0.0044	0.0144	0.0216
56	Mean	0.1170	0.2147	0.5537
	SD	0.0089	0.0127	0.0065
100	Mean	0.1470	0.1810	0.2227
	SD	0.0137	0.0035	0.0131
180	Mean	0.1527	0.1827	0.2110
	SD	0.0012	0.0172	0.0035

SD = Standard Deviation

A separate test on reference substance - 3,5-dichlorophenol was conducted in similar conditions (temperature 22.0 - 22.5°C, constant illumination from 8600-8750 lux, AAP growth medium). 3,5-dichlorophenol substance was 97% purity produced by Aldrich. The reference substance was used in 5 concentrations in the range from 0.03 to 3.2 mg/L, in three replicates for each group and control culture in six replicates. The value ErC_{50} was 1.43 mg/L and the value EyC_{50} was 1.17 mg/L and both were within the range given in literature, therefore the test conditions were acceptable.

B. Analytical verification

Iron concentrations were not measured during the test.

C. Validity criteria

Parameter	Validity criteria	Observed value	Assessment
Increase factor biomass	factor 16 in 72 h	99.5	Valid
Mean coefficient of variation of daily growth rates	max. 35%	43.7%	Not Valid
Coefficient of variation of average growth rate during the whole test period	max. 7%	1.5%	Valid

D. 72-h toxicity endpoints

Endpoint	Effect concentration (nominal) mg/L	72 hour (95% confidence limits)
Yield	E ₇ C ₅₀	66.97 (42.89-106.55)
	E ₇ C ₂₀	40.27 (9.27-56.25)
	E ₇ C ₁₀	30.82 (3.60-46.32)
	NOEC	32
	LOEC	56
Growth Rate	E ₇ C ₅₀	> 180.00
	E ₇ C ₂₀	84.66 (25.01-130.40)
	E ₇ C ₁₀	46.16 (2.42-76.90)
	NOEC	32
	LOEC	56

III. CONCLUSION

The mean concentration causing 50% inhibition of yield inhibition of *Pseudokirchneriella subcapitata* culture (ErC50/72 h) was 66.97 mg/L, and mean concentration causing 50% of average specific growth rate (EyC50/72 h) was above 180.00 mg/L.

The LOEC values were 56.0 mg/L for yield and average specific growth rate. The NOEC values were 32.0 mg/L for yield and average specific growth rate based on nominal concentrations of the preparation after 72 h.

B.9.14. RISK ASSESSMENT FOR OTHER TERRESTRIAL ORGANISMS (FLORA AND FAUNA)

The carbon transformation test is not a data requirement under 283/2013 or 284/2013. Data on carbon transformation were submitted as part of this application, therefore they were evaluated. There was no indication from these data that there is an effect on carbon transformation as a result of exposure to Iron containing products. No quantitative risk assessment has been carried out as this is not deemed necessary. No further consideration of the risk to carbon transformation is required.

B.9.15. REFERENCES RELIED ON

Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
B.9.1.1/1	██████	2008	Slug and Snail Killer Acute Oral Toxicity Test with Japanese Quail (<i>Coturnix coturnix japonica</i>) Study code: G/53/08	Y				
B.9.1.1/2 -1	Pawlina, I. and Proulx, G.	1996	Study of House Sparrow (<i>Passer domesticus</i>) feeding preference to natural color [sic] and guard coat blue coated seeds	Y	N	n/a		N
B.9.1.1/2 -2	Lockie, J	1956	The food and feeding behaviour of the Jackdaw, Rook and Carrion Crow	Y	N	n/a		
B.9.1.1/2 -3	Holyoak, D.	1968	A comparative study of the food of some British Corvidae	Y	N	n/a		
B.9.3.1/1	██████	2018	Determination of short term toxicity of Final Bite – 0402206 against <i>Daphnia magna</i> STRAUS according to OECD 202 resp EU C.2.	N	Y	n/a		N

Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
B.9.3.1/2	██████	2018	Determination of the toxicity of Final Bite – 0402206 against <i>Desmodesmus subspicatus</i> according to OECD 201 resp EU C.3	N	Y	n/a		N
B.9.5.2/1	██████ █	2018	Final Bite - 0402206: Effects on the Reproduction of Rove Beetles <i>Aleochara bilineata</i> - Extended Laboratory Study - Dose Response Test -	N	Y	n/a		N
B.9.5.2/2	██████ █	2018	Final Bite - 0402206: Effects on the Carabid beetle <i>Poecilus cupreus</i> L. - Extended Laboratory Study --Dose Response Study	N	Y	n/a		N
B.9.5.2/3	██████ █	2018	Final Bite - 0402206: An extended laboratory test to determine effects on spiders of the genus <i>Pardosa</i> (Araneae, Lycosidae) when exposed to granules applied to a natural soil substrate	N	Y	n/a		N
B.9.7.1.1/1	██████	2018a	Final Bite - 0402206: Effects on Reproduction	N	Y	n/a		N

Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
			and Growth of Earthworms <i>Eisenia andrei</i> in Artificial Soil					
B.9.7.1.1/2	██████	2018b	Final Bite - 0402206: Effects on Reproduction and Growth of Earthworms <i>Eisenia andrei</i> in Artificial Soil	N	Y	n/a		N
B.9.7.1.2/1	██████	2019	Final Bite - Blue – A Field Study to Investigate Effects on the Earthworm Fauna in Central Europe	N	Y	n/a		N
B.9.7.1.3/2	Edwards, CA <i>et al.</i>	2008	The relative toxicity of metaldehyde and iron phosphate-based molluscicides to earthworms	N	N	n/a		N
B.9.7.2/1	██████	2018c	Final Bite - 0402206: Effects on Reproduction of the Collembola <i>Folsomia candida</i> in Artificial Soil	N	Y	n/a		N
B.9.7.2/2	██████	2018d	Final Bite - 0402206: Effects on Reproduction of the Predatory Mite <i>Hypoaspis aculeifer</i> in Artificial Soil	N	Y	n/a		N
B.9.9.1	██████	2008	Slug & Snail Killer Soil Microorganism	N				N

Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
			s: Nitrogen Transformation Test					

Appendix 1**Position Papers submitted by the applicant in support of the Bird and Mammal Risk assessment following discussion with HSE****Literature Review on Iron Physiology in Birds & Mammals****Search methods****INTRODUCTION**

In a response to the submitted document on 'Final Bite (a.s. elemental iron), Literature review regarding iron uptake and regulation in birds and mammals' (RIFCON Report No. R2260023, ADAMA reference No. 000110088) CRD requested a more detailed background document on the targeted literature search. The original statement does refer to a literature review process, but in order to be fully transparent, it was requested that a report be provided describing the process (e.g., search terms, results, rapid relevance criteria, etc.) in more detail to ensure that the search was carried out with a sound scientific and comprehensible methodology.

This document describes the literature selection in more detail. However, a standard literature review could not be conducted as previously discussed and agreed with CRD and a targeted literature search was considered more appropriate for compiling literature on iron physiology.

LITERATURE REVIEW ON IRON PHYSIOLOGY

A targeted literature review was conducted as part of the risk assessment of elemental iron to birds and mammals. It was agreed with CRD that a systematic literature review is not possible and that a different approach will be taken for the review of iron physiology. The rationale for this alternative approach is explained below.

REASONS FOR THE ALTERNATIVE APPROACH

The main difference in the approach chosen from a standard literature search for a typical active ingredient is that iron is not a novel active ingredient with its own use in crop protection, but a vital nutrient that can also be used as an active ingredient in crop protection. Thus, the present case is not about the ecological effects of a novel organic pesticide, but about the effects that a vital element might have on birds and mammals when used as a molluscicide.

The basic physiological effects of the nutrient iron have long been researched and known²². Of course, there are always new findings and publications that add to the existing knowledge about iron physiology. However, recent publications on iron metabolism relate to modern research interests such as the use of iron in dietary supplements to cure iron deficiency symptoms. For example, frequently cited topics deal with issues such as epidemiological research on the global burden of iron deficiency, clinical aspects of iron deficiency anaemia, or the effects of iron deficiency on children.

In order to present a risk assessment for elevated iron levels in the diet of birds and mammals due to the use of iron as a molluscicide, the goal is to present the basic knowledge of iron homeostasis in order to evaluate the physiological responses to elevated concentrations of a vital trace element. Therefore, a regular literature search based on new publications would be counterproductive as current research on iron focusses on different topics (e.g., iron as a dietary supplement), compared to the basic information that has been investigated in the past.

SEARCH METHODS

The literature search was aimed to provide information on physiological iron homeostasis when animals are exposed to elevated iron levels. Therefore, to gather information for a higher-level risk assessment, a general

²² Research into the basic physiological effects of the nutrient iron has a long history (see Sheftel et al. 2011 from which the following is taken). Vincenzo Menghini (1704-1759) first established that iron is concentrated in red blood cells. Louis René Lecanu discovered in the 1830s that red blood cells contain the iron-containing pigment "hematosin." Fontès and Thivolle (1925) determined serum iron concentration and found that it is decreased in iron deficiency. In the 1930s, 40s, and 50s, important principles of iron homeostasis were discovered (crystallization of the iron-storing protein ferritin by Vilém Laufberger (1937), retention of iron by McCance and Widdowson (1938), discovery of the iron-binding plasma protein transferrin (Holmberg and Laurell 1945), uptake of iron from plasma and incorporation into hemoglobin (Finch et al. 1949), iron administration leads to stimulation of ferritin synthesis in the liver (Fineberg and Greenberg, 1955). In 1981, Morgan published the first comprehensive review of transferrin and discussed the mechanisms of cellular iron uptake and regulation. This (non-systematic) enumeration of some milestones in iron research shows how long the basic principles of iron physiology have been known.

literature search with predefined search terms does not seem appropriate. Also because of the massive amount of hits when using 'iron' as a search term. In addition, a literature search such as that conducted as part of the Annex I process is limited to a specific time period, usually 10 years, the period of the most recent renewal. This approach is useful for tracking down current information on an active ingredient after the last renewal. However, it is problematic for long-established areas of knowledge and data in avian and mammalian risk assessment, where case-specific information is needed regardless of when it was published.

Understanding the functioning of animal iron balance has long been a concern of researchers in physiology and medicine (see the report entitled *"The Long History of Iron in the Universe and in Health and Disease"* (Sheftel et al. 2011)). The basic mechanisms of iron are well understood and described in detail. The information on the physiological process of iron in birds and mammals is often more than a decade old (see foot note 1). Therefore, a stepwise approach was taken to obtain the most relevant facts from the published literature on physiological iron homeostasis that give insight into how animals cope with elevated dietary iron levels.

As a first step, secondary scientific literature from book publications and review articles on avian and mammalian iron physiology was searched and considered to provide information on the current state of knowledge of iron physiology. Based on this, specific information was searched for when needed in standard search engines such as Google, Google Scholar, and PubMed, as well as in RIFCON's own library on toxicology, pesticides, and relevant organisms (focal species). In addition, cross-references in retrieved publications by specific authors and research groups working on iron physiology in birds and mammals were considered and followed. The advantage of this approach is that it is not limited to a specific period and results in a list of case-specific literature.

Search engines

The available open search engines such as PubChem, PubMed, and Google scholar were searched for information on the physiological function of iron in birds and mammals.

PubChem is the world's largest collection of freely available chemical information. It provides information about chemicals by name, molecular formula, structure, and other designations.

PubMed is a free search engine accessing primarily the MEDLINE database of references and abstracts on life sciences and biomedical topics.

Google Scholar is a freely accessible web search engine that indexes the full text of scholarly literature across an array of publishing formats and disciplines. Google Scholar index includes most peer-reviewed online journals of Europe and America's largest scholarly publishers.

In addition, RIFCON's own library is available with more than 11,000 references with contexts on toxicology, pesticides, and relevant organisms.

Search terms

The search terms were adapted to the project and the desired information.

The search terms presented in Table 1 have been investigated and were used in various combinations. The search terms related to the specific references are shown in Table 4 below.

Table 1: Search terms used for the targeted literature search

Substance	Physiology	Exposure	Toxicity	Species
Iron	Metabolism	Exposure	Toxicity	Birds
	Physiology	Concentration	Effect	Avian
	Homeostasis	Diet	Mode of action	Poultry
	Regulation	Food	Detoxification	Mammals
	Transport	Environment		Mammalian
Pellets	Supplementation	Soil		Rat
Granules	Deficiency	Overload		Mice
Molluscicide	Absorption	Bioavailability		Vertebrates
Metals	Uptake			Invertebrates
	Excretion			Slugs
	Requirements			Snails
	Trace element			Plants
	Micronutrient			
	Nutrition			

To be more specific, the primary search term "iron" was combined with terms such as "physiology," "trace element," "homeostasis" to form "iron physiology," "trace element iron," "iron homeostasis," and so on. Iron is one of the elements that organisms, from simple bacteria to birds and mammals, depend on. The result set of hits obtained was therefore narrowed down using the terms "mammals", "mammalian", "birds" or "avian" and/or terms such as e.g. "review article".

The search was restricted to single years, 5-year intervals, decades, etc. For example, searches were done for studies conducted during the last year, last 5 years or between 2022 and 2012, and further back if necessary to find the most recent publications first and then go back in time.

The number of possible combinations of search terms quickly grows immeasurably. Therefore, only individual examples are given here (Table 2) to illustrate the process (e.g.: **Search engine:** pubmed; **Search terms:** iron, physiology, mammal; **Article type:** Review; **Publication date** 10 years - reveals 4,451 results). In this way, several recent review articles were found containing a wealth of information on chemical and physical properties, biological activities, safety and toxicity information, patents, and more.

Evaluation of sources (relevance criteria)

The literature citations found in this way offered a variety of articles on chemical and physical properties of iron, its biological activities, safety and toxicity information, patents, and more (Table 2). Therefore, decision criteria were used to select relevant articles for the selection process.

Table 2: The number of records retrieved from scientific literature in bibliographic databases*

	PubChem	PubMed	Google scholar
Iron	244,201	247,740	4,710,000
Iron metabolism	100,410	139,051	2,840,000
Iron metabolism bird	85	1,346	62,400
Iron metabolism mammal	36	92,327	43,100

* The number of combinations of search terms is growing rapidly to unmanageable numbers. Therefore, only examples are given here. The set of hits was first pre-sorted based on the titles in order to take a closer look at the summaries only for the relevant matches. Only literature types aimed at a biological, agricultural, or medical audience were considered, such as textbooks, review articles, substance-related studies, and peer-reviewed scientific literature.

Before a citation was included in this iron physiology literature review, sources were evaluated using the rapid relevance criteria (accuracy, relevance, authorship, objectivity, timeliness, and completeness) to determine if they contained valuable and pertinent information. The criteria for the selection process of "why the selected publications are considered relevant" are presented below.

Table 3: Selection process: rapid relevance criteria (accuracy, relevance, authorship, objectivity, timeliness, and completeness)

Criteria	Questions	Rating
Accuracy	Is the information reliable? Is the information free of errors? Is the information based on proven facts? Can the information be verified against other reliable sources?	If one of the questions was answered with "no", the citation was excluded (Score: include, exclude)
Relevance	Is the information relevant? Does the information covered meet the information need? Is the information contained valuable and pertinent? Is it basic or in-depth coverage?	The relevance is rated based on a scale (Score: 1: relevant (The text contains relevant information), 2: partially relevant (Parts of the publication contain relevant information), 3: irrelevant) (The information is not relevant to the topic)
Authority	Are the authors qualified to write on this topic? Are they associated with a reputable organization (university, research institutes) in this field?	Authors publishing in the field of agriculture, animal physiology, medicine, and veterinary medicine (Score: yes, no)
Objectivity	What is the purpose of the information? Is the information biased?	Publications aimed at a specialist agricultural, biological, toxicological, or medical audience were included (Score: yes, no)
Timeliness	When was the information published? Is the information current or outdated?	In the case of similar focal points of the information compilation (e.g., general iron physiology), more recent publications were preferred (Score: current c, outdated o)

Accuracy, relevance, authorship, objectivity, timeliness, and completeness are the five basic criteria used in this process to evaluate information from relevant sources. Internet sources are considered particularly critical in this

process. Therefore, all Internet sources that could not be traced to a reliable source (e.g., a peer-reviewed publication) were excluded.

Since the task was to compile basic information on iron metabolism, the search was terminated when a manageable number of current articles were found this way. Extending the search to further (older) review articles was not considered useful.

Klimisch score

The Klimisch score describes a method for assessing the reliability of toxicological and similar studies in a regulatory context. The following four scores are possible: 1 = reliable without limitations, 2 = reliable with limitations, 3 = not reliable, 4 = cannot be determined.

However, this procedure is not intended for the evaluation of review articles. The extent to which a result is considered reliable is judged, for example, by the informative value of the method used, the presentation of the results, and the conclusion. It is important to have a procedure that is as standardised as possible and a description of the experimental procedure and results. This information is usually lacking in review articles. In this respect, the Klimisch score was not considered suitable as a method for assessing the reliability of the articles and literature used.

RESULTS

During the literature search, it became apparent that there are many textbooks and reviews on iron physiology in vertebrates due to the reasons mentioned above. Understanding the functioning of the human and animal organism has long been a concern for researchers in physiology and medicine. Overall, the physiology of iron uptake and homeostasis in vertebrates is a well-researched area, both scientifically and medically. Numerous review articles summarise the current state of knowledge with varying emphases. Iron deficiency and overload are well-known diseases in humans (and animals), on which there is a wealth of literature from the medical (and veterinary) context. The selected review articles and textbooks summarise the mechanisms of iron homeostasis. Articles such as those by Aggett (2012), Schümann et al. (2007), Waldvogel-Abramowski et al. (2014), Coffey and Ganz (2017) are well suited to provide an overview of iron metabolism in mammals. Accordingly, there are several books (e.g., Bezzel and Prinzinger 1990, Brue 1994, and Powell 2015) that summarise the current knowledge on iron metabolism in birds.

For individual aspects, a partial search was conducted for articles addressing corresponding sub-aspects, such as Nemeth and Ganz (2009) on the role of hepcidin in iron metabolism.

The toxicological aspects of iron are discussed in detail in various sources (e.g., metals in general: Marquardt and Schäfer 1997, iron specifically: Ponka et al. 2007, or specifically on iron fortification in food/iron substitution by appropriate preparations: Schümann et al. 2007).

When searching for information on the natural iron content of foods, it was found that there are hardly any surveys on the diet of wild animals. There is, however, a large amount of data that comprehensively capture and present the iron content of foods in relation to human diets (e.g., data from the USDA database <https://fdc.nal.usda.gov/>). It can be assumed that the iron content of natural foods is not fundamentally different from those consumed by humans, so these data (e.g., Mwangi et al. 2018 Insects as sources of iron and zinc in human diets) can also be used as a proxy for wildlife (e.g., insectivorous birds and mammals). For aspects of wildlife exposure following application, e.g., from contaminated snails, refer to efficacy studies.

Specific toxicological aspects of the formulation with elemental iron, can be taken from the review publications of the manufacturers of corresponding preparations, here it comes to bear that the same and similar substances are used for iron replacement in iron deficiency in humans. Toxicological information can be found, for example, in a technical data sheet on Ferronyl® carbonyl iron powder from ISP, which also contains information on bioavailability and toxicity compared with other iron salts.

Overall, this compilation is based on about fifty references, which are mainly review articles or textbook chapters summarizing a wide range of literature sources. The selected literature is evaluated in the table below, with systematic and objective criteria for the selection process provided to describe the process of why the selected publications are considered relevant. A short description of the relevant information in the chosen references as well as the respective search terms are presented in the table as well.

REFERENCES

Sheftel AD, Mason AB, Ponka P. The long history of iron in the Universe and in health and disease. *Biochim Biophys Acta*. 2012;1820(3):161-187. doi:10.1016/j.bbagen.2011.08.002

Table 4: Literature evaluation assessed against rapid relevance criteria (accuracy, relevance, authorship, objectivity, timeliness, and completeness)

Reference	Reason for choosing the reference	Search terms	Literature type ¹	Accuracy ²	Relevance ³	Authority ⁴	Objectivity ⁵	Timeliness ⁶
1. Äckerlein, W. 1993. Die Ernährung des Vogels. 2nd Extended Edition Verlag: Ulmer Stuttgart. P. 37.	Book chapter with information on iron in avian nutrition	Iron physiology, nutrition, birds	A	I	2	Y	Y	C
2. Aggett, P.J. 2012. Iron. Chapter 33. In: Present knowledge in nutrition – 10th ed. / edited by John W. Erdman Jr., Ian A. Macdonald, Steven H. Zeisel. Wiley-Blackwell. ILSI Press, Washington, DC. p. 506-520. https://pkn10.org/	Book chapter summarising the current state of knowledge on iron balance in humans	Iron metabolism, iron physiology, iron absorption, iron homeostasis, nutrition, iron deficiency, iron bioavailability	A	I	1	Y	Y	C
3. Axmann 2019. Final Bite - Blue – A Field Study to Investigate Effects on the Earthworm Fauna in Central Europe, Report No S18-02597, Sponsor Code R-39838	Earthworm field study conducted with Final Bite contains information on potential exposure to the granules	Iron pellets, exposure to pellets	C	I	1	Y	Y	C
4. Bairlein, F. 1996. Ökologie der Vögel. Gustav Fischer Verlag. Stuttgart. (p. 20-24).	Book chapter with information on avian nutrition	Avian nutrition	A	I	2	Y	Y	C
5. Bezzel, E. and R. Prinzinger 1990. Ornithologie. Ulmer, Stuttgart. P. 174	Book chapter summarising the current knowledge on iron metabolism in birds	Iron metabolism, iron physiology, nutrition, micronutrient, bird	A	I	2	Y	Y	C
6. Boyd, E.M. and M.N. Shanas, 1963. The Acute Oral Toxicity of Reduced Iron, Canad. Med. Assn. J. 89, 171-175.	Information on the acute toxicity of reduced iron in rats	Iron toxicity, rat, mammals	C	I	1	Y	Y	C
7. Brue, R. N., 1994. Nutrition. Chapter 3. In: Avian Medicine, B. W. Ritchie, G. J. Harrison, L. R. Harrison. Wingers Pub. Lake Worth, Fla.: (p.63-95).	Book chapter with information of iron in avian nutrition	Iron metabolism, nutrition, birds	A	I	2	Y	Y	C
8. Campbell, T.W. 1994. Haematology. Chapter 9. In: Avian Medicine, B. W. Ritchie, G. J. Harrison, L. R. Harrison. Wingers Pub. Lake Worth, Fla.: (p.177-198)	Book chapter with information on avian hematology	Iron physiology, iron deficiency, birds	A	I	2	Y	Y	C

9. Cerundolo, D. L., S. F. Coons, E. D. Gibson, and C. P. Loreti. 1988. 6 - MATRIX ELEMENTS AND LIGANDS. Pages 6-1-6.12-13 in I. Bodek, W. J. Lyman, W. F. Reehl, and D. H. Rosenblatt, editors. Environmental Inorganic Chemistry. Pergamon, Amsterdam.	Book chapter with information on the occurrence of iron in the environment and concentrations of iron in the different compartments	Iron in the environment, iron concentration in soil	A	I	1	Y	Y	C
[REDACTED]	[REDACTED]	[REDACTED]	D	I	2	Y	Y	C
11. Coffey, R. and T. Ganz. 2017. Iron homeostasis: An anthropocentric perspective. Journal of Biological Chemistry 292:12727-12734. DOI:https://doi.org/10.1074/jbc.R117.781823	Review article on iron homeostasis	Iron metabolism, iron physiology, iron absorption, iron homeostasis, iron deficiency. V. mammals	B	I	1	Y	Y	C
12. Cowan, P. and M. Crowell. 2017. Visual and taste cues for minimising native bird interactions with toxic 1080 baits – A review of current practices. New Zealand Journal of Ecology 41.	Review article on exposure of birds and mammals to pellets investigating repellent effects of e.g. different bait colours	Exposure to pellets, birds, mammals, rat	B	I	2	Y	Y	C
13. Domke A., R. Grossklaus, B. Niemann, H. Przyrembel, H. Richter, and E. Schmidt, A. Weissenborn, B. Wörner, and R. Ziegenhagen. 2004. Risk Assessment of Iron in Use of Minerals in Foods – Toxicological and nutritional-physiological aspects, Part 2, Federal Institute for Risk Assessment pp. 145-188	Summary from BfR on minerals in food	Iron metabolism, nutrition, iron physiology, iron toxicity, mammals	n.a.	n.a.	1	n.a.	n.a.	n.a.
14. EFSA 2009. Guidance of EFSA - Risk Assessment for Birds and Mammals. EFSA Journal 7: 1-139.	Standard EU Guidance document for risk assessment on birds and mammals	Birds, mammals, toxicity	n.a.	n.a.	1	n.a.	n.a.	n.a.
15. Flachowsky, G. 2000. Mineralstoffe. In: Engelhardt, W. V., Breves, G. (Ed.) Physiologie der Haustiere Ferdinand Enke Verlag. Stuttgart, p. 606-620	Book chapter with information on the	Iron metabolism, iron absorption, iron	A	I	2	Y	Y	C

	physiology of domesticated animals	homeostasis, birds, mammals						
16. Frazer, D. M., Wilkins, S. J., Becker, E. M., Murphy, T. L., Vulpe, C. D., McKie, A. T., and Anderson, G. J. 2003. A rapid decrease in the expression of DMT1 and Dcytb but not Ireg1 or hephaestin explains the mucosal block phenomenon of iron absorption. <i>Gut</i> , 52(3), 340–346. https://doi.org/10.1136/gut.52.3.340	Information on iron absorption in mammals	Iron metabolism, iron absorption, rats, mammals	D	I	I	Y	Y	C
17. Früh, R. 2009. Schafe auf dem Grünland gesund und leistungsfähig halten- Mineralstoffversorgung Thüringer Landesanstalt für Landwirtschaft, Jena http://www.tll.de/www/daten/nutztierhaltung/schafe_ziegen/shgn0110.pdf	Information on iron availability from plants in mammals (sheeps)	Iron requirements, nutrition, iron absorption, iron bioavailability, mammals	n a	I	2	Y	Y	C
[REDACTED]	[REDACTED]	[REDACTED]	C	I	I	Y	Y	C
19. Hansen, S. L., J. W. Spears. 2009. Bioaccessibility of iron from soil is increased by silage fermentation <i>Journal of Dairy Science</i> 92(6), 2896-2905	Information on bioavailability of iron in the diets of cattle	Iron concentration in the diet, iron absorption, iron bioavailability, mammals	D	I	I	Y	Y	C
[REDACTED]	[REDACTED]	[REDACTED]	D	I	I	Y	Y	C
21. Harvey, J. W. 2008. Chapter 9 - Iron Metabolism and Its Disorders. Pages 259-285 in J. J. Kaneko, J. W. Harvey, and M. L. Bruss, editors. <i>Clinical Biochemistry of Domestic Animals</i> (Sixth Edition). Academic Press, San Diego.	Book chapter on iron metabolism and disorders in domestic animals	Iron metabolism, iron homeostasis, iron physiology, iron absorption, birds, mammals	A	I	I	Y	Y	C
[REDACTED]	[REDACTED]	[REDACTED]						

23.	Henderson, I.F., Briggs, G.G., Coward, N.P., Dawson, G.W. and Pickett, J.A. 1989. A new group of molluscicidal compounds. In: Henderson, I.F. (ed.) Slugs and Snails in World Agriculture. Monograph No. 41, British Crop Protection Council, Thornton Heath, pp. 289-294.	Book chapter about innovative (at that time) molluscicides including iron based molluscicides and explanation of its effect on slugs and snails	Iron molluscicide, iron mode of action, iron effect on slugs and snails	A	I	1	Y	Y	C
24.	Johnson, L. R., 2001. Iron absorption, in Gastrointestinal Physiology, Mosby. P151-153	Book chapter explaining the details of iron adsorption in the gastrointestinal tract	Iron absorption, iron excretion, iron uptake	A	I	1	Y	Y	C
25.	Knutson M.D., Walter P.B., Ames B.N., Viteri F.E., 2000. Both iron deficiency and daily iron supplementation increase lipid peroxidation in rats. J Nutr; 130:621—8.	Article providing and overview on the effects of iron supplementation in rats	Iron homeostasis, vertebrates, mammals, rat, iron concentration in diet, nutrition, iron effect	D	I	1	Y	Y	C
26.	Kobayashi, T., Nozoye, T., & Nishizawa, N. K. (2019). Iron transport and its regulation in plants. Free Radical Biology and Medicine, 133, 11-20.	Review article on iron transport and its regulation in plants	Iron, plants, iron regulation, iron transport	B	I	2	Y	Y	C
27.	Marquardt, H. and Schäfer, S.G., (ed.), 1997. Lehrbuch der Toxikologie. Spektrum Akademischer Verlag, Heidelberg	Textbook providing an overview about toxicology and toxicological aspects of iron	Iron toxicity, metals toxicity, iron metabolism, iron detoxification, vertebrates	A	I	2	Y	Y	C
				D	I	1	Y	Y	C
29.	McDonald, D. (2006) “Nutritional Considerations - Section I: Nutrition and Dietary Supplementation”, Clinical Avian Medicine. Available at: https://www.ivis.org/library/clinical-avian-medicine/nutritional-	Book chapter providing an overview about nutrition	Iron nutrition, iron uptake, iron concentration in	A	I	2	Y	Y	C

considerations-section-i-nutrition-and-dietary (Accessed: 11 March 2022).	and dietary supplementation for birds	diet, iron physiology, iron absorption, iron metabolism, birds, avian						
30. Mete, A., R. Jalving, B. A. van Oost, J. E. Van Dijk, and J. J. Marx. 2005. Intestinal overexpression of iron transporters induces iron overload in birds in captivity. <i>Blood Cells Mol. Dis.</i> 34: 151 – 156.	Article about effects of iron overload in birds (birds in captivity)	Iron uptake, iron in diet, iron concentration, iron effect, iron toxicity, iron overload, iron absorption, birds, avian	D	I	1	Y	Y	C
[REDACTED]	[REDACTED]	[REDACTED]	D	I	1	Y	Y	C
32. Moyo, V. M., E. Mandishona, S. J. Hasstedt, I. T. Gangaidzo, Z. A. Gomo, H. Khumalo, T. Saungweme, C. F. Kiire, A. C. Paterson, P. Bloom, A. P. MacPhail, T. Rouault, and V. R. Gordeuk. 1998. Evidence of genetic transmission in African iron overload. <i>Blood</i> 91:1076-1082.	Information on iron overload	Iron, iron overload	D	I	1	Y	Y	C
33. Muir A, and U. Hopfer 1985. Regional specificity of iron uptake by small intestinal brush-border membranes from normal and iron-deficient mice. <i>Am J Physiol</i> 248(G Pt 1):G376-9. doi: 10.1152/ajpgi.1985.248.3.G376. PMID: 3976894.	Information on iron uptake and absorption in mice	Iron, uptake, iron deficiency, iron absorption, mammals, mice	D	I	1	Y	Y	C
34. Mutschler, E., G. Geisslinger, H. K. Kroemer, and M. Schäfer-Korting. 2001. <i>Arzneimittelwirkungen Lehrbuch der Pharmakologie und Toxikologie</i> . Wissenschaftliche Verlagsgesellschaft mbH Stuttgart. P.:481-485	Textbook with information on iron metabolism (including iron resorption, transport, use, storage, requirements, deficiency)	Iron metabolism	A	I	2	Y	Y	C
35. Mwangi M.N., D.G.A.B. Ooninex, T. Stouten, M. Veenbos, A. Melse-Boonstra, M. Dicke, J.J.A. van Loon. 2018. Insects as sources of iron and zinc in human nutrition. <i>Nutr Res Rev.</i> 31, 248-255	Information on iron concentrations in insects	Iron, nutrition, food, diet	D	I	1	Y	Y	C
36. National Research Council, 1994. <i>Nutrient Requirements of Poultry</i> . Ninth Revised Edition, 1994. Washington, DC: The National	Information on nutrient requirements	Iron, nutrition, iron deficiency	D	I	2	Y	Y	C

Academies Press. https://doi.org/10.17226/2114 .	ts in poultry diet	y, poultry, bird						
37. Nemeth, E. and T. Ganz, 2009. The Role of Hepcidin in Iron Metabolism. <i>Acta Haematol</i> 2009;122:78–86. DOI: 10.1159/000243791	Information on the role of hepcidin in iron metabolism	Iron metabolism, iron homeostasis, iron overload	D	I	1	Y	Y	C
38. Nemeth, E., S. Tuttle Marie, J. Powelson, B. Vaughn Michael, A. Donovan, M. Ward Diane, T. Ganz and J. Kaplan, 2004. Hepcidin Regulates Cellular Iron Efflux by Binding to Ferroportin and Inducing Its Internalization. <i>Science</i> 306:2090-2093	Information on the role of hepcidin in iron metabolism	Iron metabolism, iron homeostasis, iron overload	D	I	1	Y	Y	C
[REDACTED]	[REDACTED]	[REDACTED]	D	I	2	Y	Y	C
40. Papanikolaou, G. and K. Pantopoulos, 2005. Iron metabolism and toxicity. <i>Toxicology and Applied Pharmacology</i> 202:199-211	Review article on iron metabolism and toxicity	Iron metabolism, iron toxicity, iron homeostasis, iron bioavailability, mammals	B	I	1	Y	Y	C
41. Penzlin, H., 1991. Der Transport der Atemgase, <i>Lehrbuch der Tierphysiologie</i> . Gustav Fischer, Jena. P. 310-326	Textbook on animal physiology	Iron physiology, vertebrates, birds, mammals, invertebrates	A	I	2	Y	Y	C
[REDACTED]	[REDACTED]	[REDACTED]	D	I	1	Y	Y	C
43. Ponka, P., M. Tenenbeim, and J. W. Eaton, 2007. 'Chapter 30. Iron', in <i>Handbook on the Toxicology of Metals</i> (Third Edition), ed. by Gunnar F. Nordberg, Bruce A. Fowler, Monica Nordberg and Lars T. Friberg (Burlington: Academic Press, 2007), pp. 577-98.	Book chapter on iron	Iron metabolism, iron absorption, iron physiology, iron deficiency, iron overload, iron toxicity	A	I	1	Y	Y	C
44. Powell, F.L. 2015. Chapter 13 - Respiration. In: C. G. Scanes (ed.) <i>Sturkie's Avian Physiology</i> (Sixth	Book chapter on iron in the	Iron physiology, iron	A	I	2	Y	Y	C

	Edition). p 245. Academic Press, San Diego. P. 301-336	respiratory system of birds.	transport bird, avian						
45.	Schmidt-Nielsen, K. 1997. Oxygen transport in blood. In „Animal Physiology“. 5. edition. Cambridge University Press. P. 66-69	Book chapter on oxygen transport in blood	Iron physiology, iron transport vertebrates, invertebrates	A	I	1	Y	Y	C
46.	Schümann, K., Ettle, T., Szegner, B., Elsenhans, B., & Solomons, N. W. (2007). On risks and benefits of iron supplementation recommendations for iron intake revisited. Journal of Trace Elements in Medicine and Biology, 21(3), 147-168.	Review article on risks and benefits of iron supplementation	Iron, nutrition, iron supplementation, diet, iron deficiency, iron overload, iron toxicity	B	I	1	Y	Y	C
	[REDACTED]	[REDACTED]	[REDACTED]	A	I	2	Y	Y	C
	[REDACTED]	[REDACTED]	[REDACTED]	D	I	1	Y	Y	C
49.	Stephan, E. 2010. Zur Eisenversorgung ausgewachsener Pferde, Mecklenburger Pferde 2/2010, 50-51 https://www.hippothek.de/pdf/1302013099.0689.pdf	Information on iron concentrations in feed of horses	Iron, nutrition, iron requirements, iron concentration, diet, mammals	D	I	1	Y	Y	C
50.	Troeh F. R. and Thompson, L. M. 2005. Chapter 15. The Micronutrients. Soils and Soil Fertility, 6th ed. Wiley-Blackwell	Book chapter on iron as a micronutrient in soils and plants	Iron, micronutrient, trace element	A	I	2	Y	Y	C
51.	Waldvogel-Abramowski S, Waeber G, Gassner C, Buser A, Frey BM, Favrat B, Tissot JD. 2014. Physiology of iron metabolism. Transfus Med Hemother. 2014 Jun;41(3):213-21. doi: 10.1159/000362888	Review article on physiology of iron metabolism	Iron metabolism, iron physiology, iron transport, iron homeostasis, iron deficiency, iron overload, mammals	B	I	1	Y	Y	C

				D	I	I	Y	Y	C
53.	Whaley, C. 2022. Granular iron based molluscicides and observations relating to the behaviour of slugs and snails. i2L Research Ltd.	Internal report on granular iron based molluscicides and observations relating to the behaviour of slugs and snails	Iron granules, slugs, snails	unpublished report	I	I	Y	Y	C
54.	Zhang, A. S. and C. A. Enns. 2009. Molecular mechanisms of normal iron homeostasis. Hematology 207-214.	Review article on molecular mechanisms of normal iron homeostasis	Iron homeostasis, iron absorption, iron overload, iron deficiency, iron transport	B	I	I	Y	Y	C

1) Literature type: A Textbook, B Review article, C Study, D Scientific literature

2) Accuracy Score: include (I), exclude (E)

3) Relevance Score: 1: relevant, 2: part of information given considered relevant, 3: irrelevant

4) Authority Score: Authors publishing in the field of agriculture, animal physiology, medicine, and veterinary medicine (yes (Y), no (N))

5) Objectivity Score: Publications aimed at a specialist agricultural, biological, toxicological, or medical audience were included. (Yes (Y), no (N))

6) Timeliness Score: Current (c), outdated (o)

Appendix 2: Applicant's weight of evidence consideration of risks to birds and mammals

- Amendment to the Final Report -

Final Bite (a.s. elemental iron)

**Literature review regarding iron uptake and
regulation in birds and mammals**

Data Requirement

EU Regulation 1107/2009

Guidance of EFSA: Risk Assessment of Birds and Mammals (2009)

Authors

Jens Schabacker, Felix von Blanckenhagen

RIFCON GmbH Report No.

R2260023

ADAMA reference No.

000110088

June 26, 2023

1. INTRODUCTION

According to EU Regulation 1107/2009, the effects of crop protection products on wild mammals and birds must be assessed. Therefore, a comprehensive exposure assessment of the application is required.

Final Bite is an effective molluscicide for the control of slugs and snails in arable, horticultural, and ornamental crops. The formulation in the form of a ready-to-use bait contains 10 g/kg of elemental iron and attractants of natural origin. According to the GAP table (Chapter 3) the pellets can be used in all edible and non-edible field crops and amenity vegetation.

Birds and mammals may be exposed to the active substances by the consumption of granules or contaminated slugs and snails taken from treated fields. Therefore, birds and mammals that ingest granules as a food source or grit, or that include slugs and snails in their diet, are at risk of ingesting higher levels of elemental iron from the formulation.

Within the Guidance Document on Risk Assessment for Birds and Mammals (EFSA 2009; hereafter „GD (EFSA, 2009)”) general procedures are given for Tier 1 risk assessment as well as for possible refinement steps for higher tiered risk assessments. According to the GD (EFSA 2009) ecotoxicological risk assessments should follow the general principle that visible mortality and population effects should be in the focus of concern.

It should however be noted that the active substance of Final Bite (iron) differs from the organic molecules that are usually used as active substances in pesticides. Iron is a trace element and an essential micronutrient for all animals and plants. Trace elements may make up only a small part of the diet. However, they are important and vital for many body functions. Iron, for example, is important for the oxygen transport and energy production. An insufficient supply of iron can lead to deficiency symptoms. Nevertheless, at very high body concentrations, toxic effects may occur due to iron overload. Therefore, mechanisms have evolved to balance internal concentrations of animals.

However, the model for risk assessments as described in the GD (EFSA 2009) is designed for organic compounds with no physiological function. For such substances, the detoxification reactions in the liver and excretion via faeces and urine are the primary toxicological concerns. In contrast, because iron ion species are essential micronutrients internal levels are regulated by mechanisms that control the absorption and elimination and maintain optimum internal concentrations (homeostasis²³). These mechanisms can be expected to regulate internal iron levels to non-toxic levels in environments with fluctuating, variable iron availability, which will be discussed in detail below.

According to the GD (EFSA 2009), ecotoxicological risk assessments should follow the general principle that visible mortality and population effects should be the focus of consideration. In view of the knowledge presented here on iron regulation in the organism and the only small increase in iron intake to be expected, it can be assumed that the protection objective is met.

2. LITERATURE SEARCH STRATEGY

2.1. LITERATURE SEARCH

Initially the available open search engines such as PubMed and Google scholar and others²⁴ for information on iron physiological function and dysfunction in birds and mammals were searched.

Search engines:

Google Scholar is a freely accessible web search engine that indexes the full text of scholarly literature across an array of publishing formats and disciplines. Google Scholar index includes most peer-reviewed online journals of Europe and America's largest scholarly publishers.

²³ Homeostasis is the property of a system, such as a living organism, to regulate its internal environment so that a stable, constant state is maintained regardless of changing external conditions. Homeostatic regulation allows an organism to function effectively under a wide range of environmental conditions, such as widely fluctuating availability of iron.

²⁴ E.g., as listed under <https://www.nlm.nih.gov/toxnet/index.html>

PubMed is a free search engine accessing primarily the MEDLINE database of references and abstracts on life sciences and biomedical topics.

Substance-specific search terms include iron, iron metabolism, iron homeostasis, iron substitution, iron overload, and more.

In combination with: Birds, mammals, avian species (e.g., chickens, ducks, pigeons, and quail...) mammal species (e.g., hare, rabbit, mouse, wood mouse, vole, rats...), wildlife etc.

The search was restricted to decades, such as studies carried out between 2020 and 2010 or 2000 and 2010, and further backwards, if necessary, to find most recent publications first and then work backwards in time.

2.2. RESULT

It quickly turned out that a variety of textbooks and review papers studying iron physiology in vertebrates are available. Overall, the physiology of iron uptake and homeostasis in vertebrates is a scientifically and medically well-researched field. Numerous review articles summarize the current state of knowledge with different emphases. Iron deficiency and overload are known diseases in humans (and animals) with a body of literature from veterinarian context available.

Therefore, we searched for recent review articles that summarize the current state of knowledge on iron balance. Articles such as those by Aggett (2012), Schümann et al. (2014), Waldvogel-Abramowski et al. (2014), Coffey and Ganz (2017) are well suited to provide an overview of iron metabolism in mammals. Accordingly, there are several books (e.g., Bezzel and Prinzinger 1990, Brue 1994, and Powell 2015) that summarize the current knowledge on iron metabolism in birds. For individual aspects, a partial search was performed for articles dealing with corresponding sub-aspects, such as e.g., Nemeth and Ganz 2009, The Role of Hephcidin in Iron Metabolism.

The toxicological aspects of iron are dealt with in detail in various sources (e.g., metals in general: Marquardt and Schäfer 1997, iron specifically: Ponka et al. 2007 or specifically regarding iron fortification in food/iron substitution by appropriate preparations: Schümann et al. 2014).

When searching for information on the natural iron content of foods, it was found that there are hardly any surveys on the diet of wild animals. There is, however, a large amount of data that comprehensively cover and present the iron content of foods in relation to human nutrition (e.g., data from the USDA database <https://fdc.nal.usda.gov/>). It can be assumed that the iron content of natural foods is not fundamentally different from those consumed by humans, so these data (e.g., Mwangi et al. 2018 Insects as sources of iron and zinc in human diets) can also be used as a proxy for wildlife (e.g., insectivorous birds and mammals). For aspects of wildlife exposure after application, such as from contaminated slugs, reference is made to studies on the efficacy of the application.

Specific toxicological aspects of the formulation with elemental iron, can be taken from the overview publications of the manufacturers of corresponding preparations, here it comes to pass that the same and similar substances are used for iron substitution in iron deficiency in humans. Toxicological information can be found, for example, in a technical data sheet on Ferronyl® carbonyl iron powder from ISP, which also contains information on bioavailability and toxicity compared with other iron salts.

In total, this compilation is based on about 30 references, which are usually review articles or textbook chapters summarizing a wide range of literature sources.

3. USE PATTERN

The application of Final Bite, a ready-to-use granular formulation containing elemental iron considered in this document is against slugs and snails (Table 2).

Table 2: GAP of Final Bite against slugs and snails

Crop and/ or situation	Pests or Group of pests controlled	growth stage and season	number min - max	interval between applications	kg product /ha	kg as/ha
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All edible and non-edible crops	Molluscs	n.a.	1-6	Minimum 5 days	8 kg product/ha	0.08 kg a.s./ha
Amenity Vegetation	Molluscs	n.a.	1-6	Minimum 5 days	8 kg product/ha	0.08 kg a.s./ha

3.1. RELEVANT FOR RISK ASSESSMENT

Final Bite is a granular molluscicide containing the active ingredient elemental iron²⁵ (iron powder 10 g/kg) and [REDACTED]. Another ingredient important for considerations is [REDACTED].

The formulation is to be applied by soil-based methods for the control of slugs and snails in edible and non-edible arable crops, ornamental, and horticultural plants. According to the GAP table 6 applications (5d interval) are scheduled with 8 kg product/ha which corresponds to 0.8 g product/m². The scheduled single application rate of the active substance iron is 0.08 kg a.s./ha (1% a.s. content in the product), and a maximum seasonal application rate of 0.48 kg a.s./ha.

With a granule weight of 13.3 mg, a granule density of 60 granules/m² can be expected. The granule loading is 0.133 mg a.s./granule.

It is not possible to specify a BBCH growth stage for application, as slug pellets are applied after infestation. The granules should be scattered in the evening hours, as slugs and snails are mainly active and feeding at night or in the early morning hours. Reapplication is necessary only when the number of molluscs is still high after the use.

3.2. EXPOSURE

Birds and mammals may be exposed to the active substances by the consumption of granules or contaminated slugs and snails taken from treated fields. Therefore, birds and mammals that ingest granules as a food source or grit, or that include slugs and snails in their diet, are at risk of ingesting higher levels of elemental iron from the formulation.

To get an idea of the possible exposure of wild animals eating slugs, it is necessary to look at the behaviour of the slugs after they have encountered slug pellets. Detailed behaviour observations to determine parameters such as the rate at which the slugs ate the pellets, the duration of the first "meal," and the number of pellets consumed by an individual slug until death were conducted (Whaley 2022). Observations of the efficiency show that once the molluscs ingest the granules, they immediately stop eating, crawl away and die in their hiding places. Therefore, due to the nature and mode of action of the active ingredient of the pellets, almost all poisoned snails would be below the soil surface and not readily accessible to birds and foraging animals. Furthermore, the iron uptake of snail-eating birds and mammals when consuming poisoned snails is considered low. The results of the study show that less than one pellet containing 10 g/kg elemental iron (0.133 mg a.s./granule) was sufficient to induce mortality in adult grey field slugs (*Deroceras reticulatum*) (Whaley 2022).

For animals ingesting pellets, it should be noted that the pellets have little nutritional value, as their main mass is [REDACTED]. Therefore, there is little reason to favour the intake of pellets in the amount of natural food available. Blue is a rather unattractive colour for birds and mammals and used as a visual cue for minimising interactions with toxic baits (Cowan and Crowell 2017). The pellets are preferably spread in humid conditions where slugs become active. Therefore, the pellets are expected to swell and soften quickly and soon disintegrate. In an earthworm field study, 6 applications were made (5 days apart) and the dissipation of the pellets was documented by photographs (Axmann 2019). From this study, conclusions can be drawn about the dissipation of the pellets without slug infestation (mean reduction = 59.4% in 5 days).

It should be noted that this reduction was estimated in a study without slugs feeding on pellets. Slug infestation on a field will reduce the number of pellets further. Therefore, assessments for long-term exposure (covering the

²⁵ Elemental iron (Fe⁰, CAS No. 7439-89-6), in the form [REDACTED].

²⁶ Data from the USDA database <https://fdc.nal.usda.gov/>

possible exposure for weeks and month after the applications) are not relevant due to short presence of pellets before disintegration.

In the soil, the granules disintegrate, and the active substance (elemental iron) enters the natural soil cycle of this element. The degradation products, if bioavailable, are a natural nutrient for plants. Iron is essential for plant growth and is generally considered to be a micronutrient (Troeh and Thompson, 2005). Iron is considered the key metal in energy transformations needed for syntheses and other life processes of the plant cells. Consequently, plants regulate internal levels by mechanisms that maintain optimum concentrations (Kobayashi et al. 2019). Hence, an animal feeding on plants from a treated area after the application is only exposed to levels in plant diet items that are already controlled by plant homeostatic mechanisms. The uptake of iron via plant parts is therefore not considered to be toxicologically relevant.

4. TOXICITY OF ELEMENTAL IRON

4.1. EFFECT ON MOLLUSCS – BIOACTIVATION

Slugs attracted to the scattered pellets ingest the active ingredient, powdered elemental iron. According to the manufacturer, the mechanism of action is snail-specific and can be summarized as follows. Final Bite uses elemental iron (Fe0). The active substance is enhanced [REDACTED], here the [REDACTED]. The two substances are contained separately in the formulation and do not react with each other, even when the bait pellets are soaked by rain (both substances are not water soluble). When both substances are present together in the digestive tract of the slug, mortality occurs. The fluids in the digestive tract of the slugs are needed to make the elemental iron soluble. Only when the iron dissolves at a correspondingly low pH (in molluscs gut at pH < 3.0) the [REDACTED] is created *in situ* ($\text{Fe0} + \text{low pH} \Rightarrow \text{Fe}^{2+}$). The [REDACTED] forms a soluble [REDACTED] in the stomach and in the lobes of the digestive gland.

The [REDACTED] induces oxygen free radicals and oxidative stress (i.e., an imbalance between the formation of reactive oxygen species and the ability of a biological system to detoxify the reactive intermediates or repair the resulting damage) through the Fenton reaction. The nature of the toxicity of iron based molluscicides in molluscs is mainly considered to be due to the disruption of oxygen uptake by haemocyanin. Haemocyanin is the blood pigment of the haemolymph of molluscs (Penzlin 1991, Schmidt-Nielsen 1997). In contrast to the red, iron-containing haemoglobin of vertebrates, the oxygen in haemocyanin is bound by copper ions. Compared to haemoglobin, the binding of oxygen is stronger, but haemocyanin has a lower oxygen binding capacity, resulting in an overall lower O₂ transport capacity. However, at low temperatures haemocyanin is superior to haemoglobin in poikilothermic snails (Penzlin 1991, Schmidt-Nielsen 1997).

When oxygen is released from oxyhaemocyanin, the temporarily shared electron is recaptured by the copper atom, returning to cuprous (Cu⁺) state²⁷. For binding to occur the copper atoms must be in cuprous state. The difference between haemocyanin and methaemoglobin is that the copper atoms in methaemoglobin molecule are in cupric (Cu²⁺) and are therefore incapable of binding oxygen. The formation of methaemoglobin from haemocyanin is an oxidative process that also occurs when haemocyanin is exposed to highly reactive molecules (e.g., free radicals). The effects of free radical mediated oxidation are not limited to the haemocyanin molecule. However, haemocyanin is particularly sensitive to oxidative stress.

The toxicity of iron enhanced by [REDACTED] is primarily explained due to the disruption of oxygen uptake by haemocyanin (Clark et al. 1995). Ingestion of iron-based slug pellet does not cause paralysis in molluscs, but it does interrupt food intake. Iron based products have the advantage over metaldehyde and carbamate products in that the extent of mortality is independent of the water conditions of the molluscs and thus does not depend on the prevailing moisture conditions in the environment (Henderson et al. 1989). The oxygen binding profile of haemocyanin is affected by the presence of iron ions, which interfere with oxygen uptake by haemocyanin, like how carbon monoxide interferes with oxygen uptake by mammalian haemoglobin. As a result, ingestion of iron-based products by molluscs leads to interruption of feeding and locomotion due to anoxia (Henderson et al. 1989).

This mode of action is specific to molluscs, which have haemocyanin as a blood pigment. Since haemocyanin as a blood pigment does not exist in vertebrates, such effects cannot occur in birds and mammals. In the environment

²⁷ Copper is named as cuprous or cupric based on the electronic configuration. The main difference between cuprous and cupric is that cuprous is copper +1 cation whereas cupric is copper +2 cation. When copper is reacted with oxygen, two stable compounds Cu₂O and CuO form.

of use, such effects are specific to slugs and are therefore not considered toxicologically relevant for exposure of birds and mammals.

4.2. TOXICITY OF THE A.S. ELEMENTAL IRON IN VERTEBRATES

The active ingredient is highly pure iron (purity 97%) in the form of [REDACTED] elemental iron (Fe^0).

Other forms of high-purity iron are also known [REDACTED] but they essentially differ only in their chemical production. [REDACTED]

[REDACTED] However, the toxicological properties of the high-purity iron are comparable, as the iron in both forms is in elemental form and not, for example, as in iron-containing salts.

Toxicological information on [REDACTED] [REDACTED] which also includes information on bioavailability and toxicity compared to other iron salts.

As stated on the technical data sheet, the main advantage of elemental iron over iron salts is its lower toxicity and thus its greater safety in case of accidental ingestion. This results from the rate-limiting transformation of elemental iron into ferrous ions by gastric acid. Due to the slow gastrointestinal oxidation, higher doses are required, and it takes longer for the toxic effects of exposure to [REDACTED] iron to occur.

Acute oral toxicity studies conducted in various laboratory animals show that elemental iron powder is 30-150 times less toxic at the same iron dosage (Table 2). This indicates a direct correlation of toxicity with the increase in free plasma iron and that the oxidation of elemental iron to iron(II) ions and the subsequent transport of the iron through the mucosal cell is much slower than the transport of iron(II) ions from iron salts.

Table 3: Acute toxicity of FeSO_4 , Fe^{2+} and elemental iron. For the same iron dosage, elemental iron powder is less toxic to mammals (taken from a technical data sheet on [REDACTED] iron powder by [REDACTED])

Species	FeSO_4 LD_{50} (mg/kg)	Fe^{2+} LD_{50} (mg/kg)	Elemental Iron LD_{50} (mg/kg)	Study ⁶
Rat	1490-5000	298-1000	30000	Shelanski, 1950; Boyd and Shanas, 1963
Guinea Pig	1500-1750	300-350	20000	Shelanski, 1950; Boyd and Shanas, 1963
Dog	800	160	>25000	GAF, 1990
Young Rat	950	190	19000	ISP, 1997a

In a study on young rats referred to in the technical data sheet on [REDACTED] iron, the time to death of animals receiving a lethal dose of iron was recorded. When exposed to 1000 mg/kg ferrous iron from FeSO_4 , 60% of the animals died within 2 hours and the remainder within 4 hours. Animals treated with 100 times the amount of carbonyl iron survived at least 4 hours, half survived 24 hours, and some survived 5 days (Table 3). This time difference is directly related to the increase in free plasma iron and suggests that the oxidation of [REDACTED] to ferrous ions and subsequent transport through the mucosal cell is much slower than the transport of ferrous ions from ferrous sulphate.

Table 4: Time course of toxic effects from elemental iron and iron(II) sulfate. The toxic effect of iron overdose takes longer to develop than those of ferrous salts despite the higher dosage (taken from a technical data sheet on [REDACTED] iron powder [REDACTED])

Time (Hours)	Mortalities/Treated	Mortalities/Treated
--------------	---------------------	---------------------

	100,000 mg elemental iron/kg	1000 mg as FeSO ₄ /kg
1	0/10	1/10
2	0/10	6/10
4	0/10	10/10
24	5/10	—
48	8/10	—
72	9/10	—
120	10/10	—
T ₅₀	24.57 hrs	1.4 hrs

Elemental iron enters the organism more slowly than ferrous ion. According to a study on rats given an LD₉₀ dose of iron referred to in the technical data sheet on [REDACTED], individuals treated with [REDACTED] iron had lower plasma iron levels after 1 hour and showed no visible erythema in the gastrointestinal tract ([REDACTED]) despite using iron doses 100 times (males) and 290 times (females) higher than those in animals treated with ferrous sulphate (Table 4). This confirms that elemental iron enters the organism more slowly than iron(II) ions from FeSO₄.

Table 5: The low toxicity of elemental iron forms the controlled release of iron into the blood stream (taken from a technical data sheet on [REDACTED] iron powder [REDACTED])

Iron Source	Iron Dose (mg/kg)	Plasma iron (µg/dl) 1-hour post-dosing	Stomach and Intestine Gross Pathological Examination
FeSO ₄	Male 240 (1200 FeSO ₄) Female 220 (1100 FeSO ₄)	993 ± 638	Erythema "slight pronounced"
Elemental iron	Male 23000 Female 64000	438 ± 22	No Erythema "black granular [test] material"

From this data it is evident, that elemental carbonyl iron is far less toxic than soluble iron salts (i.e., FeSO₄), it causes fewer side effects and the slow absorption from the gastrointestinal tract provides time for possible reactions e.g. to reduce the iron uptake capacity in the gut, to build up buffer capacities (see below Chapter 5.2) or also behaviourally by avoiding further uptake of slug pellets because e.g. the organism does not send signals to look for special iron-containing food.

[REDACTED]

[REDACTED]
[REDACTED]
[REDACTED]
[REDACTED]
[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

5 THE METHOD OF RISK ASSESSMENT OF THE GUIDANCE DOCUMENT (EFSA, 2009) IS INAPPROPRIATE FOR NON-ORGANIC MICRONUTRIENTS

Iron is an essential micronutrient in animals as well as in plants. Due to its function as essential micronutrient, organisms have developed mechanisms to use iron in a positive way for benefit and to cope with increased iron concentrations to a certain extent (homeostasis). Organisms might be capable of tasting and actively regulating their iron uptake from diet items. Iron has a well-known, distinctive taste, which can be noticed, for example, in juices enriched with iron. Rodents, such as rats, have innate licking behaviour and their responses to good and aversive taste stimuli are comparable to those of humans. [REDACTED] evaluated the taste of an iron-[REDACTED] dissolved in both water and chewable and orodispersible tablets using a human taste panel (n = 6) and a rat BATA model (n = 6). The leak frequency of pure water was set at 100%. A higher leak frequency of up to 86% was obtained for the iron-[REDACTED] solution with a concentration of 1 mg/mL, indicating that the solution did not have a very bad taste. Almost 50% inhibition of leakage frequency was obtained for iron-[REDACTED] solutions with a concentration of 5 mg/mL compared to water, while solutions with a concentration of 10 mg/mL elicited a lower leakage frequency of only up to 36%, indicating a higher inhibition due to the high concentration of the metallic-tasting iron-[REDACTED]. The rats thus showed a concentration-dependent decrease in leak frequency. The organism can thus distinguish between different food qualities (e.g., iron content) based on taste and select according to its physiological needs.

Nutritional/substance intake is found in essential foods such as iron but not in the organic compounds commonly used as pesticides. The model for risk assessments as described in EFSA (2009) is designed for organic compounds with no physiological function. It must therefore be concluded that the standard method of risk assessment as described in the guidance document (EFSA, 2009) does not properly reflect the situation in non-organic micronutrient iron when used as a molluscicide.

Furthermore, organic pesticides undergo processes of detoxification by metabolism and excretion. A controlled absorption does not take place. These substances enter the body due to their solubility in the hydrophilic and lipophilic layers of the intestinal cells. In many cases, these substances are lipophilic and cannot be easily transported or excreted in the aqueous compartments of the body such as blood or urine. To convert these substances into a form that can be excreted, many tissues, but especially the liver, are capable of biotransformation (Marquardt and Schäfer 1997, Mutschler et al. 2001). The process of biotransformation can be divided into two or three phases.

1. In phase I (transformation reactions), functional groups (-OH; -SH) are added to nonpolar molecules. The variation of the cytochrome P450 enzymes is the basis for the great diversity of catalysed reactions and the range of possible substrates.
2. In phase II (conjugation reactions), polar, negatively charged endogenous molecules are bound to lipophilic molecules. This binding (e.g., glucuronidation, sulfation, glycosylation, glutathione conjugation) significantly increases the water solubility of the xenobiotics. The molecules are conjugated to water-soluble molecules often via the functional groups added in phase I and can then be excreted either via the kidneys or via the bile.
3. The third phase of biotransformation involves the active transport of the transformed substances across the cell membrane e.g., of the hepatocytes into the canaliculi biliferi for the final export from the organism. Special carriers (e.g., ABC transporters) are engaged in the transport of uncharged lipophilic substrates (MDR1/P-glycoproteins) or anionic conjugates (multidrug resistance protein or MRP1, MRP2 or MRP3).

The xenobiotic metabolism is not able to "recognize" toxic or biologically active substances as such and converting them into non-toxic or inactive substances for the organism. The process of biotransformation is based on enzymes that have relatively low substrate specificity, i.e., they catalyse reactions involving a whole group of similar substances. This generally leads to detoxification or inactivation of chemical substances (Marquardt and Schäfer 1997, Mutschler et al. 2001).

However, for organic pesticides considered in the classical risk assessment scheme of the GD (EFSA 2009), the detoxification reaction in the liver and excretion via faeces and urine are the most important toxicological aspects.

In contrast, iron is an essential micronutrient in all plants and animals that derive a benefit from dietary iron uptake through regulated adsorption, metabolism, and excretion. Dietary iron levels vary naturally due to differences in iron concentrations in different food items (e.g., plants, arthropods). Internal iron levels are therefore regulated by mechanisms that maintain optimal concentrations (homeostasis). Many highly specific proteins are involved in the uptake, transport, storage, and physiological use of iron. Iron metabolism and excretion are controlled by the processes described in Chapter 6 below, where excessive iron is bound or excreted. Homeostasis, a process in which uptake and excretion are balanced, is critical to the functioning of the organism. Animals are therefore adapted to cope with natural iron concentrations and their fluctuations in the diet.

Since animals can cope with the natural Fe content in the diet, they are not expected to be affected by iron residues from anthropogenic sources if the total available iron content is of the same order of magnitude.

6 IRON BIOLOGY

As mentioned above, the separate administration of iron and [REDACTED] provides some protection against the increased absorption of iron from the formulation. However, this is not the only fact that protects animals from excessive iron intake.

Iron is an essential trace element for many biological processes, including oxygen transport and storage, oxidative phosphorylation, and catalysis of many metabolic redox reactions. Because iron ions are essential micronutrients in plants and animals, internal concentrations are regulated by mechanisms that control absorption and excretion and maintain optimal internal concentrations (homeostasis). It can be assumed that the same mechanisms regulate the internal iron concentrations to a non-toxic level even in the case of increased iron availability through the use of iron preparations. This is discussed in detail in the following chapter.

6.1 IRON AS SOIL NUTRIENT FOR PLANTS

Iron is the fourth most abundant of all elements and the most abundant transition metal on the Earth's surface and in all living organisms (Ponka 2007, Domke, et al. 2004). Iron makes up more than 5% of the earth's crust and is therefore found in varying concentrations in virtually all soils and waters. In the soil, iron is present for the most part in the bound form. Common iron compounds in arable soils are, for example, hematite, iron hydroxide and goethite (Ponka 2007).

The soil iron usually derives from weathered bedrock. Typical iron concentrations in soils range from 0.2% to 55% (20,000 to 550,000 mg/kg) (Cerundolo et al. 1988). Concentrations can vary considerably even within a given area due to soil type and varying iron sources. In general, soil iron content may be elevated near iron ore

deposits. In contrast, inputs from iron-containing pesticides such as Final Bite are small. For example, at 8 kg product/ha (1% content), iron input from Final Bite application is about 0.08 kg per hectare (see GAP table). In addition, elemental iron is not readily bioavailable to plants in the form in which it is applied.

Iron is a vital trace element for plants and has a significant influence on plant growth and crop yield. In alkaline soils the availability of iron can be reduced. Under Fe-deficient conditions, leaves show chlorosis because Fe is required for chlorophyll biosynthesis and photosynthesis. Long-lasting iron deficiency will cause the plant to stop growing, and eventually leads to plant death (Kobayashi et al. 2019).

6.2 IRON-HOMEOSTASIS IN VERTEBRATES

In vertebrates, iron is important for energy metabolism, oxidase systems, nervous system development and function, connective tissue synthesis, hormone synthesis, and antioxidant activity (Aggett 2012). Iron serves both as an electron donor and as an acceptor in redox reactions and electron transport processes. Therefore, iron is found as a cofactor in many proteins. The most important group of iron-binding proteins are the heme molecules, which contain iron in their prosthetic groups. The iron-sulphur proteins are another important group of iron-containing proteins (Penzlin 1991, Schmidt-Nielsen 1997, Mutschler et al. 2001). Birds and mammals use iron in the hemoglobin of red blood cells and in the myoglobin of muscle cells to transport and store oxygen (Penzlin 1991, Brue 1994, Schmidt-Nielsen 1997, Powell 2015).

As with many other substances, maintaining adequate concentrations in the body is critical: too low iron levels lead to deficiency symptoms (e.g., anaemia, skin lesions, immune system impairment), while too high levels lead to toxicity (e.g., oxidative damage, kidney, and liver lesions) (Brue 1994, Papanikolaou and Pantopoulos 2005, Sherry et al. 2006, Aggett 2012). Accordingly, iron homeostasis, where absorption and excretion are in balance, is crucial for the functioning of the organism.

Information on iron homeostasis is largely based on data from laboratory mammals and human studies, because of the medical importance of iron deficiency diseases. However, iron metabolism in vertebrates, where iron economy is dominated by red blood cell production and turnover, is extremely conservative. For example, iron metabolism in laboratory mice, the most important model for the study of iron metabolism, is generally considered to be similar to that in humans (Coffey and Ganz 2017). The available information on birds shows no significant differences in iron metabolism compared with the better-studied mammals. Therefore, results obtained in mammals can be extrapolated to avian species. For iron requirements in birds, see Bezzel and Prinzinger (1990), Äckerlein (1993), Brue (1994) and Powell (2015).

Iron deficiency is the most common nutrient deficiency disorder, affecting approximately 2 billion humans worldwide and impairing the function of iron-dependent enzymes and proteins. Iron deficiency anaemia occurs when the undersupply of iron in the bone marrow leads to iron-deficient hematopoiesis (Mutschler et al. 2001, Schümann et al. 2014). This condition is not limited to humans but is also known from other vertebrates (Sherry et al. 2006, Campbell 1995). Hence, iron deficiency has well-known effects in the mammal species considered in the field of risk assessment for plant protection products. In iron deficient rodents, anaemia develops, and bone morphology (strength, density) is compromised, and calcium restriction exacerbates the condition. Iron has further been shown to be essential for reproduction in vertebrates. E.g., in rats, iron deficiency during pregnancy leads to growth retardation, decreased serum triglycerides, cardiac hypertrophy, and high blood pressure in the adult offspring. Maternal iron restriction during pregnancy caused a decrease in nephron number in rat offspring (Sherry et al. 2006). Birds are similarly affected, e.g., low iron diet leads to low hemoglobin levels in birds of prey (Äckerlein 1993). Iron deficiency in chickens and turkeys results in anaemia, in which red blood cells are reduced in size and contain little hemoglobin. Red chickens fed a low-iron diet lack pigmentation (National Research Council 1994).

6.2.1 Iron concentrations in the diet

Iron is the most common transition metal on the Earth's surface and in living organisms. However, with the advent of aerobic, oxygen-producing photosynthesis about 2.7 billion years ago, Fe^{3+} became the predominant form of iron. Since Fe^{3+} is far less soluble than Fe^{2+} (solubility products: $\text{Fe}^{3+} = 10^{-18} \text{ mol/L}$ compared to $\text{Fe}^{2+} = 0.1 \text{ mol/L}$), the availability of iron declined by this process (Schümann et al. 2014). Despite its overall large amount, bioavailable iron is therefore rather scarce.

The bioavailable iron concentration in the food items for birds and mammals varies greatly from iron-rich foods, such as red meat, to iron-poor foods like milk. Meat is a high-quality food with a high content of easily absorbable

iron. It contains a most of its iron bound in the blood pigment (hemoglobin). The organism can absorb the iron from hemoglobin particularly well (Ponka et al. 2007). But even in carnivorous diets the iron content varies widely. Some examples from the prey of carnivorous birds and mammals: pheasant (meat and skin) contains 1.2 mg iron, pigeon (meat and skin) 3.5 mg iron and quail (meat and skin) 4 mg/ 100 g. Liver and other offal contain are especially rich in iron (goose liver 30.5 mg, pork liver 23.3 mg per 100 g²⁹). However, even within carnivore meat diets, iron availability is highly variable. Compared to small mammals, day-old chicks have a markedly low iron content. Therefore, birds of prey and owls fed exclusively on day-old chicks have low hemoglobin levels (Äckerlein 1993).

As for the diet of insectivorous bird and mammal species, the amount of iron in the insect diet of these species (wagtails and shrews as examples of the GD EFSA 2009) varies greatly (McDonald 2006, Mwangi 2018). A study on insects as a source of dietary iron and zinc reviewed available information on iron in insects (Mwangi 2018). Analyses of iron concentrations in 17 insect species revealed mean iron contents ranging from 4 to 62 mg/100 g dry matter (DM). Yellow mealworm (*Tenebrio molitor*) larvae have lower Fe content (3.3-10.0 mg/100 g DM) than those of migratory locust (7.8-21.7 mg/100 g DM). Large differences in iron content (1.0-75.0 mg/100 g DM) were found in three termite species of the genus *Macrotermes* (Isoptera: Termitidae). Factors contributing to differences in Fe concentrations include species, developmental stage, and their diet (Mwangi 2018).

Table 6: Iron content for a selection of insect species consumed by humans (Mean values and standard deviations where available) The first 11 species are globally mass-reared; the last six species are collected from nature (Table 1 in Mwangi 2018).

Species	Scientific name	Fe (mg/100g DM)	
		Mean	SD
House cricket	<i>Acheta domesticus</i>	9.1	4.47
Jamaican field cricket	<i>Gryllus assimilis</i>	17.93	
Two-spotted cricket	<i>Gryllus bimaculatus</i>	9.66	
Tropical house cricket	<i>Gryllobates sigillatus</i>	4.23	
Migratory locust	<i>Locusta migratoria</i>	13.7	4.35
Desert locust	<i>Schistocerca gregaria</i>	8.38	
Lesser mealworm	<i>Alphitobius diaperinus</i>	21.80	
Yellow mealworm	<i>Tenebrio molitor</i>	5.3	2.16
Superworm	<i>Zophobas morio</i>	3.8	1.00
Silkworm	<i>Bombyx mori</i>	15.9	7.23
Waxworm	<i>Galleria mellonella</i>	5.0	1.75
Termite	<i>Macrotermes subhyalinus</i>	61.9	8.61
Arboreal termite	<i>Nasutitermes spp.</i>	24.6	
Palm worm	<i>Rhynchophorus palmarum</i>	5.1	1.25
Sago grub	<i>Rhynchophorus ferrugineus</i>	9.0	0.55
Longhorn grasshopper	<i>Ruspolia differens</i>	14.8	1.80
Cornfield grasshopper	<i>Sphenarium purpurascens</i>	18.0	

Earthworms, which are an important component of the diets of many thrushes and shrews (e.g., scenarios for earthworm feeding in GD EFSA 2009), have exceptionally high iron content (earthworm, wild 1110 mg, commercial (raised on peat humus soil) 580 mg/100 g) (McDonald 2006).

Table 7: Iron Content of Invertebrates

Invertebrate	Diet	Iron mg/kg
Earth Worm, wild	Local soil	11,100
Earth Worm, commercial	peat humus soil	5,800

A study by Ancuceanu and colleagues reviewed the iron content in different parts of 1228 plant species and its absorption from herbal products, based on data collected from the literature. Descriptive and inferential statistics were used to compare iron levels in different plant parts (whole plant, roots, stems, shoots, leaves, aerial parts, flowers, fruits, seeds, wood, bark, other parts), and exploratory analyses were performed by taxonomic groups and life forms. Examination of the iron content of various organs or parts has revealed large inter- and intra-species variations. In general, iron appears to be highest in roots, lowest in green organs (leaves, stems, aerial parts), even lower in fruits and seeds, and lowest in bark and wood (Ancuceanu et al. 2015).

²⁹ Data from the USDA database <https://fdc.nal.usda.gov/>

There are large differences in the iron content of seed and fruits and berries regarding granivorous and frugivorous animals, too. Sesame seeds have the highest iron content of all seeds common for human nutrition. They contain 14.6 mg in 100 grams. Others such as pumpkin seeds, hemp seeds, and chia seeds have about 7-9 mg of iron per 100 grams. Flaxseeds and sunflower seeds have the lowest iron content at 5.7 mg and 5.3 mg, respectively.

Table 8: Examples of iron content of nuts, seed, cereals, and pulses ³⁰

Food items	Iron content in mg/100g
Pine nuts	9.2
Almonds	4.1
Hazelnuts	3.8
Brazil nuts	3.1
Walnuts	2.5
Peanuts	2.4
Chestnuts	1
Pumpkin seeds	11.2
Sesame	14.6
Flaxseeds	5.7
Sunflower seeds	5.3
Linseed	8.2
Millet	9
Quinoa	8
Amaranth	7.6
Oats	4.2
Green spelt	4.2
Buckwheat	3.5
Millet	3
Barley	2.8
Rice	1.7
Lentils	6.9
Beans	6.1
Peas	5

Regarding fruit-eating animals (e.g., starlings, dormice in GD EFSA 2009), all fruits provide some iron, but the content is comparatively low (plum 0.2 mg, cherry 0.3 mg, grapes 0.4 mg per 100 g). Fruits with comparatively high iron content can reach about 5-10 times higher iron levels (e.g., elderberry 1.5 mg, persimmon 2.5 mg per 100 g³¹).

Table 9: Examples of iron content of fruits ³²

Food items	Iron content in mg/100g
elderberry	1.5
persimmon	2.5
Black currants	1.3
Red currants	1.2
Blackberries	1.0
Raspberries	0.7
Gooseberries	0.6
Blueberries	0.5
Mirabelles	0.5
Apricots	0.4
Strawberries	0.4
Cherries	0.4
Peaches	0.3

³⁰ Data from the USDA database <https://fdc.nal.usda.gov/>

³¹ Data from the USDA database <https://fdc.nal.usda.gov/>

³² Data from the USDA database <https://fdc.nal.usda.gov/>

Grapes	0.4
Plums	0.2

Herbivorous species such as voles also find different iron contents in their diets. For green plant parts eaten by herbivores, the following table shows the iron content of wild herbs (Tab 8).

Table 10: Examples of iron content of herbs ³³

Food items	Iron content in mg/100g
Land cress	2.3
Nettle	4.1
Franciscan herb	4.8
Orache	6.1
Ground ivy	3.7
Mallow	4.1
Lesser celandine	3.5
White deadnettle	3.2
Chickweed	4.6
Snakeroot	2.4

According to Fröh (2009), the Fe content in herbs and legumes is higher than in grasses and varies with soil type. At a low/acid soil pH of 4.5, the iron can be efficiently taken up by the plants. The higher the pH, the less well the iron can be taken up by the plant (Fröh 2009, Stephan 2010). Furthermore, the iron content decreases with the age of the plants. The Fe content in grass (hay) depends on the time of cutting and contamination. It varies between 13.4 and 23.8 mg Fe/100 g DM (Stephan 2010) but can also assume significantly higher values with higher soil/sand contamination. Hay often has very high iron concentrations due to soil contamination. However, its bioavailability is low due to the presence of chelating agents in the feed. Moreover, it is mostly present in trivalent form (Fe^{3+}) and must first be reduced to the divalent form (Fe^{2+}) in the intestine (Hansen and Spears 2009).

The iron absorption from food also depends on the composition of the overall diet, as different foods may influence each other. The body can absorb animal iron about three times better than plant iron. Chelators or ligands in the food matrix can bind with non-heme iron (e.g., from plant sources) to either inhibit or enhance its absorption. Examples of absorption enhancers for non-heme iron include ascorbic acid, citric acid, fructose, sorbitol and amino acids. Inhibitors of iron absorption include phytates, polyphenols, oxalic acid, calcium, manganese, calcium phosphate salts and zinc (Sherry et al. 2006). As stated above, tannins are also a well-known inhibitor of iron absorption in rats.

Animals are thus confronted with very different iron levels and iron availabilities in their food, depending on what they eat. A blackbird, for example, which eats numerous earthworms in summer, has high iron availability in its diet, while it must cope with lower iron levels in fall, when a large part of its diet consists of fruit. Similarly, garden warblers feed mainly on insects and other arthropods during the breeding season (high iron availability). During fall migration, however, they feed primarily on fleshy fruits (Bairlein 1996) which contain relatively low iron levels. The vertebrate organism is apparently able to buffer the different iron contents in the diet well.

In diets with foods that vary widely in iron content, a regulatory mechanism is required to maintain internal concentrations in equilibrium. Several dynamic equilibrium adjustments and regulatory mechanisms allow an animal to maintain its internal iron concentrations above deficiency levels but below the toxicity threshold. Both mammals and birds regulate their iron levels by co-ordinately controlling the absorption, recycling and mobilization of iron. Intestinal iron absorption is tightly controlled and depends on the body's iron requirements (see below). Within certain limits the body adapts to current iron requirements: When iron stores are empty, it can absorb significantly more iron from food (Aggett 2012, Brue 1994, Sherry et al. 2006, Coffey and Ganz 2017). The following chapter presents an overview of the complex mechanisms involved in iron regulation.

6.2.2 Iron homeostasis and the damaging potential of iron

³³ Source data and table values from: Dr. Niklas, Joachim, from MEDIZIN-WELT, "Wildwachsende Gemüse und Salate", redaktion@medizin-welt.info

Iron is an essential element for vertebrates and other organisms, necessary for many physiological processes (Penzlin 1991). In humans, for example, a daily dose of 10-20 mg is required, and in women the requirement is about twice as high as in men due to the loss of iron during menstruation. Similarly, pregnant mammalian females or egg-laying birds have higher requirement for iron because of iron need in offspring production (Harvey 2008; e.g., recommended diet concentration for egg laying poultry: 50-120 mg Fe/kg, National Research Council 1994). Iron is supplied to the body in various forms and amounts from the natural content in food (Schümann et al. 2014, Waldvogel-Abramowski et al. 2014, Coffey and Ganz 2017).

There are 2 main forms of iron, iron (*ferrous* iron, Fe^{2+}) and oxidized iron (*ferric* iron, Fe^{3+}). During physiological processes, the iron frequently alternates between these two forms. This property of iron is used metabolically by many organisms (bacteria, plants, animals) for electron transport and oxygen transport and is mediated by specific enzymes and metalloproteins (Harvey 2008, Schümann et al. 2014, Waldvogel-Abramowski et al. 2014, Coffey and Ganz 2017).

Therefore, iron deficiency is often associated with anaemia and, subsequently, impaired physical strength and mental performance. In addition, the risk of premature birth, stillbirth, impaired development, and a weakened immune system is increased in iron deficiency (Brue 1994, Sherry et al. 2006, Aggett 2012, Schümann et al. 2014). However, at the same time, iron has a toxic potential in the presence of oxygen, as it catalyses the formation of highly reactive OH radicals in a Fenton or Haber-Weiss reaction (Ponka et al. 2007, Schümann et al. 2014, Coffey and Ganz 2017).

Under these circumstances, complex mechanisms have evolved to maintain iron status with the aim on the one hand, supplying the organism with sufficient iron and, on the other hand, protecting sensitive structures from iron-mediated oxidative stress. Therefore, to reduce the risk of deficiency or overload, uptake and distribution of iron is homeostatically regulated. For example, full iron stores and prior high iron intake reduce iron absorption in the gastrointestinal tract (Aggett 2012, Brue 1994, Sherry et al. 2006). However, this does not provide protection against iron overload in all cases. When the capacity of these regulatory mechanisms is exceeded, symptoms of iron deficiency or iron toxicity may occur, both of which can cause severe health damage (Schümann et al. 2014). Nevertheless, the mechanisms manage to keep the iron level in balance with the naturally fluctuating iron content of food.

6.2.2.1 Iron Physiology in Vertebrates

Iron ingested with food passes through the stomach and enters the lumen of the small intestine, usually in the form of oxidized iron (Fe^{3+}). However, iron cannot be absorbed in this form and must be converted to *ferrous* iron (Fe^{2+}). This occurs at the brush border membrane of enterocytes by an enzyme called ferrireductase or duodenal cytochrome B (dCytB) that reduces trivalent iron to divalent iron. It is mainly expressed on the luminal side of duodenal enterocytes. This reduction is important in iron absorption because only divalent iron can enter enterocytes via the divalent metal transporter 1 (DMT-1), which is also located on the apical side of enterocytes. The DMT-1 is a cotransporter for various metals. Through the cotransport mechanism, protons are simultaneously taken up into the cell (Sherry et al. 2006, Harvey 2008, Zhang and Enns 2009, Schümann et al. 2014, Waldvogel-Abramowski et al. 2014, Coffey and Ganz 2017).

In intestinal cells, *ferrous* iron (Fe^{2+}) can be oxidized to *ferric* iron (Fe^{3+}) and stored as ferritin (Fe^{3+}). *Ferrous* iron (Fe^{2+}) can also be transported through the blood to the liver, bone marrow and other parts of the body. For this purpose, iron leaves the cell at the basal side of the cells through the ferroportin transporter (Ireg1) (Harvey 2008, Zhang and Enns 2009, Schümann et al. 2014, Waldvogel-Abramowski et al. 2014, Coffey and Ganz 2017).

To be transported in the body, the *ferrous* iron (Fe^{2+}) must be converted back into *ferric* iron (Fe^{3+}). In the bloodstream, iron is transported bound to carriers such as transferrin, which binds 2 irons (Fe^{3+}). Therefore, outside the enterocytes there is a corresponding converter in the form of hephaestin, which oxidizes divalent iron to trivalent iron, which can then bind to apo-transferrin, resulting in the charged transferrin (Harvey 2008, Schümann et al. 2014, Waldvogel-Abramowski et al. 2014, Coffey and Ganz 2017).

Most of this iron (about 75%) goes to erythropoiesis in the bone marrow to form hemoglobin for oxygen transport in the erythrocytes (Harvey 2008). Transferrin delivers a smaller portion (10-20 %) to the liver, where it is bound to the transferrin receptor and taken up into the cell. This mechanism is crucial for iron homeostasis (Brue 1994, Sherry et al. 2006, Aggett 2012, Schümann et al. 2014, Waldvogel-Abramowski et al. 2014).

When two transferrin bind to transferrin receptors of the liver cells a vesicle is formed and taken into the cell. The influx of H^+ lowers the pH in the lumen of the vesicle, which leads to the detachment of iron from transferrin. Ferric iron leaves the vesicle in cotransport with H^+ via the DMT and the iron is reduced back to Fe^{2+} in the cytoplasm and eventually, after re-oxidation is stored as ferritin (Fe^{3+}). The transferrin is released (unloaded as apo-transferrin) back into the blood. The liver forms an iron store from which the iron can be released back into the bloodstream via the ferroportin transporter if required (Aggett 2012, Schümann et al. 2014, Waldvogel-Abramowski et al. 2014, Coffey and Ganz 2017).

Iron homeostasis is regulated and controlled to ensure a largely constant supply of iron in the event of iron deficiency or excess. Hepcidin is the major regulator of plasma iron concentration. It is produced and secreted by the liver in response to the availability of iron in iron stores. It inhibits the iron transporter ferroportin and thus both intestinal iron absorption and iron release from the reticuloendothelial system (RES; Nemeth and Ganz 2009, Zhang and Enns 2009, Coffey and Ganz 2017). The RES, composed mainly of monocytes and tissue macrophages, recycles iron from aging red blood cells and serves as an important store for excess iron. Iron recycling by the RES represents the major pathway for iron efflux in the body (Schümann et al. 2014, Waldvogel-Abramowski et al. 2014). Hence, hepcidin acts on renal macrophages to regulate the recycling cycle of iron from hemoglobin from old erythrocytes. Hepcidin blocks ferroprotein, preventing the release of iron from renal macrophages. Hepcidin further also reduces the uptake of iron from the small intestinal lumen (Nemeth and Ganz 2009, Schümann et al. 2014, Waldvogel-Abramowski et al. 2014, Coffey and Ganz 2017).

The release of hepcidin is stimulated by several factors. An increase in plasma iron concentration (amount of iron bound to transferrin) stimulates hepcidin production, which reduces intestinal iron uptake and release from storage (Nemeth and Ganz 2009, Schümann et al. 2014, Waldvogel-Abramowski et al. 2014, Coffey and Ganz 2017).

However, the most important regulator is the hemochromatosis protein (HFE). HFE is a protein in the cell membrane that binds to transferrin receptor 2 and causes transferrin to dissociate from it more readily, i.e., to bind weaker. This interaction is necessary for transferrin-triggered expression of hepcidin. The protein thus plays a central role in iron metabolism (Nemeth and Ganz 2009, Zhang and Enns 2009, Schümann et al. 2014, Waldvogel-Abramowski et al. 2014).

It should also be noted that dietary iron can also be present in the form of hemoglobin and myoglobin from animal sources. In this case, heme iron is effectively absorbed directly from the lumen of the small intestine via a transporter (HCP1) and can be separated in the enterocytes into bilirubin and Fe^{3+} , where it is in turn stored as ferritin. Normally, heme iron (from animal sources) is considered to be approximately 20-25% available to the animal, while nonheme, from vegetative sources are usually less than 5% available (Brue 1994, McDonald 2006, Sherry et al. 2006, Aggett 2012, Schümann et al. 2014, Waldvogel-Abramowski et al. 2014).

From the above, it is clear that a complex mechanism controls and regulates the metabolism of iron, its absorption, distribution and storage in vertebrates. The following examples illustrate how the body reacts to variability in iron availability and disturbances in iron uptake.

6.2.2.2 Homeostatic mechanisms and the iron content in different compartments.

Iron deficiency is one of the most common nutrient deficiency diseases and impairs the function of iron-dependent enzymes and proteins. Iron deficiency anaemia occurs when the concentration of iron in the bone marrow is low, resulting in iron-deficient haematopoiesis (blood formation) (Sherry et al. 2006, Aggett 2012, Schümann et al. 2014).

The risk of iron deficiency was high in phylogenesis of organisms. Therefore, effective homeostatic mechanisms have evolved that control the uptake and recycling of iron. Iron homeostasis is located in the various compartments of the body (see above). In the intestine, mechanisms that adjust iron uptake to the current demand can be found. However, the barrier function of the intestine outweighs absorption, so that much of the absorbed iron remains in the intestinal lumen and is excreted (Aggett 2012, Schümann et al. 2014).

The absorption of dietary iron is a variable and dynamic process. The amount of iron absorbed compared to the amount ingested is typically low but may range from 5% to as much as 35% depending on circumstances and type of iron. Absorption of dietary iron in iron salt form is usually between 10% and 20% of iron intake. Absorption of iron from animal and plant products, e.g., in the form of heme iron, and is more efficient, allowing absorption of from 15% to 35% of intake. In this context, the plasma iron pool is considered the hub for the distribution of iron in the body. Thus, iron is recycled from degraded erythrocytes into erythropoiesis, and absorbed iron from

the gut is distributed to tissues (Zhang and Enns 2009, Schümann et al. 2014, Waldvogel-Abramowski et al. 2014, Coffey and Ganz 2017).

Iron is not actively excreted from the body and normally there is minimal iron loss, except in menstruating primates and egg-laying birds, so the amount in the body must be controlled at the point of ingestion (Harvey 2008). However, a small amount is lost daily by gastrointestinal blood loss and shedding cells of the mucosal lining of the gastrointestinal tract or the skin. This steady loss means that animals must continue to absorb iron via the tightly regulated process that under normal circumstances protects against iron overload (Zhang and Enns 2009, Schümann et al. 2014, Waldvogel-Abramowski et al. 2014, Coffey and Ganz 2017).

The aim of the homeostatic mechanisms is to ensure an adequate supply of iron to tissues, even in situations of deficiency. However, its influence must also be considered under conditions of iron excess. Any iron that is not bound to ligands with a high complexation constant (free iron) may undergo potentially harmful redox reactions (Papanikolaou and Pantopoulos 2005, Ponka et al. 2007, Schümann et al. 2014). When an excess of iron is present in the body iron is stored as a pigment called hemosiderin a deposit of protein and iron created by macrophages. Hemosiderin is later resorbed by macrophages when the systemic iron overload has fallen back to lower levels (Schümann et al. 2014, Waldvogel-Abramowski et al. 2014, Coffey and Ganz 2017).

Since unbound iron has a high potential for damage, the amount of iron is effectively controlled at various physiological levels (e.g., cellular, blood plasma level, absorption in the intestine). Increased uptake of iron from slug pellet inputs on agricultural land is therefore largely buffered in these mechanisms. The features of the mechanism that maintains iron homeostasis in cells, plasma, and the intestine are summarized below.

6.2.2.2.1 Intracellular iron

Excess intracellular iron is bound to the oligomeric protein ferritin. Ferritin can bind up to 4500 iron ions per molecule in a non-toxic but still bioavailable form. The function of ferritin is to limit the amount of potentially harmful "intracellular labile or free iron" while storing iron in a form that can be mobilized in the event of a deficiency. Binding to ferritin is linked to demand by measuring the intracellular concentration of labile iron through the iron regulatory protein/iron responsive element (IRP/IRE) system. This system restricts ferritin expression when the intracellular concentration of labile iron is low and increases ferritin expression when the concentration is high (Schümann et al. 2014, Coffey and Ganz 2017).

6.2.2.2.2 Iron in plasma

In iron deficiency, intestinal absorption increases, while increased cellular iron uptake decreases plasma and intracellular iron concentrations. Therefore, iron status influences the maximum iron concentration after ingestion of readily bioavailable iron species to prevent damage (Ponka et al. 2007, Schümann et al. 2014, Coffey and Ganz 2017). For example, iron supplementation in rats increases markers of oxidative stress and inflammation, such as thiobarbituric acid reactive substances, in serum and urine (Knutson et al. 2000).

In serum, iron is bound to transferrin with high affinity. However, despite the high complexation constant (10⁻²⁰), 'non-transferrin-bound iron' (NTBI) is also detected in serum. NTBI is found when the iron binding capacity of transferrin in serum is exceeded, for example, in transferrin-deficient mice (Simpson 1992). Transferrin levels may be adjusted accordingly in the context of increased exposure. An increase in the amount of iron bound to transferrin in plasma stimulates hepcidin production, which reduces intestinal iron uptake and release from storage. Both mechanisms help to keep iron levels in balance (Nemeth and Ganz 2009, Schümann et al. 2014, Waldvogel-Abramowski et al. 2014, Coffey and Ganz 2017).

6.2.2.2.3 Iron resorption in the intestine

If the iron stores are only slightly filled or if the oxygen supply to the tissue is impaired (e.g., in the case of iron deficiency anaemia), iron absorption is increased. Conversely, iron absorption decreases when iron stores are full (Flachowsky 1994, Aggett 2012, Coffey and Ganz 2017). In the brush border of the duodenum, Dcyt b reduces trivalent (Fe³⁺) non-heme iron to the divalent (Fe²⁺) form. This is taken up from the lumen by the 'Divalent Metal Transporter 1' (= DMT-1), whose expression is coupled to body iron status. Similarly, hephaestin in the basolateral membrane of duodenal enterocytes oxidizes Fe²⁺ back to Fe³⁺ after export to plasma so that it can bind to transferrin (Schümann et al. 2014, Coffey and Ganz 2017). The "mucosa block" mechanism inhibits iron reabsorption after prior exposure to high iron concentrations in rats, probably by reducing the number of DMT-1 transporters (Frazer et al. 2003). Similarly, reactions are known from birds. For example, common hill myna (*Gracula religiosa*), can down-regulate iron absorption (Mete et al. 2001).

Hepcidin synthesized in the liver binds to ferroportin and inactivates the export function of this transport protein for iron from cells, presumably by internalizing the complex with subsequent degradation of ferroportin (Nemeth et al. 2004). Since ferroportin mediates iron export from duodenal enterocytes and the reticuloendothelial system to plasma, this process is thought to explain the inhibitory effect of hepcidin on duodenal iron absorption and accumulation of iron in the reticuloendothelial system. These mechanisms can effectively protect the body from iron excess (Aggett 2012, Schümann et al. 2014, Coffey and Ganz 2017)

6.2.2.2.4 Iron Overload (Haemochromatosis), causes and pathophysiology

The importance of iron homeostasis becomes clear when considering pathological conditions related to iron levels. The toxicity of iron is largely due to its ability to catalyse the formation of radicals that attack and damage cellular macromolecules and promote cell death and tissue damage. Health damage caused by iron may affect the intestine directly or be due to oxidative stress or stimulation of pathogen growth. Free plasma iron, not bound to transferrin, is also thought to contribute to atherogenesis³⁴ (Ponka et al. 2007, Schümann et al. 2014).

This is further evident in diseases with iron overload, such as hereditary hemochromatosis or transfusion-related siderosis, where excessive iron accumulation leads to tissue damage and organ failure.

Hemochromatosis as a clinical condition is known in medical diagnostics. There is a primary hereditary form and secondary form of this disease. Secondary hemochromatosis occurs when too much iron is ingested. However, due to homeostatic regulation, a very large amount is required for this to occur before significant complications develop. Mutations in certain transporters or their regulation mechanisms can promote hemochromatosis. For example, an excessive expression of ferroportin. Another cause of inherited hemochromatosis is a mutated HFE gene on chromosome 6 (HFE gene mutation in C282Y H63D). This affects hepcidin formation. When hepcidin is reduced, there is no effective inhibition of the ferroportin transporter (Ponka et al. 2007, Nemeth and Ganz 2009, Schümann et al. 2014, Waldvogel-Abramowski et al. 2014, Coffey and Ganz 2017).

Other causes of secondary hemochromatosis may be found in chronic liver diseases leading to reduced hepcidin formation or certain blood diseases (thalassaemia, sideroblastic anaemia). In these cases, an ineffective erythropoiesis leads to suppression of hepcidin (Domke, et al. 2004, Ponka 2007, Aggett 2012).

When there is too much iron in the blood, the plasma transferrin becomes saturated and the free iron binds to other proteins such as albumin, citrate, acetate (non-transferrin bound iron). In this case, the cells take up the non-transferrin bound iron e.g., in liver, heart, joints and pituitary gland, pancreas, gonads. There, the iron not bound to transferrin undergoes biochemical reactions that generate reactive oxygen species which in turn lead to tissue damage, inflammation, and fibrosis. Depending on the affected organs, this leads to e.g., liver cirrhosis, cardiac arrhythmia or dilated cardiomyopathy, arthritis in the joints. The absorption of large amounts of iron in the pituitary gland can cause secondary hypothyroidism, hypogonadism, in the pancreas it can cause diabetes, in the gonads it can cause testicular arthropathy. One of the first manifestations of hemochromatosis is pigmentation of the skin due to melanin and iron deposition (Ponka et al. 2007, Nemeth and Ganz 2009, Schümann et al. 2014, Waldvogel-Abramowski et al. 2014, Coffey and Ganz 2017).

However, iron overload due to an increased iron intake via the normal diet does practically not occur. Excess iron through contamination of foods (such as beer and cereals) with iron from the environment or from cooking and brewing facilities may occur in rare cases. However, resulting iron overload after long-term consumption of these foods has only been found in individuals with a genetic susceptibility to increased iron absorption (Ponka et al. 2007).

E.g., a classic case of dietary iron overload was the consumption of iron fortified beer by members of the Bantu tribe, which eventually led to pathological iron overload. Iron intake of more than 100 mg occurred, and the iron overload led to liver cirrhosis, diabetes, and possibly hepatoma. However, it was later recognized that this iron overload was also due to a combined effect of increased iron intake and a predisposing genetic factor (Ponka et al. 2007, Moyo et al. 1998).

Iron overload as a medical case occurs in humans and domestic animals due to an overdose of iron supplements. However, it is unknown in wild animals living in natural condition (at least no described cases could be found in the literature search).

³⁴ *Atherogenesis* is the process of atherosclerotic plaque formation, leading to coronary artery heart disease.

7 CONCLUSION

From the previous chapters, conclusions can be drawn about the exposure of wildlife to Final Bite treated fields. The active substance elemental iron is an essential micronutrient in both animals and plants. Due to its function as an essential micronutrient, organisms have developed mechanisms to use iron positively and to cope with elevated iron concentrations to a certain extent (homeostasis). In chapter 6 it was shown that organisms can actively regulate their iron intake from food and balance internal levels at various compartments of the body. This is a highly complex mechanism of its own with numerous specialised proteins and enzymes that has adapted to the fluctuating availability of iron in the diet in the course of evolution. It can be concluded that the standard risk assessment method described in the guidance document (EFSA, 2009) is not applicable to the non-organic micronutrient iron used as a molluscicide in agricultural landscapes. The application of this method will lead to an incorrect assessment of the potential risk as it is based on completely different toxicological conditions.

The mode of action of elemental iron acting together with its [REDACTED] is specific to molluscs, which have hemocyanin as a blood pigment. Since hemocyanin as a blood pigment is not found in vertebrates, such effects will not occur in birds and mammals. Only when the bait is eaten by the slugs do the two compounds combine and become activated, ultimately leading to the death of the snails - in the gut of the molluscs, the Fe(II) [REDACTED] is formed *in situ* at a pH < 3.0. Therefore, it has low toxicity in earthworms, soil microorganisms, birds and especially mammals (humans and pets). Since Fe(II) [REDACTED] is unstable and the Fe(II) ion immediately oxidises to Fe(III) on contact with air, the only way to administer Fe(II) [REDACTED] to molluscs in the form of a bait is to form this [REDACTED] *in situ* in the molluscs' digestive system. In the environment of use, such effects are specific to slugs and are therefore not considered toxicologically relevant for exposure of birds and mammals.

Dietary iron overload in wild animals due to increased amounts of iron in the diet via slug pellets can be largely ruled out. The uptake of iron from the formulation or the contaminated snails can be considered low after the use of Final Bite. Due to the nature and mode of action of the active ingredient in the pellets, almost all poisoned snails will be below the soil surface and not readily accessible to birds and foraging animals. Therefore, iron uptake by slug-eating birds and mammals eating poisoned slugs is considered to be low. In addition, as iron is essential for several vital biological processes and its deficiency or overload drives the development of several pathologies, the organism controls the dietary iron absorption by enterocytes, its recycling by macrophages and storage in hepatocytes.

Most of the body iron is contained in the hemoglobin (oxygen transport) and ferritin (iron storage protein). The liver's stores of ferritin are the primary physiologic source of reserve iron in the body. A considerable part of the body's total iron content is found in proteins that are engaged in energy-producing redox reactions (cytochromes). A relatively small amount circulates through the plasma, bound to transferrin. Because of its toxicity, free soluble iron (soluble ferrous ions Fe^{2+}) is kept in low concentration in the body.

Mammals as well as birds tightly regulate iron levels by co-ordinately regulating the absorption, recycling, and mobilization of iron. Intestinal iron absorption is tightly controlled and is dependent on body iron needs. When an excess of iron is present in the body iron is stored as hemosiderin, a deposit of protein and iron created by macrophages. Hemosiderin is later resorbed by macrophages when the systemic iron overload has fallen back to lower levels.

Iron stores in the body are regulated mostly by rate of iron absorption from the diet, with no significant role played by iron excretion. However, a certain amount is lost daily by gastrointestinal blood loss and shedding cells of the mucosal lining of the gastrointestinal tract or the skin. This steady loss means that animals must continue to absorb iron via a tightly regulated process that under normal circumstances protects against iron overload.

Iron is absorbed in the duodenum via metal transporters (e.g., divalent metal transporter DMT1) of enterocytes of the duodenal lining. Dietary iron can be absorbed as part of a protein (e.g., heme protein) or must be in its ferrous Fe^{2+} form. A ferric reductase enzyme on the enterocytes' brush border, Dcytb, reduces ferric Fe^{3+} to Fe^{2+} . Enterocytes can then either store the iron as ferritin or the cell can move it via ferroportin into the blood stream. The body regulates iron levels by regulating each of these steps. For instance, cells produce more Dcytb, DMT1 and ferroportin in response to iron deficiency anaemia. The body can substantially reduce the amount of iron it absorbs across the mucosa. However, it does not seem to be able to entirely shut down the iron transport process.

The absorption of dietary iron is a variable and dynamic process. The amount of iron absorbed compared to the amount ingested is typically low but may range from 5% to as much as 35% depending on circumstances and type

of iron. Absorption of dietary iron in iron salt form is usually between 10% and 20% of iron intake. Absorption of iron from animal and plant products, e.g., in the form of heme iron, and is more efficient, allowing absorption of from 15% to 35% of intake.

Transferrin is the iron-binding glycoproteins in the blood plasma that control the level of free iron. Iron toxicity results when the amount of circulating iron exceeds the amount of transferrin available to bind it, but the body can vigorously regulate its iron uptake. However, iron toxicity from ingestion is only very rarely observed in humans and usually the result of extraordinary circumstances (like iron tablet over-consumption).

It can therefore be concluded that additional elemental iron following application of the slug pellets will have no effect on free ranging birds and mammals. An exceedance of the capacity of the iron homeostasis due to application of elemental iron containing slug pellets is considered to be unlikely, especially under natural situations where animals have choice where and what to feed on.

8 REFERENCES

- Äckerlein, W. 1993. Die Ernährung des Vogels. 2nd Extended Edition Verlag: Ulmer Stuttgart. P. 37.
- Aggett, P.J. 2012. Iron. Chapter 33. In: Present knowledge in nutrition. – 10th ed. / edited by John W. Erdman Jr., Ian A. Macdonald, Steven H. Zeisel. Wiley-Blackwell, ILSI Press, Washington, DC, p. 506-520. <https://pkn10.org/>
- Ancuceanu R, Dinu M, Hovaneț MV, Anghel AI, Popescu CV, Negreș S. A (2015) Survey of Plant Iron Content - A Semi-Systematic Review. *Nutrients*. 10;7(12):10320-51. doi: 10.3390/nu7125535. PMID: 26690470; PMCID: PMC4690087.
- Axmann 2019. Final Bite - Blue – A Field Study to Investigate Effects on the Earthworm Fauna in Central Europe, Report No S18-02597, Sponsor Code R-39838
- Bairlein, F. 1996. Ökologie der Vögel. Gustav Fischer Verlag, Stuttgart. (p. 20-24).
- Bezzel, E. and R. Prinzinger 1990. Ornithologie. Ulmer, Stuttgart. P. 174
- Boyd, E.M. and M.N. Shanas, 1963. The Acute Oral Toxicity of Reduced Iron, *Canad. Med. Assn. J.* 89, 171 - 175.
- Brue, R. N., 1994. Nutrition. Chapter 3. In: Avian Medicine, B. W. Ritchie, G. J. Harrison, L. R. Harrison. Wingers Pub. Lake Worth, Fla.: (p.63-95).
- Campbell, T.W. 1994. Haematology. Chapter 9. In: Avian Medicine, B. W. Ritchie, G. J. Harrison, L. R. Harrison. Wingers Pub. Lake Worth, Fla.: (p.177-198).
- Coffey, R. and T. Ganz. 2017. Iron homeostasis: An anthropocentric perspective. *Journal of Biological Chemistry* 292:12727-12734. DOI:<https://doi.org/10.1074/jbc.R117.781823>
- Cowan, P. and M. Crowell. 2017. Visual and taste cues for minimising native bird interactions with toxic baits – A review of current practices. *New Zealand Journal of Ecology* 41.
- Domke A., R. Grossklaus, B. Niemann, H. Przyrembel, H. Richter, and E. Schmidt, A. Weissenbom, B. Wörm, and R. Ziegenhagen. 2004. Risk Assessment of Iron in Use of Minerals in Foods – Toxicological and nutritional-physiological aspects, Part 2, Federal Institute for Risk Assessment pp. 145-188
- EFSA 2009. Guidance of EFSA - Risk Assessment for Birds and Mammals. *EFSA Journal* 7: 1-139.
- Finch, C. 1994. Regulators of iron balance in humans. *Blood*; 84:1697—702.
- Flachowsky, G. 2000. Mineralstoffe. In: Engelhardt, W. V., Breves, G. (Ed.) *Physiologie der Haustiere* Ferdinand Enke Verlag, Stuttgart, p. 606-620
- Frazer, D. M., Wilkins, S. J., Becker, E. M., Murphy, T. L., Vulpe, C. D., McKie, A. T., and Anderson, G. J. 2003. A rapid decrease in the expression of DMT1 and Dcytb but not Iregl or hephaestin explains the mucosal block phenomenon of iron absorption. *Gut*, 52(3), 340–346. <https://doi.org/10.1136/gut.52.3.340>

Früh, R. 2009. Schafe auf dem Grünland gesund und leistungsfähig halten - Mineralstoffversorgung Thüringer Landesanstalt für Landwirtschaft, Jena
http://www.tl.de/www/daten/nutztierhaltung/schafe_ziegen/shgn01110.pdf

Hansen, S. L., J. W. Spears. 2009. Bioaccessibility of iron from soil is increased by silage fermentation Journal of Dairy Science 92(6), 2896-2905

Harvey, J. W. 2008. Chapter 9 - Iron Metabolism and Its Disorders. Pages 259-285 in J. J. Kaneko, J. W. Harvey, and M. L. Bruss, editors. Clinical Biochemistry of Domestic Animals (Sixth Edition). Academic Press, San Diego.

Henderson, I.F., Briggs, G.G., Coward, N.P., Dawson, G.W. and Pickett, J.A. 1989. A new group of molluscicidal compounds. In: Henderson, I.F. (ed.) Slugs and Snails in World Agriculture. Monograph No. 41, British Crop Protection Council, Thomson Heath, pp. 289-294.

Johnson, L. R., 2001. Iron absorption, in Gastrointestinal Physiology, Mosby. P151-153

Knutson M.D., Walter P.B., Ames B.N., Viteri F.E., 2000. Both iron deficiency and daily iron supplementation increase lipid per-oxidation in rats. J Nutr; 130:621—8.

Kobayashi, T., Nozoye, T., & Nishizawa, N. K. (2019). Iron transport and its regulation in plants. Free Radical Biology and Medicine, 133, 11-20.

Marquardt, H. and Schäfer, S.G., (ed.), 1997. Lehrbuch der Toxikologie. Spektrum Akademischer Verlag, Heidelberg.

McDonald, D. (2006) "Nutritional Considerations - Section I: Nutrition and Dietary Supplementation", Clinical Avian Medicine. Available at: <https://www.ivis.org/library/clinical-avian-medicine/nutritional-considerations-section-i-nutrition-and-dietary> (Accessed: 11 March 2022).

Mete, A., R. Jalving, B. A. van Oost, J. E. Van Dijk, and J. J. Marx. 2005. Intestinal over-expression of iron transporters induces iron overload in birds in captivity. Blood Cells Mol. Dis. 34, 151 – 156.

Moyo, V. M., E. Mandishona, S. J. Hasstedt, I. T. Gangaidzo, Z. A. Gomo, H. Khumalo, T. Saungweme, C. F. Kiire, A. C. Paterson, P. Bloom, A. P. MacPhail, T. Rouault, and V. R. Gordeuk. 1998. Evidence of genetic transmission in African iron overload. Blood 91:1076-1082.

Muir A. and U. Hopfer 1985. Regional specificity of iron uptake by small intestinal brush-border membranes from normal and iron-deficient mice. Am J Physiol.;248(3 Pt 1):G376-9. doi: 10.1152/ajpgi.1985.248.3.G376. PMID: 3976894

Mutschler, E., G. Geisslinger, H. K. Kroemer, and M. Schäfer-Korting. 2001. Arzneimittelwirkungen Lehrbuch der Pharmakologie und Toxikologie. Wissenschaftliche Verlagsgesellschaft mbH Stuttgart. P.:481-485

Mwangi M.N., D.G.A.B. Oonincx, T. Stouten, M. Veenenbos, A. Melse-Boonstra, M. Dicke, J.J.A. van Loon. 2018. Insects as sources of iron and zinc in human nutrition. Nutr Res Rev. 31, 248-255

National Research Council, 1994. Nutrient Requirements of Poultry: Ninth Revised Edition, 1994. Washington, DC: The National Academies Press. <https://doi.org/10.17226/2114>.

Nemeth, E. and T. Ganz., 2009. The Role of Heparin in Iron Metabolism. Acta Haematol 2009;122:78–86. DOI: 10.1159/000243791

Nemeth, E., S. Tuttle Marie, J. Powelson, B. Vaughn Michael, A. Donovan, M. Ward Diane, T. Ganz, and J. Kaplan, 2004. Heparin Regulates Cellular Iron Efflux by Binding to Ferroportin and Inducing Its Internalization. Science 306:2090-2093.

Noorjahan, A., A. Bajaj and K. Singh. 2014. *In vivo* evaluation of taste masking for developed chewable and orodispersible tablets in humans and rats, Pharmaceutical Development and Technology, 19:3, 290-295. DOI: [10.3109/10837450.2013.778870](https://doi.org/10.3109/10837450.2013.778870)

Papanikolaou, G. and K. Pantopoulos, 2005. Iron metabolism and toxicity. Toxicology and Applied Pharmacology 202:199-211

Penzlin, H., 1991. Der Transport der Atemgase, Lehrbuch der Tierphysiologie. Gustav Fischer, Jena. P. 310-326

- Ponka, P., M. Tenenbein, and J. W. Eaton, 2007. 'Chapter 30. Iron', in Handbook on the Toxicology of Metals (Third Edition), ed. by Gunnar F. Nordberg, Bruce A. Fowler, Monica Nordberg and Lars T. Friberg (Burlington: Academic Press, 2007), pp. 577-98.
- Powell, F.L. 2015. Chapter 13 - Respiration. In: C. G. Scanes (ed.) Sturkie's Avian Physiology (Sixth Edition). p 245. Academic Press, San Diego. P. 301-336
- Schmidt-Nielsen, K. 1997. Oxygen transport in blood. In „Animal Physiology“. 5. edition. Cambridge University Press. P. 66-69
- Schümann, K., Ettle, T., Szegner, B., Elsenhans, B., & Solomons, N. W. (2007). On risks and benefits of iron supplementation recommendations for iron intake revisited. *Journal of Trace Elements in Medicine and Biology*, 21(3), 147-168.
- Sherry M. Lewis, Duane E. Ullrey, Dennis E. Bamard, and Joseph J. Knapka, 2006. 'Chapter 9 - Nutrition', in The Laboratory Rat (Second Edition), ed. by Mark A. Suckow, Steven H. Weisbroth and Craig L. Franklin (Burlington: Academic Press, 2006), pp. 219-301
- Simpson RJ, Cooper CE, Raja KB, Halliwell B, Evans PJ, Aruoma OI, Singh S, Konijn AM. 1992. Non-transferrin-bound iron species in the serum of hypotransferrinaemic mice. *Biochim Biophys Acta*. ;1156(1):19-26. doi: 10.1016/0304-4165(92)90090-h. PMID: 1335284.
- Stephan, E. 2010. Zur Eisenversorgung ausgewachsener Pferde, Mecklenburger Pferde 2/2010, 50-51 <https://www.hippothek.de/pdf/1302013099.0689.pdf>
- Troeh F. R. and Thompson, L. M. 2005. Chapter 15. The Micronutrients. Soils and Soil Fertility, 6th ed. Wiley - Blackwell.
- Waldvogel-Abramowski S, Waeber G, Gassner C, Buser A, Frey BM, Favrat B, Tissot JD. 2014. Physiology of iron metabolism. *Transfus Med Hemother*. 2014 Jun;41(3):213-21. doi: 10.1159/000362888
- Whaley, C. 2022. Granular iron based molluscicides and observations relating to the behaviour of slugs and snails. i2L Research Ltd (unpublished report).
- Zhang, A. S. and C. A. Enns. 2009. Molecular mechanisms of normal iron homeostasis. *Hematology*:207 - 214.

Appendix 3 – HSE evaluation of key literature studies reviewed for the bird and mammal weight of evidence assessment

Ancuceanu R., Dinu M., Hovanet M.V., Anghel A.I., Popescu C.V. & Negres S. (2015). A survey of plant iron content – A semi-systematic review. *Nutrients* 2015, 7, 10320–10351.

GLP: No, published study

Methods and materials

This is a review publication which includes information on iron concentrations in plants. The authors adopted a semi-systematic literature search approach, based on a long list of plant genera randomly sampled in a stratified manner. Searches for information on plant iron concentrations were performed in Pubmed, Proquest Central, Google Scholar and the “Plants for the future” database. Studies were only included if they contained information on iron concentrations in a specified plant species and plant part. Where multiple studies were available for the same species/part, the range of values has been reported. Leaves were the most common plant part collected and analysed for iron concentration and were therefore used as a reference for comparison with other plant parts. Statistical comparison were made using R and included Shapiro-Wilks tests for normality and Mann-Whitney (Wilcoxon rank sum test) and Kruskal-Wallis tests for comparing iron concentrations between variables. A search for papers using Pubmed was also conducted for the absorption of non-heme iron from plants.

Results and discussion

In total 1272 publications were identified reporting iron content in various plant species and organs. Iron contents values for a 1228 species, 5 subspecies and 5 varieties were collected. This represents about 0.35% of the 350,699 species with “accepted” status included in The Plant List. Overall data are summarised in the following table per plant part.

Table 1. Synthetic overview of the data collected in our review including iron concentration variation among different plant parts.

Plant Part	Number of Species ^a	Number of Families	Minimum Iron Conc. (mg/kg, dwb ^b)	Maximum Iron Conc. (mg/kg, dwb ^b)	Median Iron Conc. (95% CI) (mg/kg, dwb ^b)	Mean Iron Conc. (95% CI) (mg/kg, dwb ^b)
Root	66	33	1.9	111,200.0	502.4 (259.3–691.0)	5706.0 (2750, 11,560)
Stem	60	34	7.3	25,650.0	171.0 (69.2–313.4)	1431.0 (829, 2696)
Shoot	32	22	20.2	9418.0	91.0 (72.7–101.5)	513.5 (227.1–1113.8)
Bark	41 ^c	19	3.6	1585.0	45.0 (35.0–57.0)	106.8 (74.3–188.8)
Leaf	632 ^d	155	0.1	24,070.0	167.0 (155.2–186.6)	489.4 (401.8–618.4)
Aerial parts	295	89	0.0	27,100.0	240.1 (216.5–263.3)	596.9 (468.4–900.8)
Flower	28	15	15.7	5139.0	159.9 (91.2–194.1)	426.1 (187.5–1008.1)
Fruit	200 ^e	62	0.0	8424.0	72.6 (61.0, 87.7)	257.9 (195.2–393.3)
Seed	104	42	0.0	11,610.0	70.2 (53.8–90.0)	522.6 (333.0–894.4)
Whole plant	41	25	11.4	70,480.0	156.0 (89–747)	2785.0 (1072–9184)
Wood	35	15	0.0	35.0	0.0 (N/A)	3.4 (1.9–6.5)
Other parts ^f	30	28	0.7	3730.0	141.0 (80.0–215.0)	293.1 (179.3–657.2)

^a Given that, for some species, iron values were available for several plant parts, whereas in the case of others iron values were available only for one or two parts, the total in this column adds up to 1562 and not 1228. The same reason explains the apparent discrepancy regarding the number of subspecies and varieties (one organ was reported for the species, while a different organ for a subspecies or variety of the same species); ^b dwb = on a dry weight basis; ^c + 1 subspecies; ^d + 2 subspecies + 3 varieties; ^e + 1 subspecies; ^f aril, bud, bulb, calyx, false fruit, leaf pulp etc. (see Figure S22).

For all plant parts analyzed, the distribution of iron concentration was positively skewed, i.e., with a posterior tail and the majority of concentrations relatively low. Roots were found to contain the highest concentrations of iron (statistically significant difference from leaves). Levels of iron in stems and leaves were similar, with no statistically significant difference. The authors concluded that no particular plant family was characterised by either high or low iron concentrations. Results for different taxonomic groups are presented in the following table, with iron concentrations shown in terms of mg/kg dry weight.

Table 2. Synthetic overview of iron concentration variation by taxonomic groups.

Plant Part	Pteridophytes (Median) (95% CI) (n ^a)	Gymnosperms (Median) (95% CI) (n)	Magnoliids (Median) (95% CI) (n)	Dicots (Median) (95% CI) (n)	Monocots (Median) (95% CI) (n)	Relevant Statistical Comparisons
Root	296.5 NA 2	NA NA 0	259.3 194.0–37856.4 3	426.5 186.0–985.9 44	573.9 394.3–1100.0 17	M ^b versus D ^c : $p = 0.443$ (Mann-Whitney)
Stem	42.0 27–50 3	175.65 NA 1	7783.7 NA 1	140.8 59–441 39	325.0 115.1–550.0 16	M ^b versus D ^c : $p = 0.584$ (Mann-Whitney)
Leaf	200.0 109.5–238.6 33	133.6 109.0–155.0 42	253.2 166.6–277.5 34	163.0 152.0–193.0 438	188.0 141.0–240.0 82	G ^d versus D: $p = 0.017$; G versus M: $p = 0.038$; G versus Mag ^e : $p = 0.005$; *
Shoot	119.2 53.4–128.0 4	NA NA 0	NA NA 0	94.4 52.15–134.50 20	89.0 72.7–92.0 8	M ^b versus D ^c versus P ^d : $p = 0.941$
Aerial parts	156.0 109.0–223.0 25	353.5 NA 2	487.0 NA 2	225.0 200.0–243.0 186	305.0 220.0–522.0 64	M vs. D: $p = 0.022$ (nparcomp) (Hedges's g 0.416)
Flower	NA NA 0	NA NA 0	2631.45 NA 2	159.9 88.3–193.6 26	NA NA 0	NA
Fruit	NA NA 0	NA NA 0	96.42 77.85–155.00 9	69.9 58.00–87.70 178	67.8 37.60–186.20 13	Kruskal Wallis: $p = 0.486$
Seed	NA NA 0	7.2 1.5–41.1 5	14.5 0.80–264.60 5	80.5 60.0–99.8 83	59 4.0–70.0 11	M vs. D: $p = 0.098$ (Mann-Whitney)
Whole plant	83.0 45.0–106.5 3	35.2 NA 1	NA NA 0	427.0 83.2–1317.5 26	118.0 72.5–3041.0 9	M vs. D: $p = 0.7976$ (Mann-Whitney)
Wood	NA NA 0	1.2 0.0–3.1 15	175 NA 2	0 NA 18	NA NA 0	NA
Bark	NA NA 0	60.5 37.0–120.0 15	14.5 9.85–123.65 3	42.0 24.0–45.7 23	20.0 NA 1	G vs. D: $p = 0.089$ (Welch t on ranks)
Other parts	240 NA 1	35.6 15.40, 37.15 2	2065 NA 1	117.9 71.1–190.8 18	248.0 45.0–317.0 8	NA

Bryophytes species ($n = 19$) not included in the table. ^a n = number of unique species (number of data points is in most cases larger); ^b M = Monocots; ^c D = Dicots; ^d G = Gymnosperms; ^e Mag = Magnoliids. * Kruskal-Wallis for all groups, $p = 0.015$; all other intergroup comparisons nonsignificant (p between 0.215 and 0.999) (nparcomp).

The search regarding absorption of non-heme iron from plants by humans returned 1392 papers in total, of which, 382 were found to be relevant. The review found that it has been historically understood that heme iron is more easily absorbed than non-heme iron. Based on the results, the authors determined a number of factors related to the food matrix influencing non-heme iron absorption. These include the presence of phytic acid, phytates, polyphenols or tannins in the plant source or from other food, iron mineral competitors (e.g. Zn, Ca), ascorbic acid and vitamin A. The authors also state that EDTA facilitates iron absorption in humans, referring to a paper by Cercamondi et al. (2014), though they note the body of research is less extensive than for ascorbic acid.

HSE comments

This literature review followed a clear and well-described search strategy. An extensive amount of data was collected on iron concentrations in a range of plant species and plant parts. This information is useful in understanding potential background exposure levels of herbivorous/omnivorous bird and mammal species to iron via their diets. It is noted however that the particular form of iron in plant tissues has not been presented in this paper due to the information seldom being available in the underlying studies.

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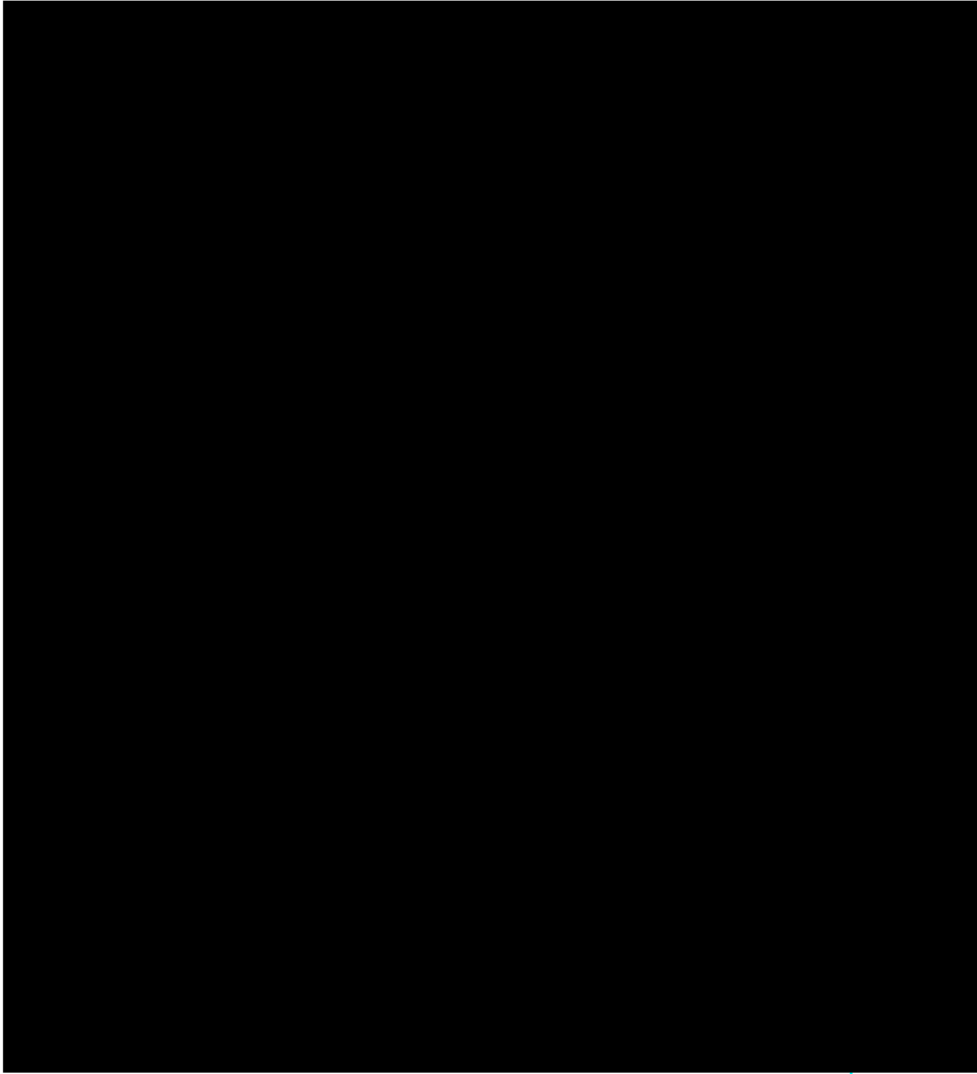
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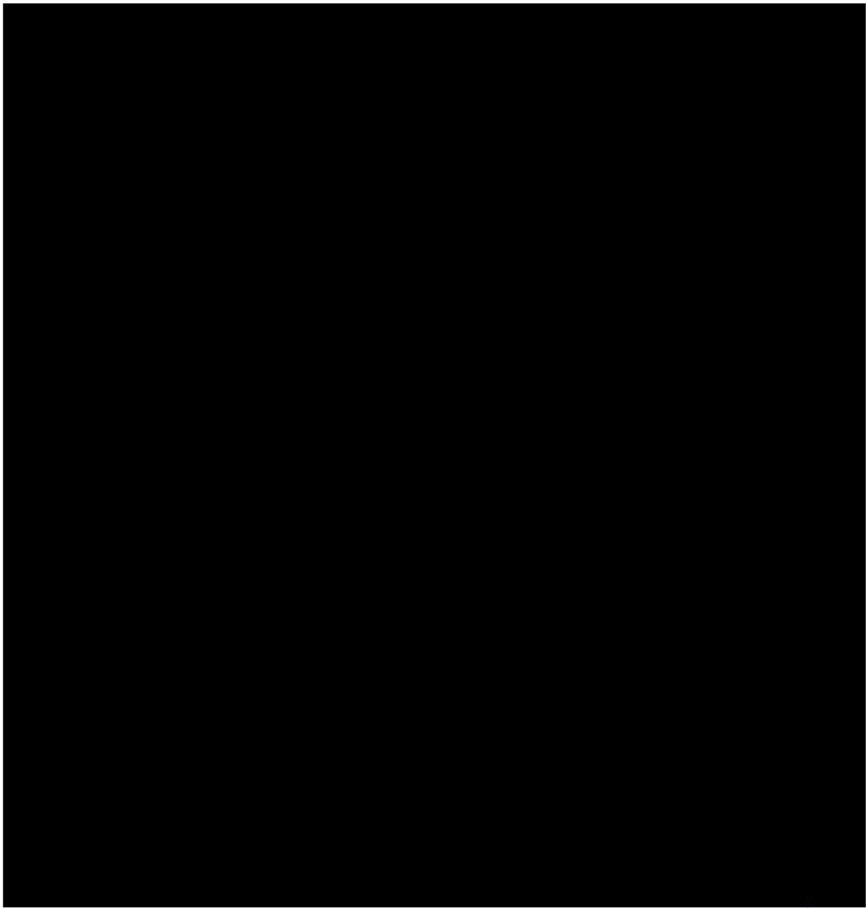
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Cork S.C., Alley M.R. & Stockdale H.G. (1995). A quantitative assessment of haemosiderosis in wild and captive birds using image analysis. *Avian Pathology*, 24, 239-254

GLP: No, published study

Methods and materials

Twelve 1-day-old male White Leghorn chickens were housed in cages. After 2 weeks they were divided into four groups of 3 birds. In each group one bird acted as a control and the other 2 were injected with 10 mg iron dextran on day 1. Birds were sacrificed 24 h, 48 h, 6 days and 10 days following treatment. Gross findings were noted following euthanasia necropsy. Samples of spleen, kidney, intestine, liver, muscle, bone marrow and lung from all 12 birds were fixed. Sections were cut and stained with haemotoxylin and eosin and Perls' iron stain. The amount and distribution of stainable iron in each section of tissue was evaluated subjectively and by image analysis.

Additionally, necropsies were performed on 180 birds submitted to the New Zealand Department of Veterinary Pathology and Public Health over a period of 2 years (September 1991 to 1993). This including 40 avian species, covering 6 orders. Gross and histological findings and the relevant clinical history were recorded for each case. In each case one or more extra liver sections were cut and stained with Perls' iron stain. Representative sections of liver tissue were examined visually and subjected to image analysis to allow a quantitative assessment of the extent and distribution of hepatic iron stores.

Results and discussions

No chickens died during the live experimental phase. At necropsy the only abnormality detected was that livers of treated birds were dark coloured. Iron was observed in the liver, in all eight of the treated birds, but not in the spleen, bone marrow, intestinal tract or muscle. Birds examined had stainable iron in their livers from 24 h onwards. The maximal extent of hepatic iron loading, determined subjectively and by image analysis, was evident at 48 h and had declined by day 10 following treatment. There was no stainable iron detected in the tissues of the four control birds. There was a significant amount of variability between individual birds within each treatment group but this was less than the difference between treatment groups. Compared to results for submitted birds of other species, white leghorn chickens were towards the centre of the distribution, in terms of stainable iron in the liver.

Haemosiderosis was observed as an histological finding in 50 (28%) of the 180 necropsy cases examined. The degree of stainable iron detected visually in the liver ranged from mild to severe. There was considerable variability between individuals of a given species, with little or no stainable iron in the liver or other tissues of at least one individual for 39 of the species. It is notable that introduced granivorous passeriformes, such as the house sparrow (*Passer domesticus*) and the greenfinch (*Carduelis chloris*), had little or no stainable iron in the liver.

In 79 of the 180 birds examined, infectious diseases were considered to be the cause of death. It was not possible to reliably determine whether iron accumulation in the liver contributed to the cause of death of birds. Only in the psittaciformes (parrots) was there significantly more total stainable iron in those with infectious disease than in those with non-infectious disease. There was insufficient information on dietary iron content to consider this as a variable in the analysis.

HSE comments

This study contains information on the frequency of iron accumulation in livers for a range of bird species, including wild and captive species. While the study was conducted in New Zealand, it does include some species that could be exposed to elemental iron via granules or molluscs in GB.

Crissey S., Trusk A., Block S. and McGil P. (1993). Iron storage: Effect of dietary iron on the accumulation of iron in the liver of European Starlings. Proceedings American Association of Zoo Veterinarians (August/September).

GLP: No, published study

Methods and materials

The European Starling was selected as a suitable model species, with birds used taken from Brookfield Zoo (Illinois, USA). Forty-six fledgling starlings were selected for the study and housed in 23 cages, each containing a pair of birds. Study birds were divided into two groups and were fed diets containing either 148 ppm iron (low iron diet) or 3035 ppm (high iron diet). Body weight and diet intake were monitored throughout the study. Measurements of liver iron content and liver weight were taken from half the birds on each diet at ten weeks and the remaining birds at 18 weeks. Histopathological exams which visually assess iron deposition also were performed on the livers at these times.

Results and discussion

All birds consumed similar quantities of food and maintained relatively constant body weights throughout the study. All appeared healthy upon visual examination.

At ten weeks the birds had similar iron levels in their livers, regardless of the diet they were consuming. There also was a tendency for iron to accumulate in the livers of all birds at 18 weeks, regardless of diet. However, the quantity of dietary iron did have a significant affect on liver iron accumulation. At 18 weeks, those consuming the high iron diet had much greater quantities of iron in their livers. Additionally, birds on the high iron diet also had larger livers when compared to those on the low iron diet.

HSE comments

This study contains information on the accumulation of iron in the livers of European starlings fed diets containing known quantities of iron. While the study contains potentially useful information, the level of reporting of study methods and results is very brief. The nature of the diet used and conditions of the study are not stated. Detailed study results are also not reported, with no information on absolute levels of iron in livers, variability or the statistical significance of any comparisons.

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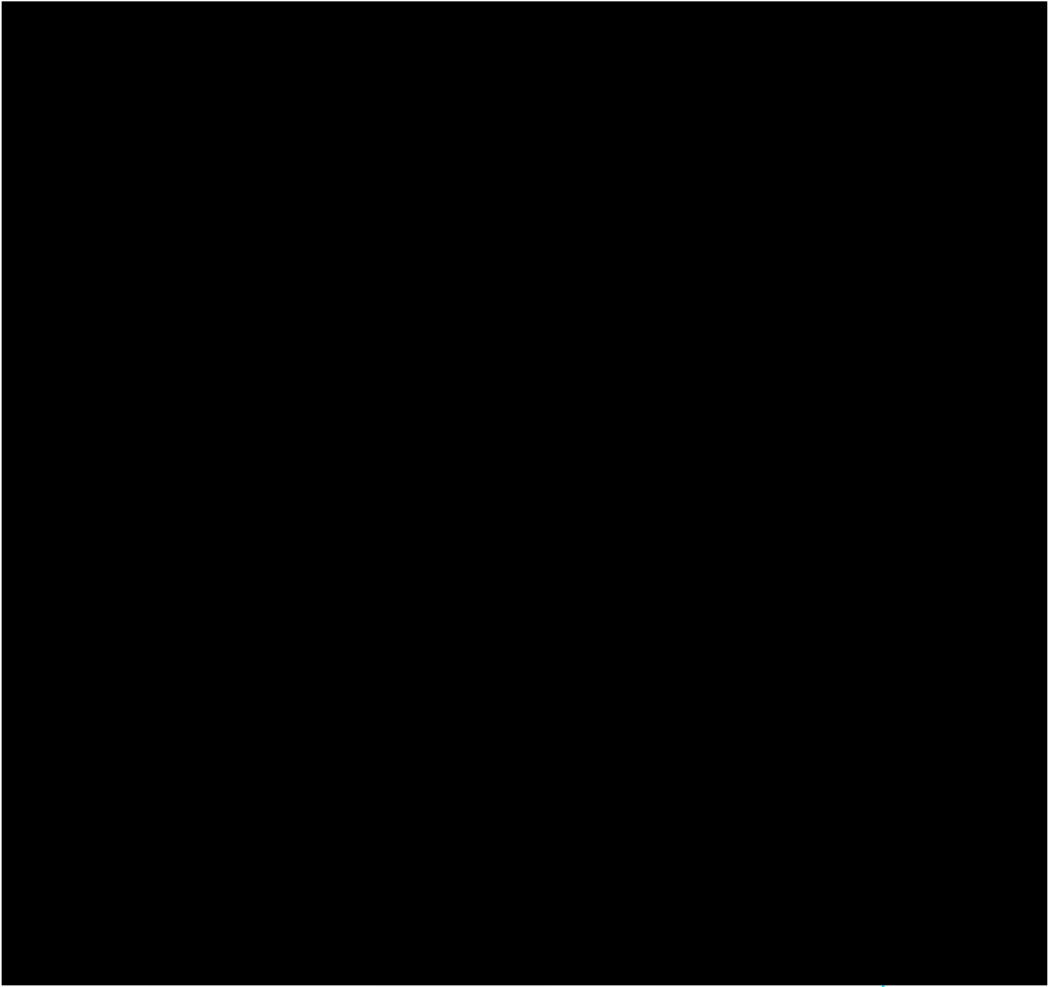
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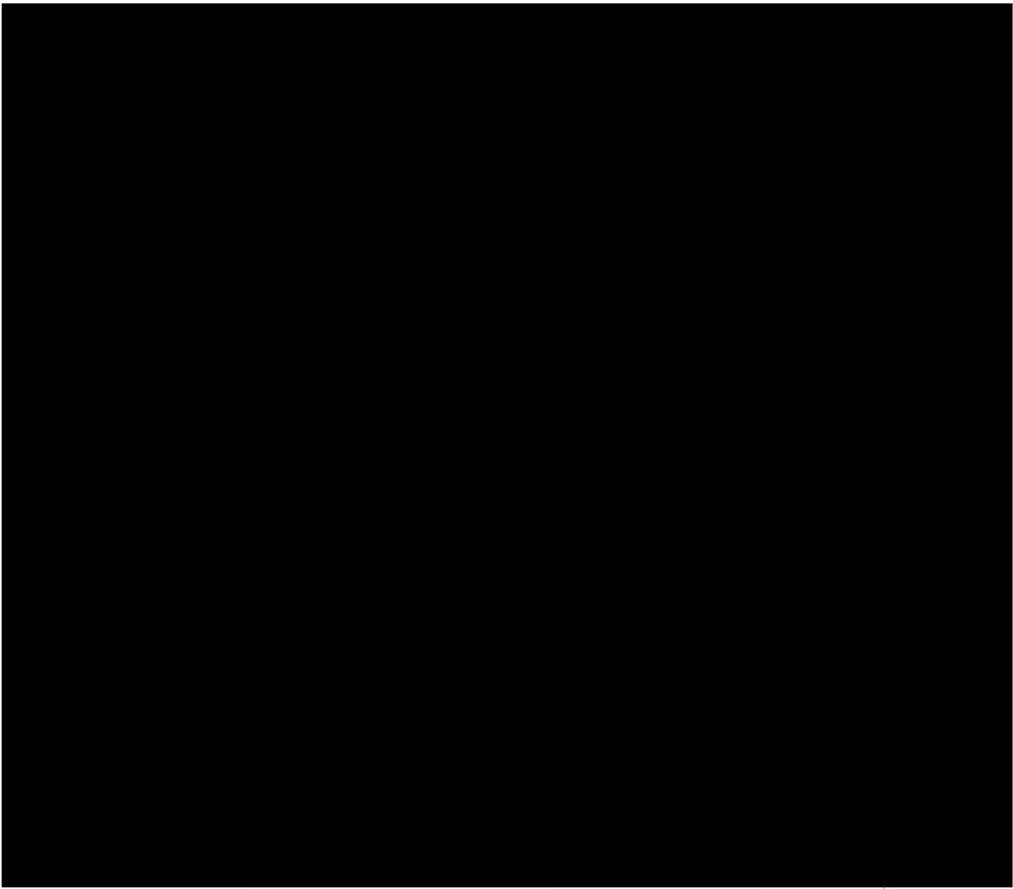
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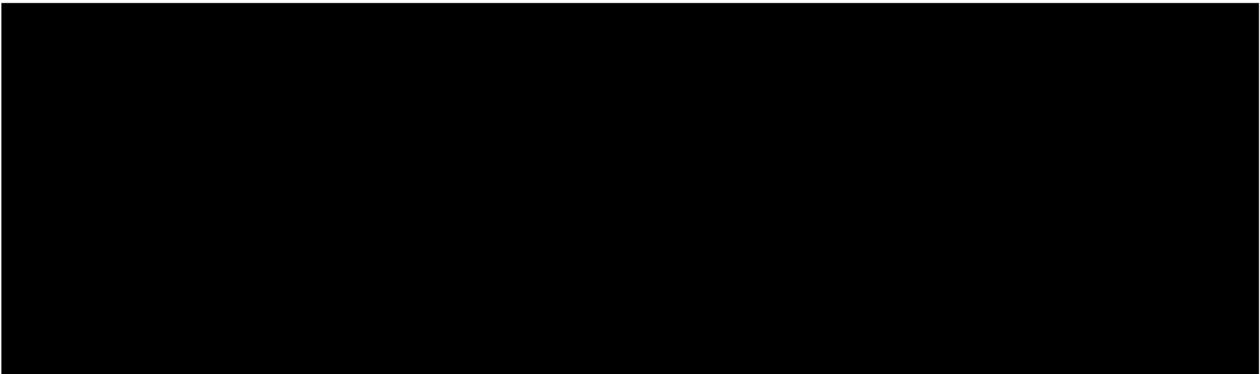
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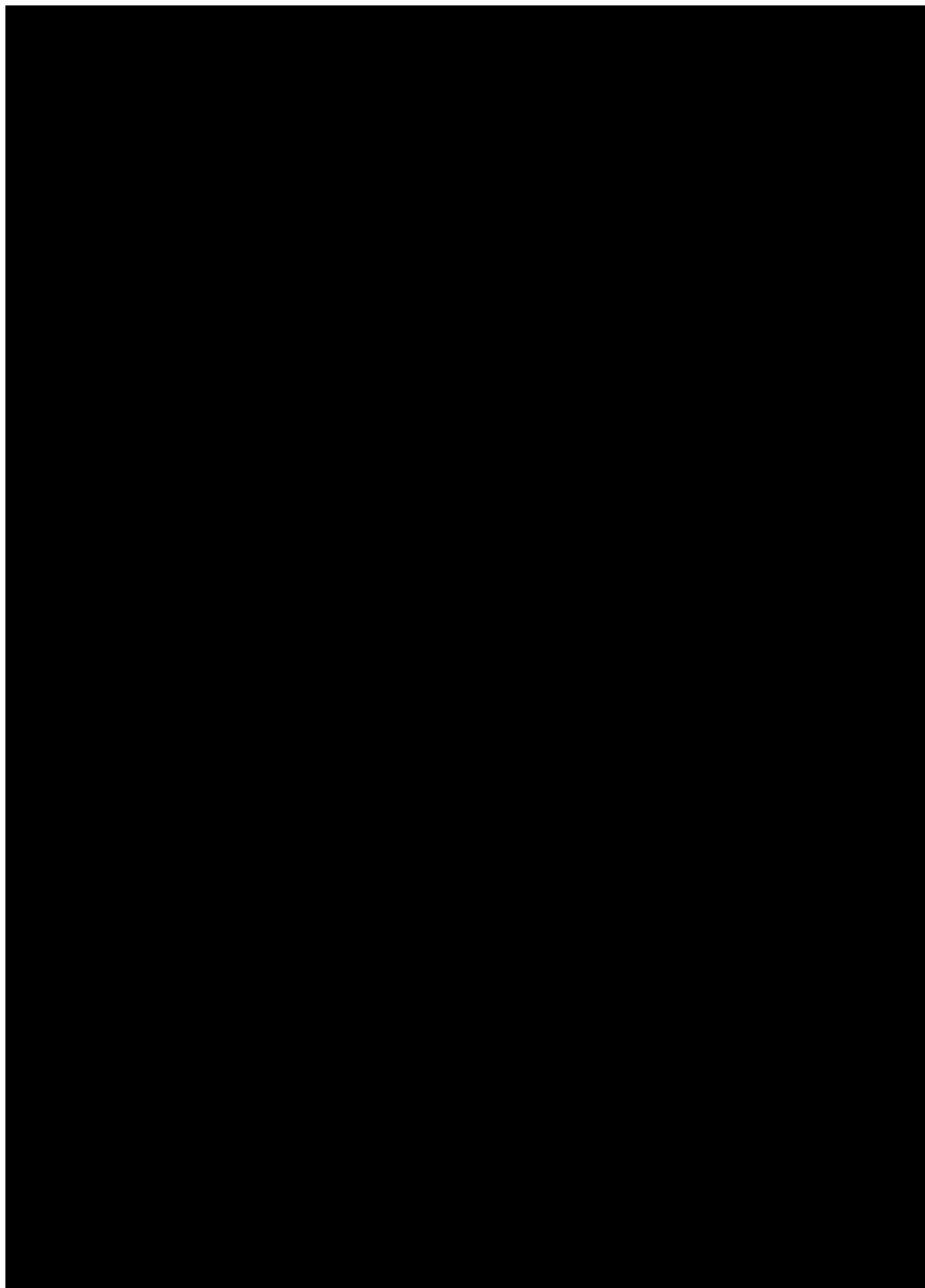
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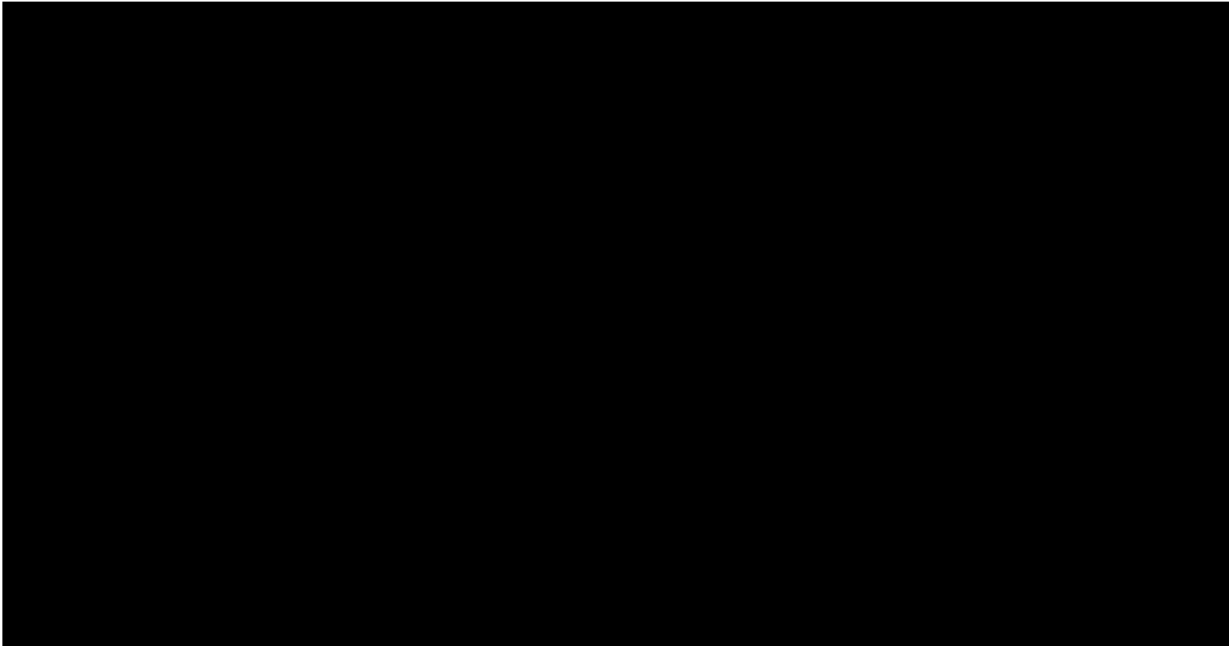
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Kozłowski J., Jaskulska M. & Kozłowska M. (2014). Evaluation of the effectiveness of iron phosphate and the parasitic nematode *Phasmarhabditis hermaphrodita* in reducing plant damage caused by the slug *Arion vulgaris* Moquin-Tandon. *Folia Malacologica*, 22(4) pp 293-300.

GLP: No, published study

Methods and materials

The aim of this study was to investigate the possibility of reducing the applied dose of iron phosphate and to compare the effectiveness of two methods of application of the nematode *P. hermaphrodita* in reducing the damage done to Chinese cabbage plants by *Arion vulgaris*. Only the aspects of the study relating to the control of *A. vulgaris* by iron phosphate are covered in this summary. After hatching, slugs were kept in a climate chamber at a temperature of 17°C, RH 70% ± 3%, with a 12/12 hour photoperiod.

In the tests of the effectiveness of iron phosphate, the commercial molluscicide Anti-Limaces Ferramol (0.99% a.s., Scotts France SAS, recommended dose – 5 g/m²) was applied in three different doses (1.0, 2.5 and 5.0 g/m²), while the two

molluscicides used for comparison, methiocarb (Mesuol Alimax 02 RB, 2% a.s.) and metaldehyde (Snacol05 GB, 5% a.s.) were applied only in the recommended doses (0.5 g/m² and 0.4 g/m², respectively).

No-choice tests were done on the Hilton variety of Chinese cabbage (3–4 leaf stage) growing in a 5 cm layer of soil inside containers. To each container with six plants, molluscicide granules were introduced in the appropriate dose, along with two slugs (average weight 1.97 g) which had been starved for the previous 48 hours. Containers with plants and slugs but without molluscicides were used as control. Five replicates were performed for each molluscicide and each control.

The vitality of the slugs and the degree of plant damage (five-point scale: 0 – no damage, 25, 50, 75 and 100% damaged plant surface) were assessed every two days. The weighted mean values of the amount of plant damage caused by the slugs for the particular experimental variant in each experiment were subject to statistical analysis (ANCOVA and Student's t-test).

Results and discussion

The percentage of slugs killed following the applied doses was as follows: iron phosphate, dose 1.0 g/m² – 20% (2 dead 7 days after application), dose 2.5 g/m² – 50% (4 dead after 9 days and 1 after 11 days), and dose 5.0 g/m² – 80% (2 dead after 5 days, 4 after 7 days and 2 after 9 days); methiocarb, dose 0.5 g/m² – 90% (7 dead after 7 days, 1 after 9 days and 1 after 11 days); metaldehyde, dose 0.4 g/m² – 30% (3 dead after 7 days).

The applied molluscicides were found to reduce the damage done to the plants by *A. vulgaris* after just 24 hours from their application. By that time, iron phosphate in doses of 1.0, 2.5 and 5.0 g/m², and metaldehyde in a dose of 0.4 g/m², had caused significant reduction in the plant damage, compared with the untreated (control) plants. However, damage to the plants treated with methiocarb did not differ significantly from the damage to the control plants.

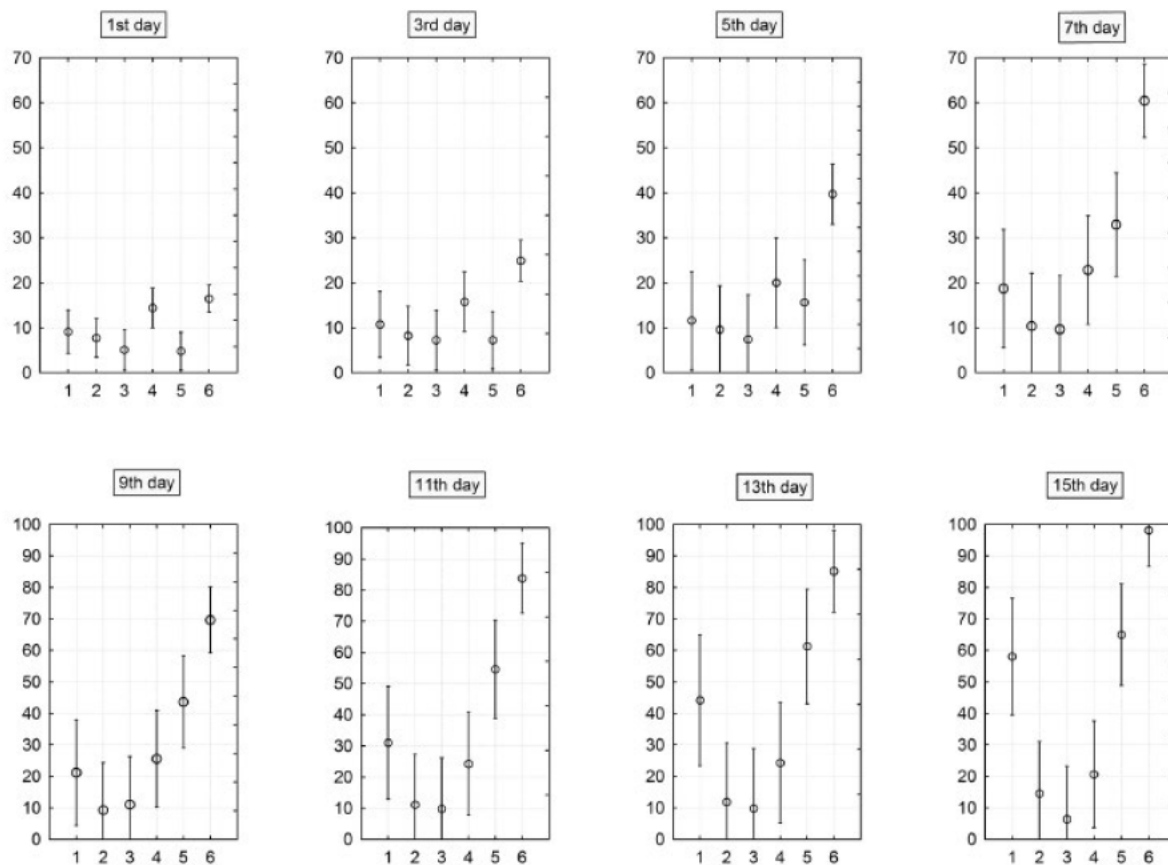


Fig. 1. Adjusted means of damage to seedlings of Chinese cabbage caused by *Arion vulgaris* after treatment with various compounds on consecutive days: axis Y – means in % from ANCOVA and confidence intervals; axis X – 1 denotes iron phosphate 0.99%, dose 1.0 g/m²; 2 – iron phosphate 0.99%, dose 2.5 g/m²; 3 – iron phosphate 0.99%, dose 5.0 g/m²; 4 – methiocarb 2%, dose 0.5 g/m²; 5 – metaldehyde 5%, dose 0.4 g/m²; 6 – control treatment

HSE comments

This study is clearly described and appears well conducted. It contains information on how rapidly consumption of ferric phosphate granules resulted in mortality in *A. vulgaris* slugs and how quickly slug damage to Chinese cabbage seedlings was reduced.

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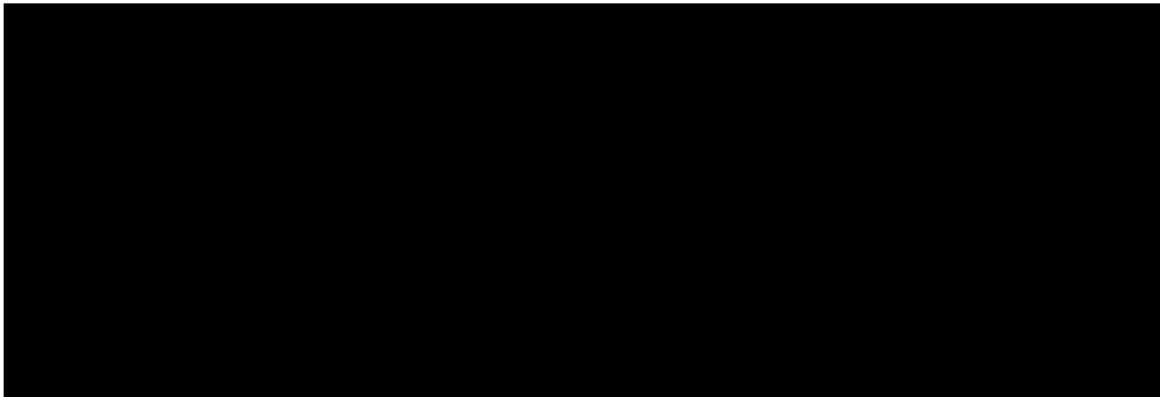
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Pavone S., Salamis S., Pecorelli I., Rossi E. & Manuali E. (2014). Deadly Outbreak of Iron Storage Disease (ISD) in Italian Birds of the Family Turdidae. J. Vet. Med. Sci. 76(9): 1209–1212.

GLP: No, published study

Methods and materials

The authors report on a mortality outbreak in captive birds belonging to the family Turdidae in Italy. From October to January, 6 adult blackbirds (*Turdus merula*), 6 adult fieldfares (*Turdus pilaris*), 20 adult song thrushes (*Turdus philomelos*) and 14 adult redwings (*Turdus iliacus*) were sent for necropsy examination. All animals came from 3 small decoy-bird breeders in central Italy. The mortality rates were 15% for fieldfares, 30% for redwings, 32% for song thrushes and 14% for blackbirds. No other species of bird held by these breeders (*Coccothraustes coccothraustes*, *Alauda arvensis* and *Fringilla montifringilla*) died. It is noted that the breeders reported a change in diet before the hunting season, switching to a commercial diet reported as containing 25 mg/kg iron.

Full necropsy examinations were conducted for all birds and representative tissue samples were stained with Masson's trichrome and Perls' Prussian blue to determine the iron content. During necropsy, fresh tissue samples from lungs, brain and intestines were collected and subjected to molecular analysis for a range of diseases. Intestinal and hepatic specimens were also aseptically collected for bacteriological examinations.

Results and discussion

The clinical histories of all the dead birds included anorexia, lethargy and finally dyspnoea and death. At necropsy, all birds showed moderate to marked congestion and enlargement of the liver and spleen. The liver was found to be dark brown in colour in around 10% of the birds. The hearts of some animals appeared enlarged, showing small well-demarcated, pale greyish areas consistent with necrosis. Microscopic examination of liver samples showed venous congestion and severe, mainly periportal, parenchymal cell degeneration. Yellow/gold to brown granular pigment in the

cytoplasm of hepatocytes consistent with hemosiderin was detected in all liver samples. Necrosis of isolated hepatocytes was also observed.

Blackbirds showed the most severe hepatic iron accumulation, while song thrushes appeared to be a less affected species. However, the high mortality rate seen in song thrushes potentially indicates this species is less resistant to iron accumulation.

Table 1. Level of hemosiderosis in the livers of four investigated species

Iron deposits in	Blackbirds	Fieldfares	Song thrushes	Redwings
Periportal	0	1	14	3
Periportal and midzonal	2	4	6	11
Panlobular	4	1	0	0

Microscopical examination of heart samples showed acute myocardial necrosis in 37% of the birds examined. Perls's Prussian blue staining revealed some granular iron deposits in scattered myocardial cells. Iron deposits were also detected in tubular epithelial cells.

Analysis for Newcastle disease, avian influenza and West Nile virus showed negative results, as did bacteriological exams. Expected clinical signs of iron storage disease (fibrosis, cirrhosis or neoplasms) were not observed in birds, though acute liver damage represented by degeneration and necrosis of hepatocytes with iron overload (sideronecrosis) was constantly found. The authors postulate that given the acute effects, there was insufficient time for chronic effects to manifest. Analysis of heavy metal contaminants in food pellets found: iron 111 mg/kg; copper 6 mg/kg; zinc 38 mg/kg; lead 0.132 mg/kg. Environmental and nutritional conditions of the three small bird-decoy breeders were uniform.

HSE comments

This study includes data on an apparent iron poisoning incident involving blackbirds, fieldfares, song thrushes and redwings. These species are potentially relevant for the elemental iron risk assessment. The incident details are well-reported and are considered further in the risk assessment section.

Wadsworth P.F., Jones D.M. and Pugsley S.L. (1983). Hepatic haemosiderosis in birds at the Zoological Society of London. *Avian Pathology*, 12: 321-330.

GLP: No, published study

Methods and materials

Post mortem examinations on birds between 1976 and 1982. The majority of birds came from the Zoological Society of London collection, with a small proportion from external sources. Five hundred and thirty-one birds from 18 different orders were examined. All the carcasses were examined for macroscopic lesions of disease and routine bacteriological and parasitological examinations were carried out as necessary. Representative sections of major organs were fixed and stained with Perls' Prussian blue to determine the extent of iron accumulation, where this was expected to have occurred. Where significant iron quantities were detected in livers, these were categorised as either 'moderate' or 'marked'.

Results and discussion

A total of 37 cases (7%) showed substantial levels of stainable iron (moderate to marked) in the liver. A high incidence of hepatic haemosiderosis was recorded in some species in the orders Ciconiiformes, Passeriformes, Cuculiformes and Coraciiformes. The macroscopic appearance of the livers of affected birds varied. In 12 cases the livers were swollen or enlarged. In 10 cases the colour of the liver was either yellow, brown or golden. Histological evidence of tissue damage associated with hepatic haemosiderosis was seen in two cases - a Black casqued hornbill and a Swainson's toucan.

Table 1. The occurrence of hepatic haemosiderosis in individual orders of birds examined at necropsy at the Zoological Society of London.

Order	Hepatic haemosiderosis (Moderate/Marked)	Number of birds examined	Incidence (%)
Rheiformes	0	4	0
Casuariiformes	0	1	0
Sphenisciformes	0	32	0
Pelecaniformes	0	4	0
Ciconiiformes	10	53	19
Anseriformes	4	65	6
Falconiformes	0	8	0
Galliformes	1	34	3
Gruiformes	1	11	9
Charadriiformes	1	17	6
Columbiformes	1	23	4
Psittaciformes	0	132	0
Cuculiformes	3	4	75
Strigiformes	0	26	0
Apodiformes	0	8	0
Coraciiformes	5	14	36
Piciformes	1	12	8
Passeriformes	10	83	12
Total	37	531	7

HSE comments

This study provides insight into which bird families are more likely to experience hepatic haemosiderosis. Whether the accumulation of iron in the livers observed resulted in the death of the bird or other health effects is difficult to determine, with the authors stating that 'in the absence of clinical chemistry data prior to death, the clinical significance of the hepatic haemosiderosis was difficult to assess'. The concentration of iron in bird diets is also unknown, so it cannot be determined what concentrations were more likely to result in hepatic haemosiderosis.

Ward R.J., Iancu T.C., Henderson G.M., Kirikwood J.R. & Peters T.J. (1988). Hepatic iron overload in birds: Analytical and morphological studies. *Avian Pathology*, 17, 451-464

GLP: No, published study

Methods and materials

Livers collected post-mortem from a wide range of avian species during a one-year period have been studied both biochemically and morphologically to investigate the prevalence of iron-overload and hepatic iron content. Liver samples were collected at post mortem examination from birds that died during the year July 1985 - June 1986 at The Zoological Society of London (ZSL). A portion was processed for light and electron microscopy, and the remaining tissue frozen at -20°C for assay of iron content. Sections were stained with haematoxylin and eosin or Perls' Prussian blue for iron. Total hepatic iron was determined by electrothermal atomic absorption. In human haemochromatosis hepatic damage is indicated by increased activities of serum liver enzymes. Therefore the serum activities of liver enzymes were investigated in birds.

Results and discussion

In 90% of the samples analysed, the iron concentration was greater than 5.4 $\mu\text{mol Fe/g}$ wet weight (which the authors considered a normal level found in rodents). There was no correlation between liver iron content and the age of the bird at death. There were differences between bird orders in terms of iron liver contents, with higher values found for *Passeriformes*, *Coraciiformes* and *Anseriformes*.

Table 1. Hepatic iron content and serum enzyme levels according to taxonomic order of birds

Order	No	Liver iron content ($\mu\text{mol Fe/g}$ tissue)		No	Serum enzymes (IU/litre)			
		Mean \pm SD	Range		LDH	ALT	AST	AP
Passeriformes	10	70 \pm 65	18 - 230	5	1460 \pm 290	35 \pm 30	334 \pm 102	497 \pm 126
Galliformes	9	5 \pm 4	2 - 11	2	503	—	390	562
Anseriformes	6	43 \pm 37	0.5 - 97	2	774	21	430	69
Coraciiformes	4	90 \pm 45	29 - 131	2	—	43	464	27
Falconiformes	2	9 \pm 1.5	8 - 10	1	—	57	539	29
Sphenisciformes	2	12 \pm 1.5	11 - 13	2	772	35	132	73
Ciconiiformes	3	11 \pm 5	6 - 15	2	1300	—	360	501
Charadriiformes	4	9 \pm 4	6 - 13					
Strigiformes	2	42 \pm 0.5	41 - 42					
Psittaciformes	2	9 \pm 0.4	9 - 9					
Tinamiformes	2	2 \pm 0.5	2 - 3					
Cuculiformes	1	3	—					
Columbiformes	1	5	—					
Piciformes	1	36	—					
Phoenicopteriformes	1	37	—					

LDH - lactate dehydrogenase; ALT - alanine aminotransferase; AST - aspartate aminotransferase; AP - alkaline phosphatase. Reference ranges (IU/litre) from Randell *et al.* (1981): LDH, 180-420; ALT, 20-80; AST, 130-330; AP, 100-300.

Morphological investigation of samples from passerines found that at the highest iron loading there were morphological changes similar to those of the human disease idiopathic haemochromatosis when there is excessive absorption of iron from the diet. However, based on serum activities of liver enzymes, the authors concluded that despite the high iron-loading, each bird had the capacity successfully to sequester any excess iron to prevent its toxic effects.

HSE comments

This study contains information on the magnitude and frequency of iron accumulation in livers for a range of bird species. Accumulation of iron in the liver was seen in a wide range of bird species, with the Passeriformes data particularly relevant for the risk assessment. Despite the accumulation of iron, there was no indication that liver functioning was impacted.

Ward R.J., Smith T., Henderson G.M. & Peters T.J. (1991). Investigation of the aetiology of haemosiderosis in the starling (<i>Sturnus vulgaris</i>). <i>Avian Pathology</i> , 20, 225-232
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GLP: No, published study

Methods and materials

This study investigated absorption radio-labelled iron in starlings. Common pigeons and rats (Sprague Dawley) were also included for comparative purposes. Nineteen starlings were divided into 3 groups: one group of pre-moult birds (n = 4), one group of post-moult birds (n = 9), and one group of pre-moult birds injected with iron-dextran (Imferon; 5 mg Fe). Six rats were tested, split evenly between a control group and a group fed a diet containing carbonyl-iron. Common pigeons (n = 4) were also included but not pre-exposed to iron.

All animals were then dosed with radio-labelled iron, ferrous sulphate and ascorbic acid via a stomach tube to investigate absorption. Whole body counters were used to detect activity from 30 mins to 6 weeks after dosing. Microscopic examination of livers was as described in Ward et al. (1988).

Results and discussion

Table 1. Hepatic iron concentration and retention of oral ^{59}Fe in starlings (*Sturnus vulgaris*), pigeons (*Columba livia*) and rats (*Sprague Dawley*)

	Hepatic iron ($\mu\text{mol Fe/g liver}$)	^{59}Fe absorption (% dose)
Starling		
Pre-moult (4)	15.8 ± 6.0^a	40.0 ± 12.4
Post-moult (9)	43.9 ± 12.9	15.1 ± 7.6
Pre-moult + Imferon (6)	42.1 ± 11.9	11.2 ± 4.0
Pigeons (4)	22.5 ± 12.8	24.8 ± 5.1
Rats		
Controls (3)	3.2 ± 1.0	14.0 ± 7.2
Carbonyl-iron-loaded (3)	20.1 ± 1.8	0.9 ± 0.1

^a Mean \pm SD. Number of animals investigated between parentheses.

Although there was individual variation in the hepatic iron concentration of starlings, the mean was approximately 2 to 3 times higher during the post-moult season than the pre-moult period. Administration of Imferon to these starlings during the pre-moult period increased hepatic iron concentration 2 to 3-fold. A moderate iron loading was found in livers of untreated pigeons. The hepatic loading of iron was increased approximately 6-fold in carbonyl-iron-loaded rats when compared with control rats. Increased iron content was not associated with increased serum enzyme activities which could indicate hepatic dysfunction.

During the iron absorption phase of the study, by 24 h approximately 50% and 70% of the administered iron had been excreted in the faeces of the starlings and pigeons, respectively, and thereafter the amount excreted rapidly declined. The hepatic iron content was considerably lower in starlings during the pre-moult season and the percentage of orally-administered radioactive iron retained was raised significantly in this group. The retention of Fe by each bird showed significant inverse correlation with hepatic iron concentrations during pre and post-moult periods.

In rats after dietary loading with 2% carbonyl-iron for 197 days, the hepatic iron concentration was 6 to 7-fold higher than rats fed control chow diet. The percentage of orally-administered Fe retained by the iron-loaded rats was only 1%, compared to 14% retained by the control animals.

HSE comments

This study contains information on the absorption of iron by starlings, pigeons and rats. These species are of potential relevance for the elemental iron risk assessment. The results showed that pre-moult starlings had a lower hepatic iron concentration and a greater proportion of the administered dose absorbed compared to post-moult birds. These results indicate temporal variability, and that where hepatic iron concentrations were relatively high, birds were able to reduce the absorption of iron in response. This latter response was also seen in rats, with a higher proportion of iron absorbed by rats fed a control diet, compared to rats fed a carbonyl iron loaded diet.

It is noted that even in animals with the highest hepatic iron concentrations, liver function did not appear to be impacted. However, the relevance of the exposure levels experienced in this study for the field exposure situation is not clear and the available information in the study does not allow for such a comparison.

References

- Aggett P.J. (2012). Iron. Chapter 33. In: Present knowledge in nutrition. – 10th ed. / edited by John W. Erdman Jr., Ian A. Macdonald, Steven H. Zeisel. Wiley-Blackwell, ILSI Press, Washington, DC, p. 506-520. <https://pkn10.org/>
- Ancuceanu R., Dinu M., Hovane M.V., Anghel A.I., Popescu C.V. & Negres S. (2015). A Survey of Plant Iron Content—A Semi-Systematic Review. *Nutrients* 2015, 7, 10320–10351.
- Anon (2022). Literature Review on Iron Physiology in Birds & Mammals: Search methods. RIFCON.
- Axmann (2019). Final Bite - Blue – A Field Study to Investigate Effects on the Earthworm Fauna in Central Europe, Report No S18-02597, Sponsor Code R-39838.
- Boling S.D. & Firman J. (1997). Rendered by-products as soybean meal replacement in turkey rations. *J. Appl. Poult. Res.*, 6 (2): 210-215.
- Brue R. N. (1994). Nutrition. Chapter 3. In: Avian Medicine, B. W. Ritchie, G. J. Harrison, L. R. Harrison. Wingers Pub. Lake Worth, Fla.: (p.63-95).
- Buxton J.M., Crocker D.R., and Pascual J.A. (1998). Portable Document Format. Birds and farming: information for risk assessment. 1998 Update Contract PN0919 Milestone Report FERA Project No M37
- [REDACTED]
- [REDACTED]
- [REDACTED]
- Clarke, J.R. (1985). The reproductive biology of the bank vole (*Clethrionomys glareolus*) and the wood mouse (*Apodemus sylvaticus*). *Symp. Zool. Soc. Lond.* 55: 33-59.
- Coffey R. and Ganz T. (2017). Iron homeostasis: An anthropocentric perspective. *Journal of Biological Chemistry* 292:12727-12734. DOI:<https://doi.org/10.1074/jbc.R117.781823>
- Cork S.C., Alley M.R. & Stockdale H.G. (1995). A quantitative assessment of haemosiderosis in wild and captive birds using image analysis. *Avian Pathology*, 24, 239-254.
- Cork S.C. (2000). Iron storage diseases in birds. *Avian Pathology*, 29, 7-12.
- Cowan P. & Crowell M. (2017). Visual and taste cues for minimising native bird interactions with toxic 1080 baits – a review of current practices. *New Zealand Journal of Ecology* (2017) 41(2): 178-185.
- Crissey S., Trusk A., Block S. and McGil P. (1993). Iron storage: Effect of dietary iron on the accumulation of iron in the liver of European Starlings. *Proceedings American Association of Zoo Veterinarians* (August/September).

- Dierenfeld E.S., Pinis M.T. & Sheppard C.D. (1994). Hemosiderosis and dietary iron in birds. *Journal of Nutrition*, 124, 26855-26866.
- Dieumou F., Adegbola T & Doma U. (2013). Growth Performance, carcass characteristics and economics of production of broilers fed diets with two sources of protein and two levels of wheat offal. *Journal of Cereals and Oilseeds*, Vol. 4(4), pp. 42-47.
- Firman J. (1991). Nutrient requirements of Chickens and Turkeys. *Nutrient Requirements of Poultry*, eighth revised edition, 1984, National Academy Press, 2101 Constitution Ave., N.W. Washington, D.C. 20418.
- Eissler C. & Firman J. (1996). Effects of feather meal on the performance of Turkeys. *Journal of Applied Poultry Research*, volume 5, issue 3, pp 246-253.
- Entezari S., Haghi S.M. Norouzkhani N., Sahebznazar B., Vosoughian F., Akbarzadeh D., Islampanah M., Naghsh N., Abbasalizadeh M. and Deravi N. (2022). Iron Chelators in Treatment of Iron Overload. *Journal of Toxicology*. Volume 2022, 4911205.
- Fröh, R. (2009). Schafe auf dem Grünland gesund und leistungsfähig halten- Mineralstoffversorgung Thüringer Landesanstalt für Landwirtschaft, Jena http://www.tll.de/www/daten/nutztierhaltung/schafe_ziegen/shgn0110.pdf.
- Gabriel F, Suen V., Marchini J. & Dutra-de-Oliveira J.E. (2012). Short bowel syndrome and anemia: Evidence beyond iron deficiency. *Iron Deficiency and its Complications*. 131-137.
- [REDACTED]
- [REDACTED]
- Greig-Smith P.W. & Rowney C.M. (1987). Effects of colour on the aversions of starlings and house sparrows to five chemical repellents. *Crop Protection* 6: 402–409.
- Greig-Smith P.W. (1989). The boxworth project - environmental effects of cereal pesticides. *Journal of RASE*, vol 150, pp 171-187.
- Gurney J.E., Perrett J., Crocker D.R & Pascual, J.A (1998). CONTRACT PN0910/PN0919 MILESTONE REPORT Mammals and farming: information for risk assessment. CSL Project No. M37.
- [REDACTED]
- [REDACTED]
- Harris, S. and Yalden, D.W. (2008). *Mammals of the British isles*. 4th edition. The Mammal Society, Southampton, UK.
- [REDACTED]
- [REDACTED]
- [REDACTED]
- Johnson I.P., Flowerdew J.R. and Hare R. (1991). Effects of broadcasting and of drilling methiocarb molluscicide pellets on field populations of wood mice, *Apodemus sylvaticus*. *Bull. Environ. Contam.*, vol 46, pp 84-91.

- [REDACTED]
- [REDACTED]
- Kohler R., Kariuki L., Lambert C. & Biesalski H.K. (2019). Protein, amino acid and mineral composition of some edible insects from Thailand. *Journal of Asia-Pacific Entomology* Volume 22, Issue 1, March 2019, Pages 372-378
- Kozłowski J., Jaskulska M. & Kozłowska M. (2014). Evaluation of the effectiveness of iron phosphate and the parasitic nematode *Phasmarhabditis hermaphrodita* in reducing plant damage caused by the slug *Arion vulgaris* moquin-andon. *Folia Malacologica*, 22(4) pp 293-300.
- Lewis S.M., Ullrey D.E., Barnard D.E. & Knapka J.J.(2006). Chapter 9 – Nutrition. In ‘*The Laboratory Rat*’ by Suckow M.A. (2006). Elsevier Science & Technology.
- [REDACTED]
- [REDACTED]
- McDonald D. (2006). Nutritional Considerations - Section I: Nutrition and Dietary Supplementation”, *Clinical Avian Medicine*. Available at: <https://www.ivis.org/library/clinical-avian-medicine/nutritional-considerations-section-i-nutrition-and-dietary> (Accessed: 11 March 2022).
- Mete A., Hendriks H.G., Klaren P.H.M., Dorrestein G.M. van Dijk J.E. & Marx J.M. (2003). Iron metabolism in mynah birds (*Gracula religiosa*) resembles human hereditary haemochromatosis, *Avian Pathology*, 32:6, 625-632.
- [REDACTED]
- [REDACTED]
- [REDACTED]
- [REDACTED]
- Moore D., Baker K., Thompson K., Blair E. & Firman J. (2003). Digestible Sulfur Amino Acid Requirement of Male Turkeys During the 12 to 18 week Period. *International Journal of Poultry Science*, volume 2, issue 1, pp 38-43.
- Mwangi M.N., Oonincx D.G.A.B., Stouten T., Veenenbos M., Melse-Boonstra A., Dicke, M. & van Loon J.J.A. (2018). Insects as sources of iron and zinc in human nutrition. *Nutr Res Rev*. 31, 248-255
- Niethammer, J. (1978). *Apodemus sylvaticus* (Linnaeus, 1758) - Waldmaus. In: Niethammer, J. and Krapp, F. (eds.) *Handbuch der Säugetiere Europas*, Vol. 1 Rodentia. Akademische Verlagsgesellschaft, Wiesbaden, Germany, p. 337-358.
- Noorjahan A., Amrita B. & Kavita S. (2014). In vivo evaluation of taste masking for developed chewable and orodispersible tablets in humans and rats. *Pharm Dev Technol*, 2014; 19(3): 290–295.

- Ojha S., Bekhit A.E.D., Grune T. & Schluter O.K. (2021). Bioavailability of nutrients from edible insects. *Current Opinion in Food Science* Volume 41, October 2021, Pages 240-248.
- [REDACTED] (2008). FINAL REPORT: SLUG & SNAIL KILLER acute oral toxicity test with Japanese quail (*Coturnix coturnix japonica*). Study code: G/53/08. [REDACTED]
- Papanikolaou G. and Pantopoulos K. (2005). Iron metabolism and toxicity. *Toxicology and Applied Pharmacology* 202:199-211.
- Pavone S., Salamisa S., Pecorelli I., Rossi E. & Manuali E. (2014). Deadly Outbreak of Iron Storage Disease (ISD) in Italian Birds of the Family Turdidae. *J. Vet. Med. Sci.* 76(9): 1209–1212.
- Ponka, P., Tenenbein M. and Eaton J.W. (2007). 'Chapter 30. Iron', in *Handbook on the Toxicology of Metals* (Third Edition), ed. by Gunnar F. Nordberg, Bruce A. Fowler, Monica Nordberg and Lars T. Friberg (Burlington: Academic Press, 2007), pp. 577-98.
- Prosser, P. (1999). Potential exposure of birds to treated seed. Project PN0907 Final milestone report, CSL, UK.
- Rodriguez-Matas M.C., Campos M.S., Lopez-Aliaga I., Gomez-Ayala A.E., and Lisbona F. (1998). Iron-manganese interactions in the evolution of iron deficiency. *Ann. Nutr. Metab.* 42, 96–109.
- Schabacker J. & von Blanckenhagen F. (2023a). Amendment to the Final Report - Final Bite (a.s. elemental iron): Literature review regarding iron uptake and regulation in birds and mammals. Report number: R2260023. RIFCON GmbH.
- Schabacker J. & von Blanckenhagen F. (2023b). Final Report - Final Bite (a.s. elemental iron): Expert statement. Report number: R2324001-03. RIFCON GmbH.
- Schabacker J. & von Blanckenhagen F. (2023c). Final Report - Final Bite (a.s. elemental iron). Expert statement - Summary of arguments and example calculation for the long-term risk to birds and mammals. Report number: R2324059. RIFCON GmbH.
- Schümann K., Ettle, T., Szegner, B., Elsenhans, B. and Solomons, N. W. (2007). On risks and benefits of iron supplementation recommendations for iron intake revisited. *Journal of Trace Elements in Medicine and Biology*, 21(3), 147-168.
- Stephan, E. 2010. Zur Eisenversorgung ausgewachsener Pferde, *Mecklenburger Pferde* 2/2010, 50-51 <https://www.hippothek.de/pdf/1302013099.0689.pdf>.
- Wadsworth P.F., Jones D.M. and Pugsley S.L. (1983). Hepatic haemosiderosis in birds at the Zoological Society of London. *Avian Pathology*, 12: 321-330.
- Ward R.J., Iancu T.C., Henderson G.M., Kirikwood J.R. & Peters T.J. (1988). Hepatic iron overload in birds: Analytical and morphological studies. *Avian Pathology*, 17, 451-464.
- Ward R.J., Smith T., Henderson G.M. & Peters T.J. (1991). Investigation of the aetiology of haemosiderosis in the starling (*Sturnus vulgaris*). *Avian Pathology*, 20, 225-232.
- Wallace D. F. (2016). The Regulation of Iron Absorption and Homeostasis. *Clin Biochem Rev* 37 (2).

- Whaley C. (2022). Granular iron based molluscicides and observations relating to the behaviour of slugs and snails. i2L Research Ltd (unpublished report).
- Whittaker P., Hines F.A., Robl M.G., Dunkel V.C. (1996). Histopathological evaluation of liver, pancreas, spleen, and heart from iron-overloaded Sprague-Dawley rats. *Toxicol Pathol.* Sep-Oct;24(5):558-63. doi: 10.1177/019262339602400504. PMID: 8923676.
- Whittaker P., Ali S.F., Imam S.Z., Dunkel V.C. (2002). Acute toxicity of carbonyl iron and sodium iron EDTA compared with ferrous sulfate in young rats. *Regul Toxicol Pharmacol.* Dec;36(3):280-6. doi: 10.1006/rtph.2002.1577. PMID: 12473412.
- Zielinska E., Baraniak B., Kara M., Rybczynska K. & Jakubczyk A. (2015). Selected species of edible insects as a source of nutrient composition. *Food Research International* Volume 77, Part 3, November 2015, Pages 460-466.
- Zhu, Q., Qian, Y., Yang, Y., Wu, W., Xie, J. and Wei, D. (2016). Effects of carbonyl iron powder on iron deficiency anemia and its subchronic toxicity. *Journal of Food and Drug Analysis* 24, 746-753.

Appendix 4

Position Papers submitted by the applicant in support of the Earthworm Risk assessment following discussion with HSE in December 2021.

HSE comments

The following report was produced on behalf of ADAMA by Dr K. Brown (2021) in response to CRD's initial assessment for the risk to earthworms. The report below is a copy of the original position paper and has not been altered or amended by CRD. The report is evaluated and discussed in the earthworm risk assessment (section B.9.8). Copies of the papers referenced by the report are available on request.

Position Paper on the UK response to: Axmann (2019): Final Bite - Blue – A Field Study to Investigate Effects on the Earthworm Fauna in Central Europe

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This earthworm field study conducted in Germany between 2018 and 2019 appears to have been well conducted in compliance with the relevant Guidelines (ISO, 2006 and ISO, 2014). All validity criteria were met with high enough earthworm abundance, the presence of key earthworm species of different ecological types and the homogeneity in abundance and species distribution at the field site. The effects observed of the reference item treatment were >50 % reduction in abundance and biomass for at least one sampling occasion and indicated the sensitivity of the earthworm population.

Due to their surface feeding activity the anecic species *L. terrestris* and *A. longa* are most likely to be exposed to Final Bite pellets (containing 10 g/kg of the active substance elemental iron formulated as ready-to-use bait (RB)) on the surface of the soil. The results for anecic worms from the Axmann (2019) study show a consistent trend of both abundance and biomass being similar to, or greater than, the control in the Final Bite treatment. The reference item shows clear and prolonged reduction in abundance and biomass of anecic worms throughout the entire post-treatment period. It is hard to see how these results give rise to a concern over possible adverse effects of Final Bite on anecic earthworms.

Dry weather conditions are cited as a major cause for concern over the reliability of the results for anecic species to represent the risk in the UK but no data or further evidence is provided to support this statement. How little rainfall and irrigation would be too dry?

There are two different but equally relevant aspects to this concern;

- a) Did the rainfall and irrigation that took place in the study result in the pellets degrading and being available to surface active earthworms in a realistic and relevant manner for the UK?

b) Were anecic earthworms sufficiently active in the post-treatment period to have been exposed to the pellets and their degraded remains in a realistic and relevant manner for the UK?

Clearly the UK experiences a very wide range of weather conditions depending on location, with higher rainfall generally occurring in the West. It would not be possible or practical to conduct an earthworm field study to match every rainfall scenario.

In the Axmann (2019) field study, treatments were applied on six occasions between 24 April and 19 May 2018. Therefore, a comparison of the study conditions and historical weather data over the months of April, May, June, July and August would appear to be most relevant in considering the concerns over excessively dry conditions in the study and the potential for non-exposure.

The UK Met Office holds publicly available records for monthly rainfall at its fixed weather stations going back as far as 1853 (see www.metoffice.gov.uk). In order to demonstrate whether the monthly rainfall plus irrigation that occurred in the field study was outside the range of what would typically occur in the UK a comparison was made of the monthly rainfall over the months of April, May, June, July and August.

Considering East Anglia as a major UK agricultural area the long-term rainfall data from the NIAB station at Cambridge for the months April-August was tabulated and the monthly average over the 41 years 1980-2020 and over the 20 years (2001-2020) was calculated. These are summarised in Table 1 below together with the rainfall plus irrigation that occurred in the Axmann (2019) field study.

Considering the question (a) above as to whether the rainfall in the field study would have caused the pellets to have been available to anecic earthworms, the natural rainfall during May at the study site (45.3 mm) was slightly higher than 41 year average for Cambridge. The addition of irrigation in May to give a total of 120 mm, almost 3 times the Cambridge average, means that the precipitation during the application period and the two weeks after it should be considered to be relevant for the UK conditions as found in Cambridge. Additionally, the soil moisture recorded in May 2018 was 32.1% at 5 cm depth and 29.5% at 20 cm depth. This would mean that

the pellets can be considered to have been degraded in an appropriate manner and that the earthworms would have been active at the time of application weeks immediately after it.

Table 1: Monthly long-term average rainfall at Cambridge UK and recorded rainfall +irrigation in the earthworm field study of Axmann, (2019)

Month	41 year monthly average rainfall (mm)	20 year monthly average rainfall (mm)	Rainfall at the study site (mm)	Irrigation at the study site (mm)	Rainfall + irrigation at the study site (mm)
April	39.3	31.6	10.7	22.5	33.2
May	43.6	46.8	74.8	45.3	120.0
June	50.4	42.5	22.0	0	22.0
July	49.0	54.0	55.0	24.3	79.3
August	53.8	61.4	80.8	13.0	93.8
Total April - August	236.0	236.3	243.3	105.1	348.3

The total rainfall (243.3 mm) occurring at the study site over the period April-August was slightly higher than the long-term average for Cambridge. The addition of irrigation (to give a total of 348.3 mm) makes the total of natural and simulated rainfall representative for parts of the UK with wetter conditions than Cambridge.

It would require a more detailed analysis to determine exactly where on the distribution of British wetness the conditions in the Axmann (2019) earthworm field study lie but they can certainly be considered be applicable to UK agricultural conditions.

The first post-treatment earthworm sampling occurred between 28 May and 1 June, after a period of above average rainfall supplemented by irrigation and two weeks after the last of the six application events. Mean numbers of the two anecic species

A. longa and *L. terrestris*, are summarised in Table 2. At this time point good numbers (mean of 13.3/m²) of *A. longa* were collected in samples from the control

plots and mean numbers of *A. longa* in Final Bite treated post were slightly lower (mean of 9.8/m²) compared to 2.3/m² in the reference item treated plots. There is no indication of any reduction in *A. longa* numbers due to the Final Bite treatment. The activity and abundance of *L. terrestris* and *A. longa* at the first post-treatment sampling addresses question (b) above and confirms that there would have been relevant and realistic exposure of anecic earthworms to Final Bite.

Table 2: Mean number (n/m²) for anecic earthworm species, standard deviation and percentage change of species earthworm abundance in the test item treatment (T) and toxic reference treatment (R) relative to the control (C)

Species / group	Timing	Mean number (n/m ²) ± standard deviation and deviation from control (%) ^a		
		C (untreated)	Final Bite – Blue (T)	Carbomax (R)
<i>Aporrectodea longa</i>	7 DBA1	7.5±1.0	8.8±4.6 (16.7)	6.8±1.7 (-10.0)
	34 DAA1	13.3±5.7	9.8±4.7 (-26.4)	2.3±2.1 * (-83.0)
	56 DAA1	7.5±2.6	9.5±4.8 (26.7)	1.0±1.2 * (-86.7)
	204 DAA1	13.8±4.6	21.0±5.8 (52.7)	2.0±2.2 * (-85.5)
	366 DAA1	3.3±1.9	3.8±2.1 (15.4)	0.0±0.0 * (-100.0)
<i>Lumbricus terrestris</i>	7 DBA1	13.3±2.6	12.3±4.7 (-7.5)	11.8±3.5 (-11.3)
	34 DAA1	6.5±4.5	8.3±5.1 (26.9)	2.0±2.2 (-69.2)
	56 DAA1	1.0±0.8	3.5±1.7 (250.0)	0.0±0.0 (-100.0)
	204 DAA1	8.0±3.7	12.0±4.1 (50.0)	1.5±0.6 * (-81.3)
	366 DAA1	7.0±2.2	5.8±3.3 (-17.9)	2.0±1.4 * (-71.4)

a) negative values (-) indicate lower earthworm numbers compared to the control; positive values (+) indicate higher earthworm numbers compared to the control

DBA1= days before application 1, DAA1= days after application 1

*significantly different from control ($p \leq 0.05$)

June 2018 was clearly a very dry month at the study site and the monthly rainfall of 22 mm was much lower than the Cambridge long-term average but not unusual, being similar to June rainfall in 2015 (19 mm), 2013 (14.2 mm) and 2006 (18.9 mm). With hindsight additional irrigation in June would have been helpful although the soil

moisture remained at 28.3% at 5 cm depth and 28.0% at 20 cm depth at the study site.

The second post-treatment earthworm sampling took place 19-26 June, 2018, (56 DAA1) and it appears that *L. terrestris* had entered diapause by this stage, being caught in very low numbers (mean of 1.0 in the control and 3.5 individuals /m² in the test treated item plots respectively). It is accepted that there is almost no statistical power in the data for *L. terrestris* at this time point but with mean numbers being slightly higher than the control compared to zero in the reference item, there is also no indication of any adverse effects due to Final Bite.

However, at this time point the second anecic species, *A. longa*, was found in adequate numbers with a mean of 7.5/m² in the control and 9.5/m² in the Final Bite treated plots. These larger anecic species are usually found in much lower numbers than the smaller species such as *A. caliginosa* and these actually represent good data for a mid-summer sampling. Sampling the anecic species alongside other earthworms often causes problems as the digging procedure and prolonged activity on the surface results in the anecic worms retreating deep into their burrows. Since this field study has one more post-treatment sampling occasion than required by the ISO 11268-3 guideline, the relative insensitivity of the June 2018 sampling (56 DAA1) is compensated for by the fact that there are three further post treatment sampling dates.

The November 2018 sampling (204 DAA1) is perhaps the most important in this field study since higher numbers of anecic worms were collected and longer-term effects would have had a chance to develop if they were going to do so. Both *L. terrestris* and *A. longa* had higher mean numbers than in the control at this time point and again there is no indication any harmful effects on anecic worms due to the Final Bite treatment.

Although not recommended for the interpretation of earthworm field studies the MDD analysis from 204 DAA1 for anecic earthworms shows a 26% difference could be detected in the data from 204 DAA1. MDD values for the two anecic species at each sampling point are presented in Table 3. Where the MDD values for anecic worms were above 50% (34 and 56 DAA1) they were only marginally so, 53% and

52% showing that the study did have a reasonable ability to detect effects on anecic earthworms. In addition, there were no signs or trends indicating any reduction in abundance or biomass relative to the control at any time point for either of the anecic species.

Table 3: Minimum Detectable Difference values (%) for earthworm abundance

	% MDD based on abundance				
Taxon	7 DBA1	34 DAA1	56 DAA1	204 DAA1	366 DAA1
<i>Aporrectodea longa</i>	59 (III)	51 (III)	66 (III)	50 (IV)	77 (II)
<i>Lumbricus terrestris</i>	43 (IV)	98 (I)	170 (0)	62 (III)	53 (III)
Anecic species combined	46 (IV)	53 (III)	52 (III)	26 (IV)	49 (IV)

Classification of MDD values according to EFSA PPR PANEL (2013): 0: >100%, I: >90-100%, II: >70-90%, III: >50-70%, IV: ≤50%

From a regulatory decision-making perspective, the November 2018 results for anecic species should be considered as being sufficient to confirm an absence of harmful effects following six applications of Final Bite. The exposure pattern of the field study represented an extreme worst-case with the full application rate, the lowest time interval between treatments and the maximum number of applications.

The results from the final sampling occasion, one year after the first treatment, confirm that there was no difference in abundance or biomass between the control and the Final Bite treatment with an MDD of 53% for adult *L. terrestris* and 49% for adult anecic worms combined. When juvenile anecic worms are included together with the adults, as shown in Figures 25 and 26 from the study report (reproduced below) there is a clear and consistent lack of effects of Final Bite on either abundance or biomass on consecutive post-treatment sampling dates. The trends over time for both abundance and biomass show numbers and weights of anecic earthworms were either very similar to the control or higher than the control at every sampling occasion in the study.

Since that the rainfall and irrigation that occurred in the study was sufficient to confirm realistic exposure, these results demonstrate that Final Bite was not harmful to the key anecic earthworm species *L. terrestris* or *A. longa*.

The author has previously conducted earthworm field studies in the relatively high rainfall area of South West England over a period of approximately 15 years. The numbers of *L. terrestris* in the Axmann (2019) study are broadly similar to those that would occur in a UK field study in a model grassland system. Numbers of *A. longa* collected in the Axmann (2019) study are higher than would typically be found in UK grass or arable systems.

Whilst a granular reference item would indeed have provided reassurance of the breakdown of the granules it is not a requirement of the guideline that the test item formulation type matches that of the reference item. The response of the reference item is in line with what would be expected following application of carbendazim and demonstrates that the study is capable of detecting effects on earthworms.

Conclusion:

The earthworm field study of Axmann (2019) was conducted in accordance with the current test guidelines and is considered to be valid. Rainfall, supplemented by irrigation, resulted in adequate exposure of earthworms to Final Bite pellets. The reference item showed expected effects across all taxa, confirming that the study would have been able to detect negative effects of the test item had they occurred. There was no indication on any of the four post-treatment sampling occasions that Final Bite led to harmful effects on anecic earthworms. Both numbers and biomass of anecic earthworms in Final Bite treated plots were similar to or higher than those in the control on each sampling occasion.

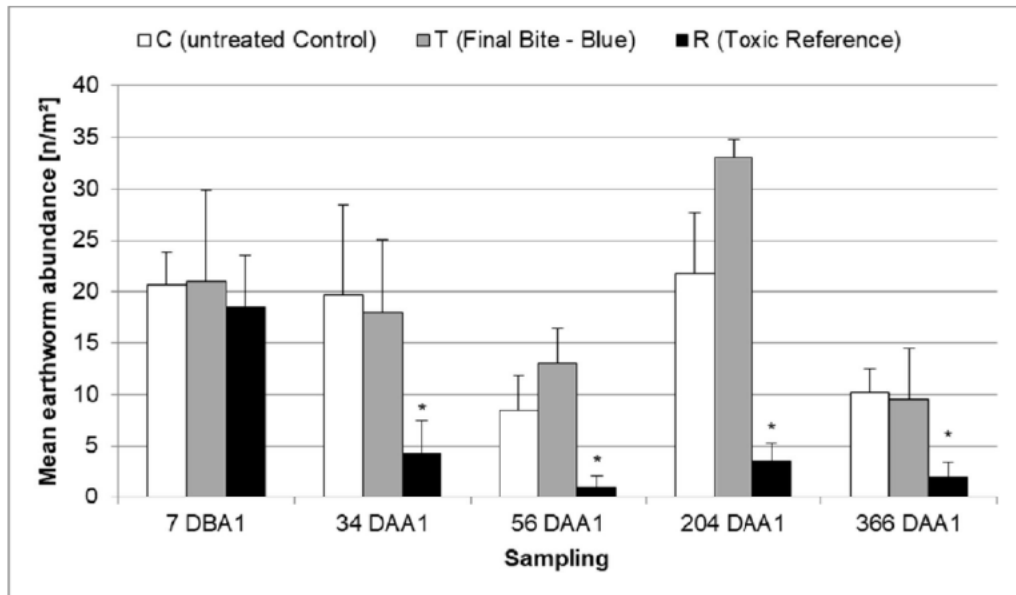


Figure 1: Mean numbers of anecic earthworms

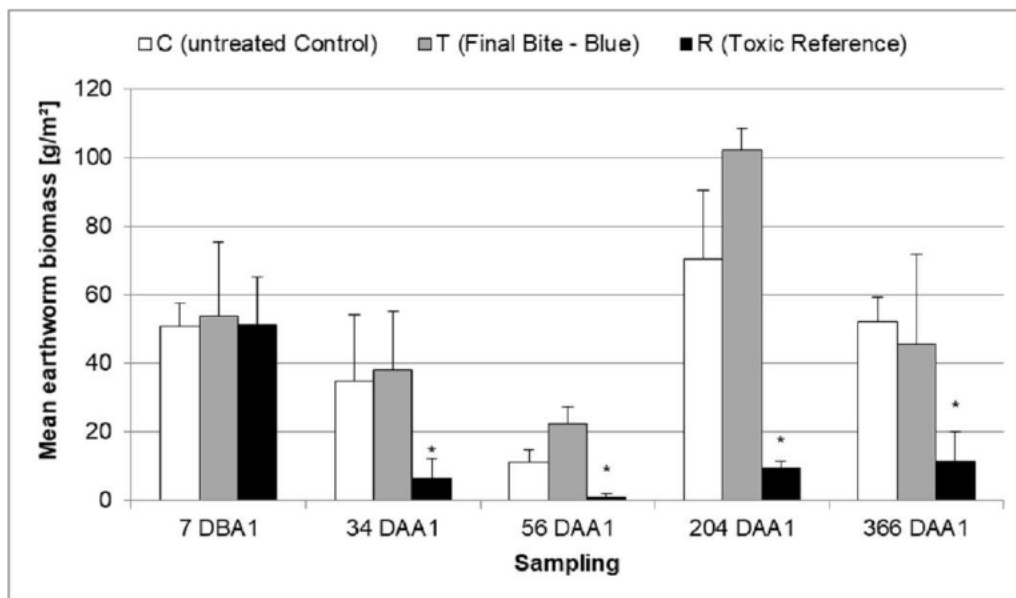


Figure 2: Mean biomass of anecic earthworms

Appendix 1: Long-term rainfall data for April to August from Cambridge, U.K. Source:

www.metoffice.gov.uk

Month	Year	Rainfall (mm)		Month	Year	Rainfall (mm)
April	1980	17.9		April	1985	31.2
May	1980	7.6		May	1985	49.0
June	1980	77.2		June	1985	107.0
July	1980	55.9		July	1985	38.5
August	1980	56.4		August	1985	50.9
April	1981	56.0		April	1986	63.6
May	1981	58.2		May	1986	74.7
June	1981	14.2		June	1986	13.2
July	1981	73.5		July	1986	43.3
August	1981	27.3		August	1986	70.9
April	1982	10.0		April	1987	42.9
May	1982	51.1		May	1987	46.9
June	1982	107.4		June	1987	93.5
July	1982	53.1		July	1987	70.2
August	1982	72.3		August	1987	71.9
April	1983	86.2		April	1988	37.2
May	1983	101.8		May	1988	38.7
June	1983	33.3		June	1988	48.1
July	1983	48.6		July	1988	94.6
August	1983	10.5		August	1988	46.8
April	1984	13.6		April	1989	75.8
May	1984	76.1		May	1989	6.1
June	1984	48.9		June	1989	40.5
July	1984	15.0		July	1989	43.0
August	1984	60.9		August	1989	28.8

Appendix 1: Long-term rainfall data for Cambridge, U.K. continued

Month	Year	Rainfall (mm)
April	1990	28.2
May	1990	6.2
June	1990	30.1
July	1990	16.9
August	1990	21.1
April	1991	42.9
May	1991	13.9
June	1991	97.6
July	1991	32.1
August	1991	41.2
April	1992	40.7
May	1992	34.3
June	1992	35.8
July	1992	54.4
August	1992	72.4
April	1993	78.4
May	1993	47.9
June	1993	86.0
July	1993	54.4
August	1993	37.5
April	1994	67.3
May	1994	44.1
June	1994	20.0
July	1994	23.0
August	1994	36.4

Month	Year	Rainfall (mm)
April	1995	10.2
May	1995	24.4
June	1995	17.3
July	1995	27.1
August	1995	5.3
April	1996	4.5
May	1996	19.8
June	1996	12.4
July	1996	40.5
August	1996	63.9
April	1997	12.5
May	1997	36.8
June	1997	151
July	1997	25.9
August	1997	60.5
April	1998	120.4
May	1998	6.4
June	1998	101.9
July	1998	25.7
August	1998	16.0
April	1999	33.0
May	1999	49.8
June	1999	89.0
July	1999	24.5
August	1999	92.5

Appendix 1: Long-term rainfall data for Cambridge, U.K. continued

Month	Year	Rainfall (mm)
April	2000	85.9
May	2000	83.8
June	2000	17.5
July	2000	60.7
August	2000	21.1
April	2001	62.6
May	2001	17.5
June	2001	22.8
July	2001	55.1
August	2001	65.8
April	2002	33.4
May	2002	53.5
June	2002	28.5
July	2002	94.6
August	2002	42.0
April	2003	24.2
May	2003	39.9
June	2003	60.7
July	2003	66.8
August	2003	2.0
April	2004	41.4
May	2004	44.5
June	2004	34.0
July	2004	59.3
August	2004	70.7

Month	Year	Rainfall (mm)
April	2005	27.7
May	2005	47.4
June	2005	47.1
July	2005	43.7
August	2005	53.3
April	2006	30.1
May	2006	62.8
June	2006	18.9
July	2006	45.1
August	2006	74.9
April	2007	1.0
May	2007	124.3
June	2007	59.0
July	2007	62.1
August	2007	51.1
April	2008	50.8
May	2008	62.9
June	2008	34.6
July	2008	52.1
August	2008	64.7
April	2009	12.6
May	2009	28.4
June	2009	40.8
July	2009	71.0
August	2009	58.6

Appendix 1: Long-term rainfall data for Cambridge, U.K. continued

Month	Year	Rainfall (mm)
April	2010	12.5
May	2010	28.6
June	2010	25.4
July	2010	10.8
August	2010	133.2
April	2011	2.0
May	2011	12.8
June	2011	53.0
July	2011	38.4
August	2011	40.2
April	2012	95.6
May	2012	42.6
June	2012	91.4
July	2012	101.4
August	2012	39.2
April	2013	26.4
May	2013	52.0
June	2013	14.2
July	2013	32.8
August	2013	48.2
April	2014	13.8
May	2014	84.6
June	2014	44.4
July	2014	49.2
August	2014	126.8

Month	Year	Rainfall (mm)
April	2015	20.2
May	2015	42.2
June	2015	19.0
July	2015	78.8
August	2015	47.4
April	2016	58.8
May	2016	39.4
June	2016	66.4
July	2016	18.2
August	2016	44.0
April	2017	12.8
May	2017	64.8
June	2017	59.0
July	2017	94.8
August	2017	64.2
April	2018	64.6
May	2018	43.8
June	2018	0.8
July	2018	12.4
August	2018	62.8
April	2019	10.8
May	2019	41.4
June	2019	79.2
July	2019	43.4
August	2019	35.8

Appendix 1: Long-term rainfall data for Cambridge, U.K. continued

Month	Year	Rainfall (mm)
April	2020	29.8
May	2020	1.6
June	2020	51.0
July	2020	50.6
August	2020	104

41 year average	(mm)
April	39.3
May	43.6
June	50.4
July	49.0
August	53.8
Total	236.0

20 year average	(mm)
April	31.6
May	46.8
June	42.5
July	54.0
August	61.4
Total	236.3

Literature review

Systematic Literature Search with respect to specific aspects of earthworm ecology and ecotoxicity of slug pellets in the UK. Meller, M. 2022.

Guidelines: EFSA Guidance, 2011; 9 (2): 2092.

Summary: Three sources of scientific peer-reviewed literature (bibliographic databases) were searched for publications with no time restrictions applied to the date span of the search. After removal of duplicates, a total of 274 publications were found. The found publications cover a time span from 1964 to 2022.

Methodology

Search terms:

In order to meet the review targets, the following three questions were proposed by the sponsors of the search:

1) *What are typical numbers of *L. terrestris* and *A. longa* in agricultural fields in the UK?*

a) abundance AND *Lumbricus terrestris* OR *Aporrectodea longa* AND UK OR England

b) abundance OR distribution AND anecic earthworms AND UK OR England

2) *What are optimum conditions for *L. terrestris* with specific respect to soil moisture and temperature?*

a) *Lumbricus terrestris* OR *Aporrectodea longa* AND soil moisture OR soil temperature

b) *Lumbricus terrestris* OR *Aporrectodea longa* AND optimum soil conditions

3) *Do earthworms feed on granules? Effects of granules on earthworms?*

a) earthworms AND granular application OR granular products

b) earthworms AND slug baits OR slug pellets

Databases searched: Web of Science (interdisciplinary citation database); PubMed (biomedical science and toxicology citation database); Science direct (interdisciplinary citation database). No other sources were used. The search terms quoted above were modified slightly to account for the differences in each database search engine and added to in order to optimise the search.

Time window of Search: No restrictions applied

Results

Results in terms of returned publications are summarised in the table below copied from the full literature review report.

Table 1: Reporting of the search process for scientific peer-reviewed open literature in bibliographic databases

	ID	Search question	Details of the searches			
Database:			Web of Science Core Collection	PubMed	ScienceDirect	Total
Justification for choosing the source:			please refer to section 3.2.1.1	please refer to section 3.2.1.2	please refer to section 3.2.1.3	
Date of the search:			21.02.2022	21.02.2022	21-22.02.2022	
Date span of the search:			unlimited	unlimited	unlimited	
Date of the latest database update included in the search:			19.02.2022	18.02.2022	Continuously updated	
Search strategies used for this data requirement:			please refer to section 3.3.2.1	please refer to section 3.3.2.2	please refer to section 3.3.2.3	
Number of records retrieved:	01	abundance of <i>L. terrestris</i> + <i>A. longa</i>	12	9	8 ⁽¹⁾	
	02	abundance of anecic earthworms	8	4	2	
	03	soil moisture/temperature	148	8	48	
	04	optimum soil conditions	6	2	9	
	05	granular application/products	58	31	20	
	06	slug baits/pellets	9	4	3	
		Total number	241	58	90	389
		Total number after removing duplicates (n=)				274

⁽¹⁾ In order to cope with the ScienceDirect database restriction of max. 8 Boolean operators, the search term combination for ScienceDirect were divided in 2 parts and the search was performed with individual queries. Retrieved records for the individual queries were pooled and duplicates were removed.

Sifting

The 274 returned publications were assessed based on their abstracts for relevance to each of the three questions posed by the sponsor. Those considered relevant based on the abstract were ordered and reviewed in full for reliability and relevance to the questions. Of the 274 from the initial search, 22 (plus an additional 2) were considered relevant and reliable for question 1, 39 were considered so for question 2 and 8 were considered so for question 3, a total of 71 publications.

These were discussed further in Brown, 2022, the report accompanying this methodology document.

HSE comments

The methodology of the literature review is in line with EFSA (2011) and the conclusions of the CRD/ADAMA meeting in December 2021, in particular by not imposing a time window restriction (EFSA 2011 standard literature searches are normally limited to the ten years prior to submission). It is noted that while the returned publications cover a timespan of 1964-2022, the majority seem to have been published within the last 20 years. It is noted that none of the search terms were iron-specific. The literature review was produced following points of concern raised by CRD with the field study Axmann (2019) viz:

- 1) Low abundance of anecic species considered most likely to be exposed to test item - resulting in low sensitivity of test to effects of test item.
- 2) Can the results of the spray reference item used be relied upon to demonstrate test sensitivity to effects of granular test item?
- 3) Representativeness of climatic conditions during period of application in study (spring/summer) versus those typical during autumnal application of slug pellets in UK – potentially greater bioavailability of test item, greater earthworm abundance and activity in this later period.

The questions addressed by this review are:

1) What are typical numbers of *L. terrestris* and *A. longa* in the agricultural fields of the UK? – This could address the first point of concern, the aim presumably being that while numbers of anecic earthworms are low in the study, they are representative of agricultural populations in the UK and so are unlikely to be improved upon. While this could help make the case that the field study situation is realistic, there is still the question over whether the population abundance would allow treatment effects to be detected statistically.

2) What are optimum conditions for *L. terrestris* with specific respect to soil moisture and temperature? – This appears to be directed at addressing the third point of concern. Potentially this helps making the argument about extrapolating the study between different times of year but only partially, since the bioavailability point and how climate could affect this remains.

3) Do earthworms feed on granules? Do granules have effects on earthworms? - This is potentially the most significant as it might demonstrate that anecic worms would have been exposed to the test item, which then gives more reassurance referring to the reference item results to show that effects of the test item could have been detected (if there were effects). This would not fully address the concerns over study sensitivity, since the effect of the reference item is large and the MDDs for *L. terrestris* in Axmann (2019) are high, meaning small to medium effects could be missed by the statistical analysis.

Further comment on these areas is discussed in the risk assessment for earthworms, following the literature review report by Brown (2022 ; below).

HSE comments

The following report was produced on behalf of ADAMA by Dr K. Brown (2022) based on the literature search evaluated above (Meller, 2022). The report below is a copy of the original position paper and has not been altered or amended by CRD. The report is evaluated and discussed in the earthworm risk assessment (section B.9.8). Copies of the papers referenced by the report are available on request.

A literature review to address the concerns of UK CRD concerning the risk to earthworms in the UK from slug pellets containing elemental iron.

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Reference No.: 000110096

Date of issue: 30 March 2022

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1. INTRODUCTION AND THE LITERATURE REVIEW PROCEDURE

Following discussions with CRD concerning the acceptability of the earthworm field study by Axmann (2019) it was agreed that a literature search would be conducted to provide peer reviewed and scientifically accepted information to support the arguments made in support of the study.

The detailed search was conducted by Ecotox Consult using terms agreed with the sponsor, the consultant and CRD. The report of the literature search itself which detailed the data bases searched and the search terms used is provided in author (year) as a stand-alone document (Meller, 2022).

Search terms were intended to address the three questions that required additional information.

1. What are optimal soil conditions for the earthworm species *Lumbricus terrestris* and *Aporrectodea longa*?
2. Is the abundance of anecic worms seen in the Axmann (2019) field study comparable with UK population levels?
3. Do foraging anecic earthworms encounter and consume slug pellets and are they affected by iron containing slug pellets?

The search output was provided in the form of an Excel file with a short abstract provided for each hit. These were read and it was decided whether they were relevant and reliable for use in this review. All selected papers were then ordered electronically and each one was read in full and scored according to reliability and relevance for each of the three questions.

The key information from each paper was included in the review and very often it was thought relevant to copy tables and graphs directly for ease of understanding.

As part of the response to the question on suitable abundance of anecic species in a field study, numerous earthworm field studies owned by ADAMA were reviewed. Unfortunately, only one of these was conducted in the UK and that was performed about 20 years ago. The control data from each sampling timepoint in each of the studies that contained either *L. terrestris* or *A. longa* were tabulated and are included in this review. This is intended to demonstrate the range of levels of abundance found with anecic earthworms across 15 field studies as well as the seasonality of anecic worms.

The climatic conditions recorded at the site in the Axmann (2019) study (maximum, minimum and mean daily soil temperature at 5 cm and 20 cm depths, soil moisture and air temperature) were plotted so as to allow a comparison with the findings of the review regarding optimal and limiting conditions for *L. terrestris* and *A. longa*.

2. WHAT ARE OPTIMAL SOIL CONDITIONS FOR THE EARTHWORMS *LUMBRICUS TERRESTRIS* AND *APORRECTODEA LONGA*?

2.1 SUMMARY

A review of the abstracts generated by the literature search revealed 39 publications with the potential to provide information on the preferred soil conditions for *L. terrestris* and *A. longa*. These ranged from laboratory studies at different selected temperatures to mesocosms where worms were held at a single temperature, deemed to be acceptable. A small number of field studies with measured soil conditions were also present in the findings. A review of the full publications found that 22 of these papers described the reaction of at least one of these two anecic earthworm species to changes in soil conditions. More papers considered *L. terrestris* than *A. longa* and the main variables they researched were soil temperature and soil moisture content. The driving force for much of this research was the desire to culture these species for release in soil remediation programmes.

One paper aimed at modelling (Moreau Valancogne *et al.*, 2013) reviewed the available published research describing the effects of soil conditions on earthworms. Whilst most of these papers were included in the literature search findings, the few that were not were subsequently added to the search and reviewed. Together with the review by Lowe and Butt (2005) this provided the most relevant and comprehensive information, although mostly derived from laboratory experiments. Most of the published research with anecic worms involved soil microcosms and the most commonly used conditions for both *L. terrestris* and *A. longa* were 15 °C and 30 % soil moisture. The most commonly measured variables to assess the suitability of soil conditions were individual body weight, development time from juvenile to adult and reproductive success, both as the number of cocoons produced and their viability.

In summary, the optimum soil moisture content for culturing anecic earthworm species was 25-30%, both *L. terrestris* and *A. longa* prefer a soil moisture content above 14%. The optimum temperature is considered to be 15 °C for *A. longa* and 15-20 °C for *L. terrestris*, with incubation of cocoons occurring best at 20 °C (Butt *et al.*, 1992). Optimal soil conditions described in each publication are summarized in Table 1 for *L. terrestris* and Table 2 for *A. longa*.

L. terrestris is considered to prefer alkaline soils, pH 6.2-10 (Sims and Gerard, 1999) and *A. longa* was reported as rejecting soils with a pH < 4.5. Bouché (1972) classified *A. longa* in France as a “neutrophile”, with a preferred soil pH = 6.7. Jefferson (1959) described also *A. longa* as preferring neutral and alkaline soils in northern England. The soil pH in the Axmann (2019) field study was 7.49, clearly suitable for a neutrophile species such as *A. longa* and in the range given for *L. terrestris*.

Table 1: Summary of optimal conditions for *Lumbricus terrestris*

Reference	Life history parameters studied	Environmental factors studied	Optimum conditions found	Limiting conditions
Berry and Jordan, 2001	Growth	Temperature Soil moisture	20 °C 30% soil moisture	Death at 30 °C after 14 days Death at 25 °C after 182 days
Butt, 1991	Growth Cocoon production Rate Survival	Temperature	20 °C optimum for cocoon development and hatchling growth	Hatchling survival: 96% at 15 °C 87% at 20 °C 11% at 25 °C
Daniel <i>et al</i> , 1996	Weight gain of juveniles	Temperature	15 – 17.5 °C At 40% soil moisture	Time from hatching to maturity: 25.7 weeks at 7.5°C 23.7 weeks at 9 °C 14 weeks at 15 °C 9.4 weeks at 20 °C
Daugbjerg, 1988	Adult and juvenile choice over 5 days in soil columns with temperature gradients	Temperature at 20% moisture	10 °C for adults	13% preferred 5 °C 60% preferred 10 °C 13% preferred 15 °C 13% preferred 20 °C <10% soil moisture induces diapause.
Holmstrup <i>et al</i> , 1996	Incubation time of cocoons	Temperature		Cocoon incubation: 48 days at 20°C 152 days at 15 °C 160 days at 10 °C
Khan <i>et al</i> , 2012	Heartbeat rate Blood oxygen level Enzyme activity	Temperature	14 °C had highest respiration and haemoglobin synthesis.	20 – 23 °C respiration rate and haemoglobin synthesis decreased. Death after a few days at 25 °C
Lowe and Butt, 2005	Growth Cocoon development	Temperature Soil moisture	Growth: 15 - 20 °C 30% soil moisture. 20 °C optimum for cocoon development.	
Miles, 1963	Mortality	Temperature	Researched lethal temp.	28 °C for 6 h 40 minutes was lethal.
Moreau Valancogne <i>et al</i> , 2013	Review of publications. Growth Cocoon development	Temperature	16.9 °C for growth 21.6 °C for cocoon development	No growth above 25.9 °C No growth below 2.5 °C No cocoon devpt below 2.6 °C
Perreault and Whalen, 2006	Weight gain Surface casting	Temperature Soil moisture	20 °C 30% soil moisture	Soil moisture a key driver, as greater weight gain at 20 °C and 30% moisture than lower temps and drier soil.

Table 2: Summary of optimal conditions for *Aporrectodea longa*

Reference	Life history parameters studied	Environmental factors studied	Optimum conditions found	Limiting conditions
Baker and Whitby, 2003	Hatchling growth Cocoon hatching	Temperature At fixed 25% soil moisture pH	15-20 °C	Development time from hatching to adults: 9 months at 15 °C, 6 months at 20 °C, 80% death at 25 °C, Requires pH >4.5
Bouché, 1972	Growth and survival	pH	soil pH = 6.7	
Daugbjerg, 1988	Adult and juvenile choice over 5 days in soil with temperature gradients	Temperature at 20% moisture	10 -15°C for adults 10 °C for juveniles	Approx.: 4% preferred 5 °C 40 % preferred 10 °C 40 % preferred 15 °C 12% preferred 20 °C Approx.: 12% preferred 5 °C 64 % preferred 10 °C 14 % preferred 15 °C 4 % preferred 20 °C
Miles, 1963	Mortality as induced by temperature	Temperature	15 °C	>25.7 °C for 12 hours is lethal threshold.
Holmstrup <i>et al.</i> , 1996	Incubation time of cocoons	Temperature		50 days at 20°C 69 days at 15 °C 125 days at 10 °C
Holmstrup, 1999	Development time for cocoons	Temperature	22.5 C	Cocoon development: 106 days at 9.6°C 54.1 days at 15 °C 37.3 days at 20 °C 32.4 days at 22.5 °C 42.1 days at 26 °C

2.2 REVIEW OF RELEVANT PUBLICATIONS

2.2.1 Laboratory or microcosm studies

Lowe and Butt (2005) reviewed conditions for culturing earthworm species in the literature and report optimum soil moisture for *L. terrestris* as being 25-30% with both the anecic species preferring moisture above 14%. The reported optimum temperature for *A. longa* was 15 °C and for *L. terrestris* was 15-20 °C, although incubation of cocoons occurred best at 20 °C (Butt *et al.*, 1992). Fig. 1 below is reproduced from Butt (1991) and shows hatchling growth at constant temperatures (in increments of 5 °C) from 5 °C to 25 °C.

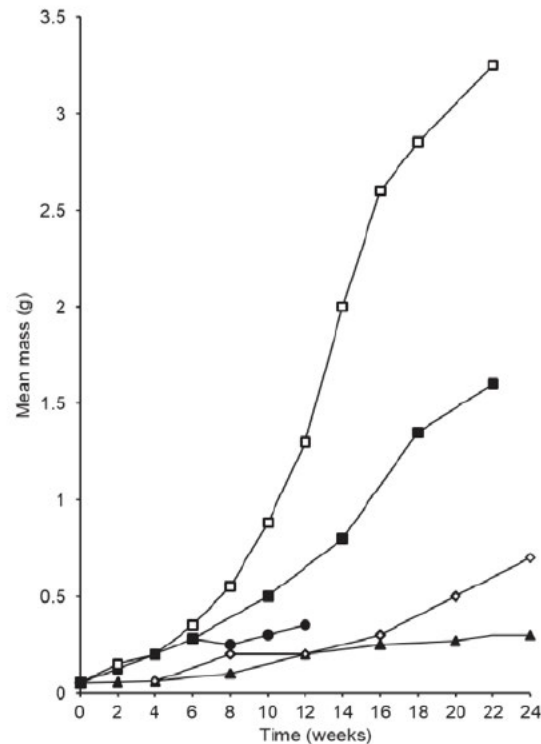


Figure 1. Mean hatchling growth of *L. terrestris* at a range of constant temperatures ($n = 8$). (▲ 5°C, ◇ 10°C, ■ 15°C, □ 20°C, ● 25°C). Adapted from Butt (1991). At 25°C *L. terrestris* suffered high mortality and all individuals were dead by week 16.

Fig. 1: Taken from Butt (1991)

The hatchling growth rates seen in the laboratory microcosms are considered by Daniel *et al* (1996) to be much faster than would occur under field conditions. This is thought to be because the microcosm worms were provided with excess food whereas field populations are thought to be constrained by the availability of sufficient food.

Daniel *et al* (1996) found that for *L. terrestris* the duration from hatching until the development of male pores was 25.7 weeks at 7.5 °C and 9.4 weeks at 20 °C. The additional time needed for the development of a clitellum was 4.7 and 2.3 weeks, respectively. It has been shown by Lofs-Holmin (1982) that *L. terrestris* developed a clitellum after about 14 weeks at 15 °C. Extrapolations of developmental rates showed a development threshold for *L. terrestris* of about 0°C. This is lower than for the compost inhabiting earthworm *Eisenia fetida* with a development threshold of 5.6 °C (Tsukamoto and Watanabe, 1977). Therefore, in contrast to *E. fetida*, *L. terrestris* may develop and gain weight at low temperatures in winter. There was no mortality before male pores appeared up to a temperature of 17.5 °C. At higher temperatures, and after male pores appeared, mortality increased and at 20, 22.5 and 25 °C, most juveniles died after 10 weeks. It seems that *L. terrestris* may only gain weight for some time at high temperatures, and is especially sensitive to high temperatures when becoming a dult.

The effects of temperature from Daniel *et al* (1996) are summarised in Fig. 2 below.

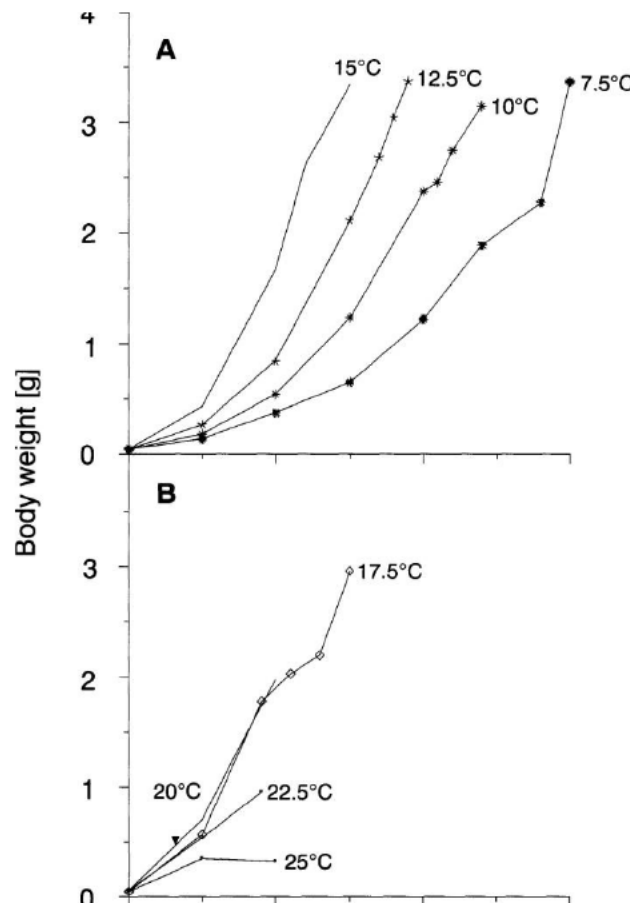


Fig. 2: Weight gain of *Lumbricus terrestris* fed ad libitum with dandelion leaves at various temperatures: (A) temperatures up to 15 °C and (B) temperatures above 15 °C. From Daniel *et al* (1996).

Butt (2011) showed how food quality affected the development of *L. terrestris* juveniles in microcosms under controlled environmental conditions of 15 °C (Fig. 3). Horse manure as a food source resulted in faster development of hatchlings and a greater adult weight than birch leaves (6.169 g in horse manure fed worms and 4.194 g in birch feed worms). Survivorship of the manure fed worms was 100% compared to 80% for the birch fed worms. Unfortunately, there was no study with the inclusion of grass as a food substrate although the mean weight of individual adult *L. terrestris* in the Axmann (2019) field study with elemental iron was 3.10 g and 3.31 g per adult worm in the control and test item treated plots at the start of the study (18 April 2018) and 6.34 g and 6.67 g per adult worm in the control and test item treated plots after 1 year (25 April 2019). These adult body weights indicate that the adult *L. terrestris* of the Axmann (2019) field study with elemental iron had an adequate supply of food throughout the year and were able to mature with a relatively high average body weight.

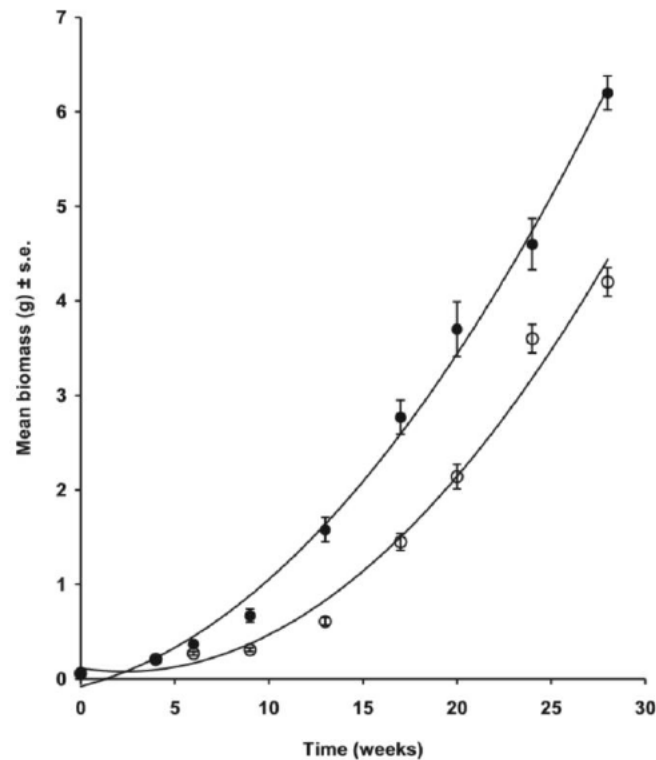


Fig. 1. Mean \pm s.e. growth of *Lumbricus terrestris* (g) from hatchling stage to maturity with feeds of horse manure (●) and birch leaves (○) above a loamy soil at 15 °C in 750 ml mesocosms (10 replicates per treatment). Trend lines are represented by the equations $y = 0.0063x^2 + 0.0503x - 0.0773$ (horse manure) and $y = 0.0066x^2 - 0.0308x + 0.1141$ (birch leaves).

Fig. 3: Taken from Butt (2011)

Euteneuer *et al* (2020) investigated the effects of four cover crop treatments (radish *Raphanus sativus* var. *longipinnatus* B. at high and low seed density, black oat *Avena strigosa* Schreb. and Sudan grass (*Sorghum sudanese* M.) on *A. longa* and *A. caliginosa* in laboratory and field experiments. In their laboratory study hatchlings of *A. longa* (mean individual mass of 48.3 ± 5.7 mg) were kept in 0.4 L plastic vessels filled with 150 g of moist soil (Kettering loam). Three hatchlings were introduced into each vessel. The crops were included in the test units as food for the earthworms, with soil only as a control. Experimental vessels were examined every two weeks and earthworm survival, mass and developmental stage were recorded before earthworms were returned to vessels. At four-week intervals, the substrate was replaced with fresh soil and feed. Four replicates per treatment were maintained and the experiment was terminated after 132 days. Their results are summarised in Fig. 4 below and show how *A. longa* juveniles developed to much greater body weights with radish as a food plant than with black oat.

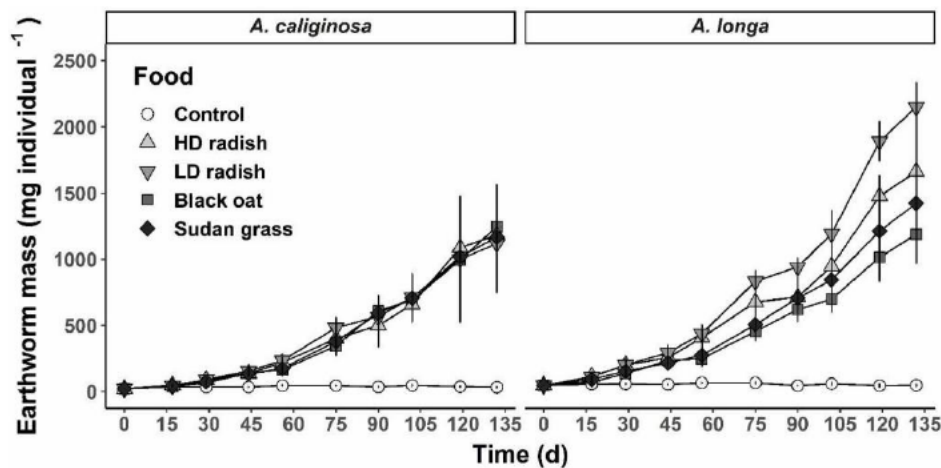


Fig. 4: Earthworm mass gain ($\text{mg individual}^{-1}$) for two species (*Aporrectodea caliginosa* and *Aporrectodea longa*) in a laboratory experiment with cover crop treatments and a control (soil only (control), HD radish (high density), LD radish (low density), black oat, Sudan grass) over 132 days. Taken from Euteneuer *et al* (2020)

Perreault and Whalen (2006) studied the effects of different soil temperatures and moisture content on earthworm burrowing activity in laboratory microcosms. Soil moisture was presented as matric potential, a more meaningful way to express biological water availability than % water content. The 5 and 11 kPa matrix potentials correspond to 30% and 25% gravimetric moisture content, respectively.

Table 3: Taken from Perreault and Whalen (2006)

Table 2. . Temperature and soil moisture effects on the surface casting and burrowing activity of juvenile *L. terrestris*.

Temperature ($^{\circ}\text{C}$)	Soil matric potential (kPa)	Change in mass (g fresh weight)	Surface cast area (cm^2)	Burrow length (cm)	Maximum burrow depth (cm)
5	-11	-0.13 ± 0.04	3.5 ± 0.8	20 ± 7	16 ± 1
10	-11	0.11 ± 0.03	6.0 ± 1.5	48 ± 8	20 ± 1
15	-11	0.06 ± 0.02	3.7 ± 1.0	70 ± 19	21 ± 1
20	-11	0.26 ± 0.08	2.9 ± 1.1	121 ± 25	28 ± 4
5	-5	0.08 ± 0.03	2.1 ± 0.6	1.4 ± 0.7	4.5 ± 1
10	-5	0.25 ± 0.08	9.2 ± 2.0	15 ± 4	17 ± 4
15	-5	0.40 ± 0.13	14 ± 1.4	30 ± 7	24 ± 2
20	-5	0.41 ± 0.13	19 ± 2.2	37 ± 3	22 ± 3
Temperature		$P = 0.0001$	$P = 0.0001$	$P = 0.0001$	$P = 0.0001$
Moisture		$P = 0.0001$	$P = 0.0001$	$P = 0.0001$	$P = 0.0238$
Temperature \times moisture		NS	$P = 0.0001$	$P = 0.0646$	$P = 0.0806$

Values are the mean \pm standard error. Significance of treatment effects was determined by ANOVA, NS = not significant ($P > 0.10$).

5 kPa = 30% moisture. 11 kPa = 25% moisture

Although burrow length and maximum burrow depth (Table 3 above) increased with increasing temperature, there was less burrowing in wetter soil (5 kPa) than drier soil (11 kPa). In addition, *L. terrestris* deposited more casts at the surface of the wetter soil than the drier soil, suggesting that they consumed more soil and organic substrates under these conditions. *L. terrestris* gained more weight in the wetter soil (5 kPa) with similar weight gain at 15 and 20 $^{\circ}\text{C}$. These results support the findings of other authors that 15-20 $^{\circ}\text{C}$ and 30% soil moisture provide good growing conditions for *L. terrestris*.

Several studies have shown that earthworm development is inversely related to temperature (Butt 1991, 1997). Evans and Guild (1948) suggested that *A. longa* may take a year to reach maturity at the temperatures it experiences in the field in the UK. Butt (1993) studied the growth and reproduction of *A. longa* in laboratory cultures in the UK with a view to developing methods to rear this species for inoculation into reclaimed land. He reared *A. longa* in moist loamy sand, with paper pulp, cattle solids and yeast for food, and reported that the complete life cycle could be achieved in six months. He found that maturity was reached, at an average of 3.9 g fresh weight, within 4 months from hatching, when the earthworms were reared at 20 $^{\circ}\text{C}$.

Baker and Whitby (2003) investigated soil pH preferences and the influences of soil type and temperature on the survival and growth of *A. longa*. *A. longa* inhabits soils in the field with pH > 4.5 (presumably measured in water) and Laverack (1961) noted that *A. longa* rejects soils and solutions with pH < 4.5. Bouché (1972) classified *A. longa* in France as a “neutrophile”, with a preferred soil pH = 6.7. Jefferson (1959) described *A. longa* as preferring neutral and alkaline soils in northern England. However, Baker *et al* (1999) introduced *A. longa*, within cages, to several sites with acid soils (pH in CaCl₂ = 4.5–5.3) in southern Australia and recorded high levels of survival at most sites five months later. The addition of lime to these soils did not affect the survival or biomass of *A. longa*. Sims and Gerard (1999) describe *L. terrestris* as preferring alkaline soils (pH 6.2–10).

In laboratory experiments Baker and Whitby (2003) found that survival of the hatchlings was high at 5–15 °C (96–100% after 6 months), moderate at 20 °C (78% after 6 months), but very low at 25 °C, with only ten individuals surviving to 6 weeks and two to 6 months at the highest temperature. However, the biomass per earthworm was greatest at 20 °C (Fig. 5). The average biomass per adult earthworm was 1.75 g (range = 1.48–2.21 g).

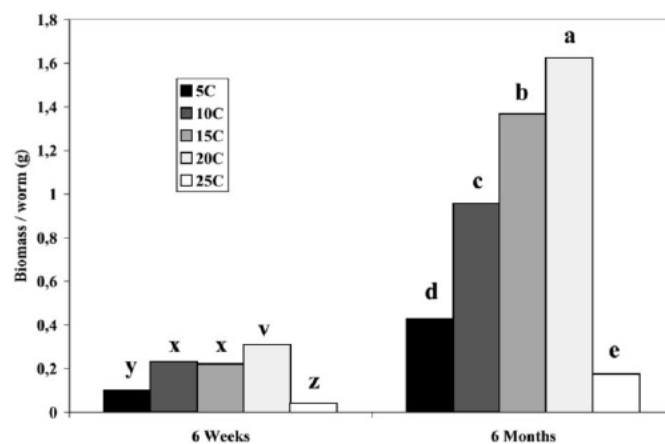


Fig. 5: Average biomass per earthworm (g) for *Aporrectodea longa* reared in the laboratory at various temperatures for 6 weeks and 6 months. Different letters above each histogram indicate significant differences ($P < 0.05$) between treatments within the one time interval. From Baker and Whitby (2003).

The optimum temperature range for culturing cocoons from was 15–20 °C (slightly higher viability at 15 °C; slightly faster development at 20 °C). Optimal growth of hatchlings occurred at 20 °C. These findings were close to Butt’s (1993) recommendation of 15 °C for rearing *A. longa*, but differ from the optimum of 10–12 °C suggested by Nordstrum (1975). Daugbjerg (1988) found that *A. longa* preferred 10–15 °C in choice tests.

Berry and Jordan (2001) conducted laboratory experiments in soils from the mid-western USA with 10 replicates each to investigate the effects of temperature and soil moisture content on the growth of *L. terrestris*. Growth only occurred in the temperature range from 10 to 20 °C (Fig. 6 below).

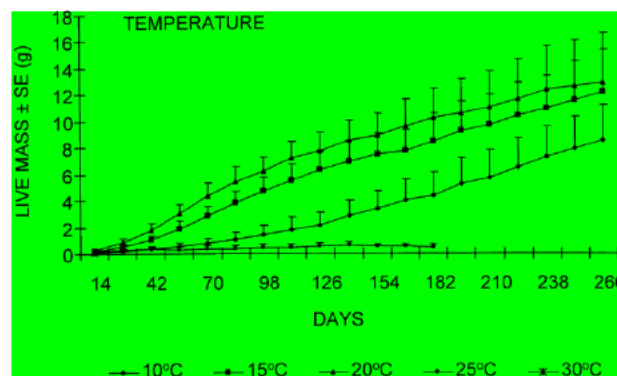


Fig. 6: Effect of temperature on live mass of *L. terrestris* from Berry and Jordan (2001). Data are the means averaged across soil moisture.

Temperatures equal to or greater than 25 °C were detrimental to *L. terrestris* which is consistent with other research. However, under controlled conditions these worms did survive for 182 d, or about six months, if the temperature was 25 °C (Fig. 6 above), which is considerably longer than other similar studies (Butt, 1993). In some environmental conditions 20 °C is the upper limit for survival of *L. terrestris* under controlled and field conditions and most of these species seem to do better at about 15 °C or below (Butt *et al.*, 1992; Daniel *et al.*, 1996; Whalen and Parmelee, 1999).

Berry and Jordan (2001) reared *L. terrestris* hatchlings at 25 °C under conditions of 20, 25 and 30% soil moisture (Fig. 7). Growth was poor at 20% and 25% moisture and was significantly greater at 30%, supporting the idea that soil moisture affects the ability of the developing worms to tolerate high temperature.

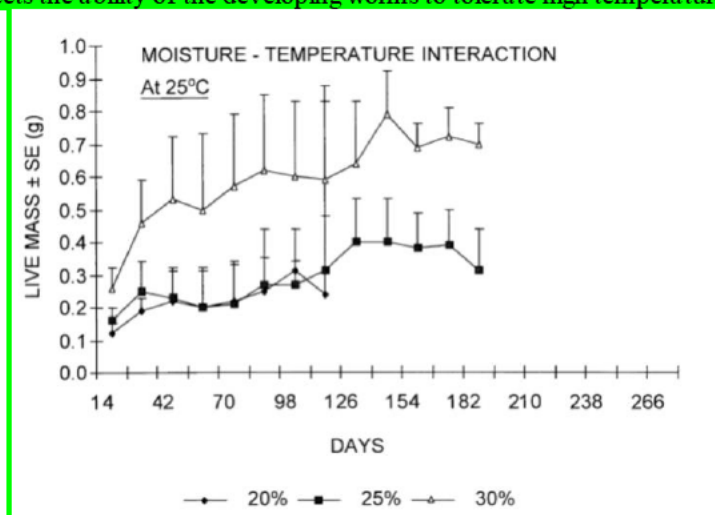


Fig. 7: Effect of moisture at 25 °C on live mass (with S.E.) of *L. terrestris* (from Berry and Jordan, 2001).

Khan *et al* (2007) investigated the effects of changing temperature on the toxicity of zinc, lead, cadmium and copper to *L. terrestris* looking at earthworm physiology. A basal oxygen consumption rate was determined at 10 °C and this was found to increase by about 5% at 10 – 12 °C, by about 38% at 14-16 °C and by 40% at 16-18 °C. However, in worms held at 20 -22 °C oxygen consumption rate decreased by 84%. Temperature also increased the blood hemoglobin (Hb) concentration, which decreased slightly in the worms held at 20–22 °C. The worms held at 20–22 °C had blood that was more hypovolemic than that of the worms from 10–12 °C worms, indicating dehydration. Pre-exposure of 10 -14 °C-acclimated worms to sublethal concentrations of zinc, copper, and lead did not significantly affect the rate of respiration. However, at higher temperatures all these metals inhibited oxygen consumption; zinc, lead, and cadmium by 11% and copper by 18% of that at 14–16 °C. At 20–22 °C, their respiration was further inhibited, 36% by copper, 18% by cadmium, and 10% by lead and zinc. Khan *et al* (2012) found that that above 20 °C adult *L. terrestris* had a high heart rate and a low blood oxygen level.

Khan *et al* (2012) looked at physiological indicators of stress in worms and found that *L. terrestris* kept at 2 and 6 °C above their average habitat temperature (of 10-12 °C) had respectively 15 and 40% higher rates of respiration than those at 10 °C. At 14 °C, the rate of respiration and blood hemoglobin (Hb) concentration both increased by 60 and 50%, respectively compared to the values at habitat temperature. At higher temperatures the rate of respiration and Hb synthesis started decreasing. At 20–23 °C, the respiration and Hb concentration decreased respectively by about 85% and 35% of that at 14 °C. Worms did not survive for more than a few days at 25 °C. It is difficult to conclude from these results which temperature range is optimal for *L. terrestris*, however highest blood oxygen levels occurring at 12-14 °C with a mean heart beat rate of 71.3 beats/minute, suggests that this may be the optimal. Given that temperature range was adjusted in increments of 5 °C in most of the other relevant publications this is not inconsistent with the lower end of the 15-20 °C range suggested by Lowe and Butt (2005) or the 16.9 °C proposed by Moreau Valancogne *et al* (2013).

Earthworm growth rates and fecundity are considered to be temperature dependent with both increasing with rising temperatures up to critical (lethal) thresholds. Daniel *et al* (1996) maintained *L. terrestris* juveniles at 7.5, 10, 12.5, 15, 17.5, 20, 22.5 and 25 °C, with highest growth rates occurring at intermediate temperatures compared with lower or higher temperatures. Earthworms deprived of food resources lost more weight at higher temperatures.

Based on the results of choice tests, offering worms a range of temperatures within a stratified microcosm, Daugbjerg (1988) suggested a temperature of 10 °C as being optimal for *L. terrestris*. Daugbjerg (1988) reported that soil moisture of less than 10% induced diapause in *L. terrestris*.

Moreau Valancogne *et al* (2013) summarised a range of publications and considered 16.9 °C to be optimal for growth of *L. terrestris* and 21.6 °C optimal for cocoon development. No growth occurred above 25.9 °C or below 2.5 °C. Cocoon development stopped below 2.6 °C.

All of the above papers consider the effects of constant soil temperatures under laboratory conditions on earthworm development or growth. However, field soil is not at a constant temperature and in summer months it is warmer near the surface and cooler at depth.

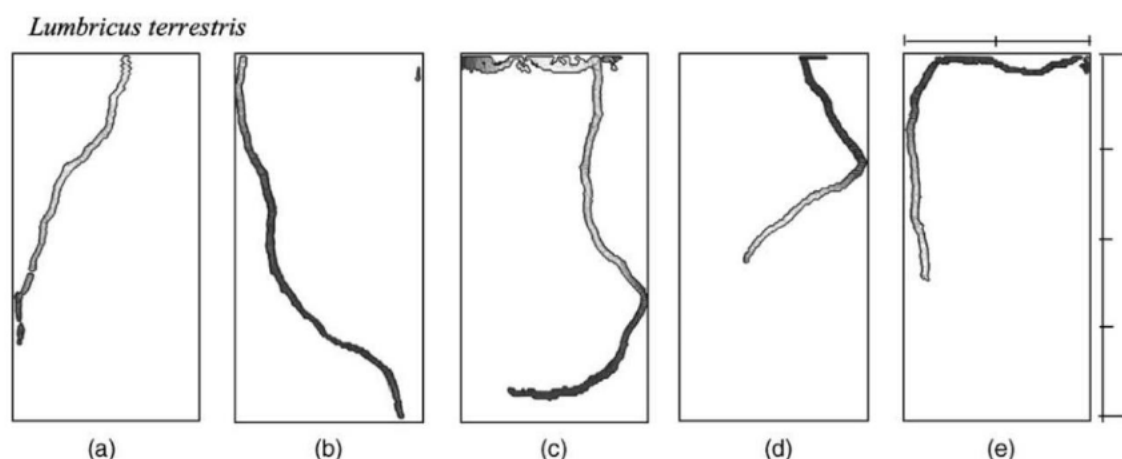


Fig. 8: Burrow systems of five individual *L. terrestris*. From Bastardie (2003) after 21 days.

Note, the original Figure had no scale but the text describes the total core depth of 40 cm so the tick marks in the above Figure indicate 10 cm units of depth.

Bastardie (2003) used X-ray computed tomography to reconstruct the cores of individual *L. terrestris* adults held in soil cores in the laboratory for 21 days. Fig. 8 above shows that over this period the adult worms made mostly vertical burrows between 20 and 40 cm deep. In such vertical burrows the adult worms would be able to move up and down to avoid unfavourable conditions. Holmstrup (1999) cites Gerard (1967) who reported that from a grass pasture, *A. longa* cocoons were found in the horizon 7.5 to 30 cm with a mean depth of 15 cm based on 150 cocoons.

2.2.2 Studies where field conditions were recorded

Only a small number of publications reported the results of field studies with earthworms in which soil conditions were also measured. Prendergast-Miller *et al* (2021) investigated the effects of introducing ley strips in arable fields on earthworm abundance in a multi-year field study in Yorkshire, England. Earthworms were sampled by digging followed by AITC (allyl isothiocyanate) application, and soil temperature and moisture were recorded over three years, 2015-17. Soil temperature never rose above 20 °C and never fell below 5 °C (Fig. 9 below). In arable fields *L. terrestris* and *A. longa* adults made up a very small percentage of the total earthworms, *A. longa* was more abundant than *L. terrestris* in both 2015 and 2017, with more anecic juveniles being found in samples than adults.

In the study of Prendergast-Miller *et al* (2021) soil moisture, temperature and bulk density varied between sampling years. Soils tended to be drier, warmer and had a higher bulk density in 2017 compared to 2016. Overall, soil moisture was lower (~ 6% drier) and soil temperature higher (~1 °C) in April 2017 compared to April 2016. This matches the weather station monitoring data, which indicated that April 2017 was drier compared to the 20-year average for the farm. Soil properties varied with land use and depth, but were not affected by distance from the field margins. Overall, mean soil moisture was significantly higher in margin soils compared to arable, hedgerow and ley soils ($P < 0.001$). Mean soil temperatures under hedgerows and margin soils were similar, and the highest mean soil temperatures were recorded in arable soils.

Fig. 9 below, taken from Prendergast-Miller *et al* (2021) shows soil temperature and moisture values from hedgerows, field margins, arable fields and leys at different sampling times over a two year period.

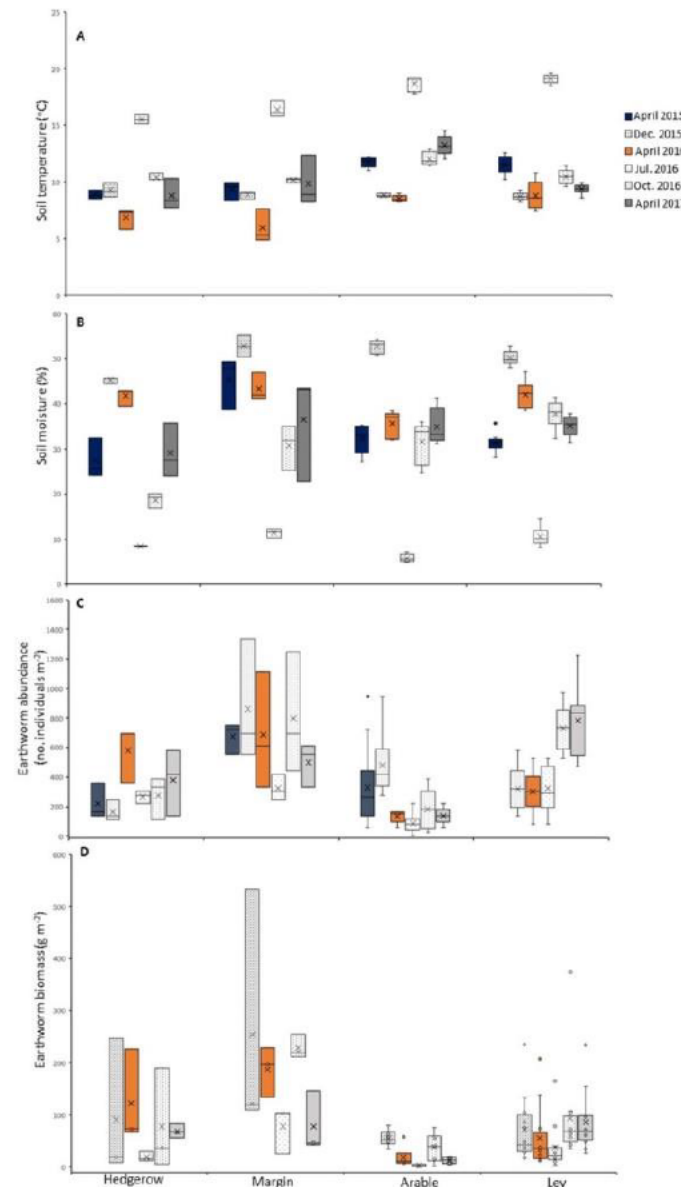


Fig. 9: Seasonal variation in soil temperature (A), soil moisture (B), earthworm abundance (C) and biomass (D) in hedgerows, field margins, arable field and new arable-to-leys strips (from Prendergast *et al*, 2021). Samples were taken in April 2015 (before new leys were set up) and again in April 2016 and April 2017, with additional seasonal sampling in December 2015, July 2016 and October 2016. For each month, $n = 3$ in hedgerow and margin soils; $n = 6$ in arable soils (for April 2015, $n = 18$ in arable soils; $n = 12$ in ley soils). Biomass was not determined in April 2015. In the boxplots, X marks the mean, with the median line dividing the box into the interquartile range. Outliers are shown beyond the maximum and minimum vertical lines.

Earthworm abundance (2017 data) showed weak correlations with soil properties. For example, abundance and biomass were negatively correlated to soil temperature and bulk density (at 5 cm depth), but showed no correlation with soil moisture (at 5 cm depth). The relationship between earthworm species composition and soil properties in each land use was analysed using earthworm, soil temperature and density data collected in April 2017 and OC, N and water holding capacity collected in April 2018. Here, soil temperature was found to be a strong factor differentiating arable soils from ley, margin and hedgerow soils.

Fig. 9 considers the total number of earthworms but Prendergast-Miller *et al* (2021) also identified their specimens to species level for adults and functional groups for juveniles. These data are more relevant for the consideration

of UK abundance levels and have been presented in Fig. 17. The temperature at 5 cm depth in this study did not rise above 20 °C but the soil moisture in July fell to approximately 10% in all the habitats. Daugbjerg (1988) considered that soil moisture levels below 10% induced diapause in *L. terrestris*.

Kanianska *et al* (2016) compared earthworm populations in grassland and arable field sites in Slovakia and looked at soil biotic and abiotic factors. The mean biomass of earthworms was also more than twice as high in permanent grasslands (68.3 g/m²) than arable land (33.3 g/m²). Results showed that the distribution and biomass of earthworms in the soil depended primarily on land use and management practices. *L. terrestris* was present in 3 of 7 arable sites and 5 of 7 grassland sites. *A. longa* was only present in 2 of 7 grassland sites and not found at all in arable sites. Unfortunately, soil conditions were reported as mean soil temperature and mean moisture. It's not possible to draw any conclusions as to how these factors might have affected earthworm densities.

2.3 HOW THE CONDITIONS OF THE AXMANN (2019) FIELD STUDY WITH ELEMENTAL IRON COMPARE TO THE PUBLISHED OPTIMAL RANGE FOR *A. LONGA* AND *L. TERRESTRIS*?

2.3.1 Soil Moisture

From the literature review the optimum soil moisture content for culturing anecic earthworm species was 25-30% and both *L. terrestris* and *A. longa* prefer a soil moisture content above 14%. The solid orange and grey lines in Fig. 10 below show the daily measured soil moisture at 0-10 and 10-20 cm depths in the Axmann (2019) field study with elemental iron.

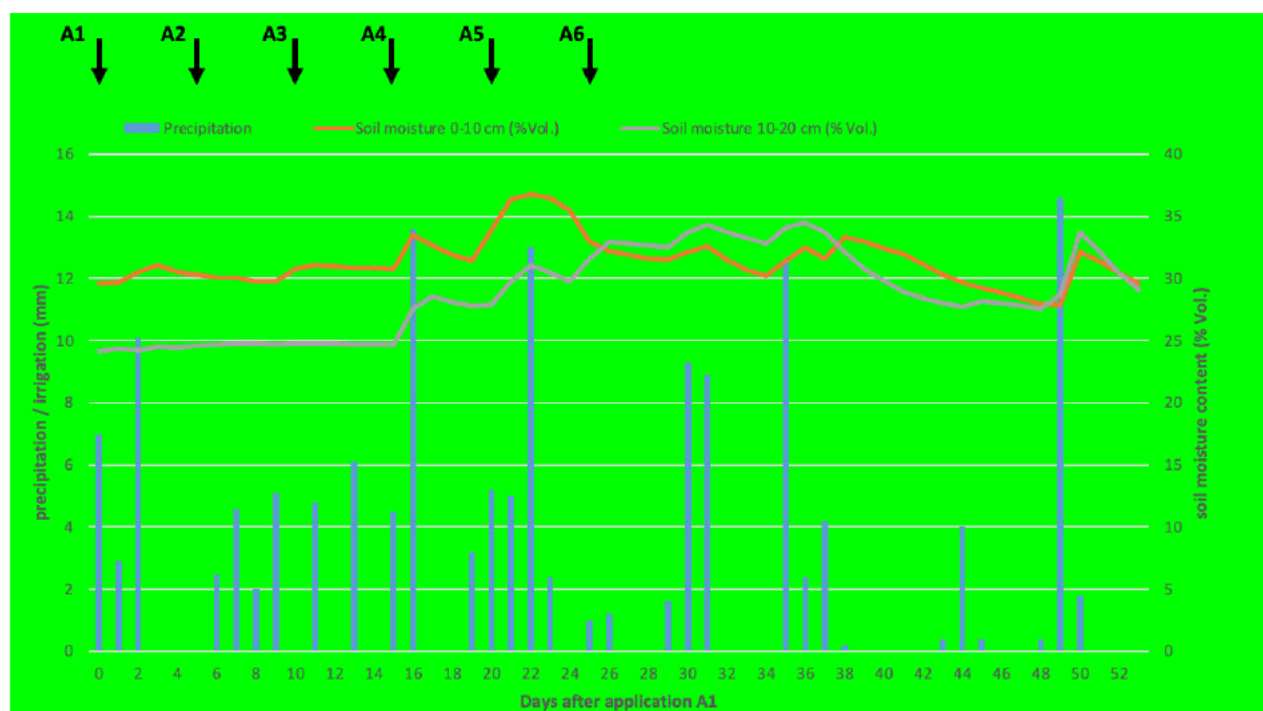


Fig. 10: Soil moisture and precipitation in the Axmann (2019) field study with elemental iron recorded at 10 cm and 20 cm depth (Arrows indicate the 6 applications A1-A6).

During the period of the six applications of treatments as well as during the month after the last application, measured soil moisture in the 0-10 cm layer was consistently at or close to 30%. At the greater depth of 10-20 cm soil conditions were drier for the first two weeks after the first application with about 25% soil moisture. It then increased steadily and for the second month after the first application it followed quite closely the soil moisture of the 0-10 cm layer.

Soil moisture was never below 20% in the two months after the first application of treatments. With respect to soil moisture the conditions during the exposure phase of the field study can be considered to have been optimal for anecic earthworm species and neither *A. longa* or *L. terrestris* would not have been adversely affected by soil moisture at the time.

2.3.2 Temperature

From the literature the optimum soil temperature is considered to be 15 °C for *A. longa* and 15-20 °C for *L. terrestris*, with incubation of cocoons occurring best at 20 °C. Figures 11 and 12 show the daily maximum (red), minimum (grey) and mean (black) soil temperatures at 5 and 20 cm depths in the Axmann (2019) field study with elemental iron.

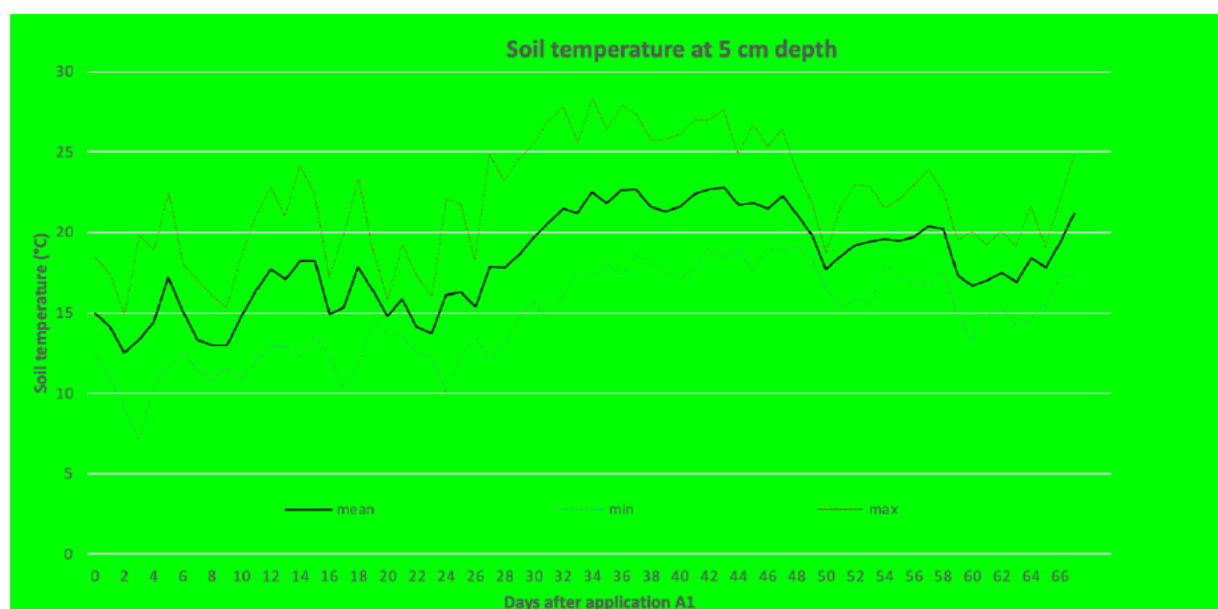


Fig. 11: Soil temperature (°C) at a 5 cm depth after applications in the Axmann (2019) field study.

As would be expected there was greater daily temperature fluctuation nearer the surface than at 20 cm depth. The soil temperature at 5 cm depth was optimal for both anecic species during the first 28 days after the first application of slug pellets, with only a few daily maxima above 20 °C and none above 25°C. For the period from 28 days to 50 days after the first application the daily maximum soil temperature at 5 cm was consistently higher than 25 °C and this would have been sub-optimal for both species. However, both *A. longa* and *L. terrestris* adults would have been present in vertical burrows (see Fig. 8 taken from Bastardie, 2003) and avoided the higher daily maximum temperatures by moving to greater depths. Sims and Gerard (1999) describe the burrows of adult *L. terrestris* as being between 1 m and 3 m deep.

The soil temperature at 20 cm depth for the first four weeks of the study, during the period when the slug pellets would have been broadcast at regular intervals, fluctuated from a minimum of close to 11 °C to a maximum of 23 °C. With a mean of close to 15 °C the temperature conditions over this crucial “exposure” period of the study can be considered to have been close to optimal for anecic earthworms.

Over a two week period, from 34 to 48 days after the first application, mean soil temperature at 20 cm was slightly higher than 20 °C but never approached the worst case lethal thresholds proposed by Miles (1963) of 25.7 °C for *A. longa* and 28 °C for *L. terrestris*. It would have been helpful to have recorded soil temperature at greater depths in the study since it is quite likely that worms with deeper burrows would simply have moved to greater depths during the day time. The high soil moisture conditions that accompanied these temperatures (close to 30%) would have reduced their effects on anecic species (Berry and Jordan, 2001).

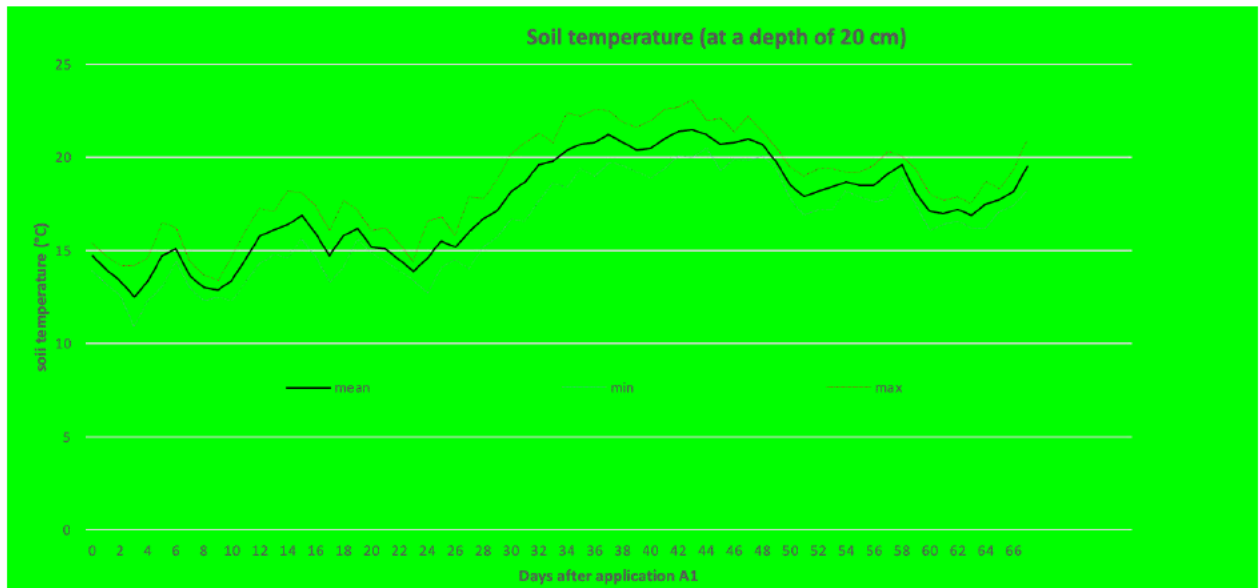


Fig. 12: Soil temperature (°C) at a 20 cm depth after applications in the Axmann (2019) field study.

Since both *A. longa* and *L. terrestris* would have foraged on the surface at night the air temperature in the Axmann (2019) field study is also relevant. Fig. 13 shows the daily maximum, minimum and mean air temperature in the Axmann (2019) study. The maximum air temperature would have occurred during the day when all earthworms would have been found in the soil. The mean air temperature only rose above 20 °C on three occasions and the minimum air temperature, presumably recorded after many hours of darkness on clear nights, was mostly between 5 and 10 °C for the first month and then between 10 and 15 °C for the second month of the study.

In their field studies in Oregon USA Gavin *et al* (2012) observed that night-time foraging by earthworms began several hours after sunset and continued throughout the night. Therefore, the foraging anecic worms in the Axmann (2019) study would have been exposed to the air temperatures between the mean and the daily minimum. Apart from two short period of cold nights at day 3 and day 60, the air temperature would have been suitable for foraging by both *A. longa* and *L. terrestris*.

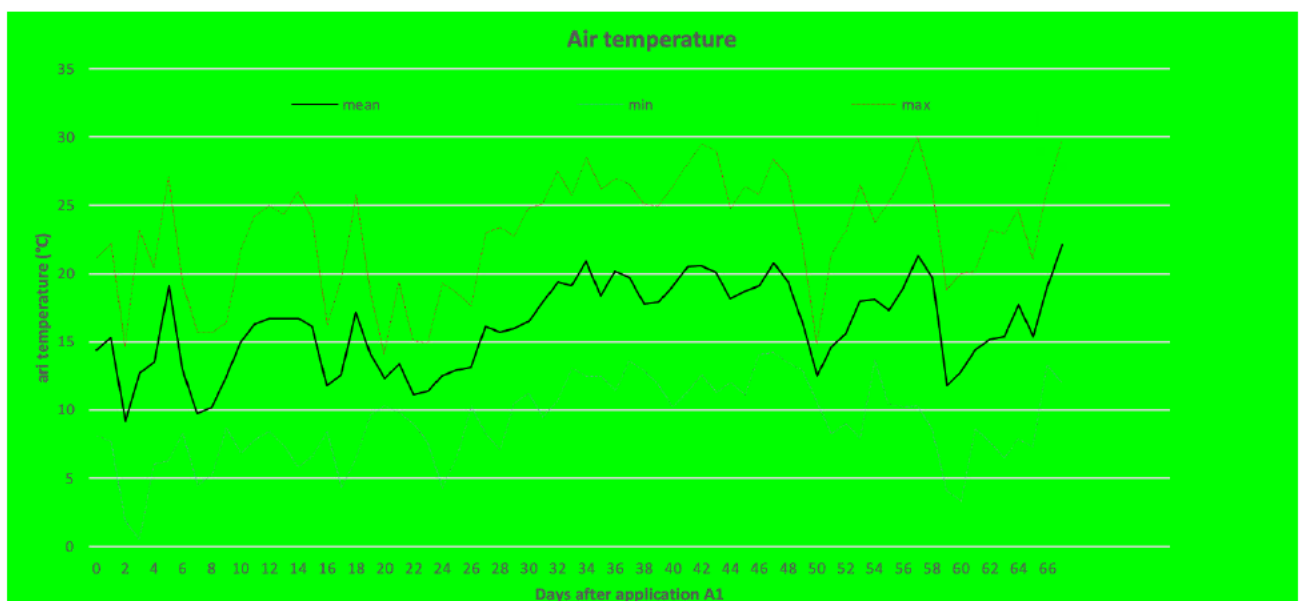


Fig. 13: Air temperature (°C) at the study site after applications in the Axmann (2019) field study.

The literature review points to similar optimal soil conditions for both *A. longa* and *L. terrestris*. In the Axmann (2019) study numbers of *A. longa* adults in the samples from the elemental iron (Final Bite) treatment remained almost the same (close to 9 individuals/m²) for the first 56 days of the study before increasing to 21/m² at 204 DAA1. There is no indication of any decline in *A. longa* numbers due to elemental iron, yet the corresponding reference item treatment shows a sharp reduction when compared to the control 34 days after the first treatment. This confirms that adult *A. longa* would have been actively foraging at the surface during this period in order to be exposed to the reference item. The adult anecic earthworm numbers from the Axmann (2019) study are reproduced below in Table 4.

Table 4: Mean number (n/m²) for adults of anecic earthworm species, standard deviation and percentage change of species earthworm abundance in the test item treatment (T) and toxic reference treatment (R) relative to the control (C) from Axmann (2019)

Species / group	Timing	Mean number (n/m ²) ± standard deviation and deviation from control (%) ^{a)}		
		C (untreated)	Final Bite (T)	Carbomax (R)
<i>Aporrectodea longa</i>	7 DBA1	7.5±1.0	8.8±4.6 (16.7)	6.8±1.7 (-10.0)
	34 DAA1	13.3±5.7	9.8±4.7 (-26.4)	2.3±2.1 * (-83.0)
	56 DAA1	7.5±2.6	9.5±4.8 (26.7)	1.0±1.2 * (-86.7)
	204 DAA1	13.8±4.6	21.0±5.8 (52.7)	2.0±2.2 * (-85.5)
	366 DAA1	3.3±1.9	3.8±2.1 (15.4)	0.0±0.0 * (-100.0)
<i>Lumbricus terrestris</i>	7 DBA1	13.3±2.6	12.3±4.7 (-7.5)	11.8±3.5 (-11.3)
	34 DAA1	6.5±4.5	8.3±5.1 (26.9)	2.0±2.2 (-69.2)
	56 DAA1	1.0±0.8	3.5±1.7 (250.0)	0.0±0.0 (-100.0)
	204 DAA1	8.0±3.7	12.0±4.1 (50.0)	1.5±0.6 * (-81.3)
	366 DAA1	7.0±2.2	5.8±3.3 (-17.9)	2.0±1.4 * (-71.4)

^{a)} negative values (-) indicate lower earthworm numbers compared to the control; positive values (+) indicate higher earthworm numbers compared to the control

DBA1 = days before application 1, DAA1 = days after application 1

* significantly different from control ($p \leq 0.05$)

Adult *L. terrestris* were present in good numbers (8.3/m²) in the samples taken 34 DAA1 from the Final bite treated plots. However, the decline to low levels seen in the samples taken 56 days after the first application in all treatments, including the control, has given rise to uncertainty over the level of activity and hence exposure to the slug pellet treatment that would have occurred between 34 and 56 days after the first application.

A plausible explanation for this comes from consideration of the sampling method used in the Axmann field study and an appreciation of where in the soil profile the adult *L. terrestris* would have been located at 56 DAA1. Given the soil temperatures at 5 and 20 cm at 56 DAA1 it is most likely that adult *L. terrestris* would have been in their burrows at depths of 20 cm or more during the daytime. The sampling method used by Axmann (2009) involved digging by hand to a depth of 20 cm and then hand sorting that soil for earthworms. After this a 0.2 % formaldehyde solution was uniformly applied at a rate of 20 -40 L/m² to the sampled areas in the excavated holes. After several minutes the first worms appeared on the soil surface in the excavated hole. The duration of the formalin extraction in the 56 DAA1 sampling was 30 minutes. It is highly likely that because adult *L. terrestris* would have been more than 20 cm deep in the soil they would have evaded the dug part of the sample and, in response to the surface vibration from the digging, would have been driven to the bottom of their burrows and failed to come to the surface with formalin sampling. On the first two sampling dates the adult *L. terrestris* may have been closer to the surface and more likely to occur in the dug samples.

The fact that *A. longa* was present at mean levels of 9.5 adults/m² at 56 DAA1, compared with 7.5/m² in the control, supports this hypothesis. If the test item had an effect on surface foraging worms at 56 DAA1 then it would have been expected to occur in the *A. longa* population too.

3. IS THE ABUNDANCE OF ANECIC WORMS IN THE AXMANN (2019) FIELD STUDY COMPARABLE WITH UK POPULATION LEVELS?

3.1 SUMMARY

A review of the abstracts from the literature search yielded 39 papers that were considered potentially relevant for evaluating the UK abundance of *L. terrestris* and *A. longa*. The full publications of these were supplied and 22 were found to be of sufficient quality and contain information relevant for the UK. In addition, two further relevant publications were identified and added to the search results. Only a small number of the papers reported results of UK surveys that were relevant for the purposes of this review.

The abundance of both *L. terrestris* and *A. longa* in UK agricultural soils appeared to be highly variable and depends largely on historical land use and the extent of cultivation. *A. longa* is more widely distributed and common in agricultural soils than *L. terrestris*. Typical levels of abundance for both anecic species ranged from 1 to 20 individuals/m² but could be as low as 0 or as high as 50/m². Populations of both anecic species are consistently higher in grassland and consistently lower in arable systems.

Numbers of adult *L. terrestris* from the Axmann (2019) study ranged from 1.0 to 13.3/m² in the control plots and 3.5 to 12.3/m² in the Final Bite treated plots. Similarly, numbers of adult *A. longa* ranged from 3.3 to 13.8/m² in the control plots and 3.8 to 21.0/m² in the Final Bite (elemental iron) treated plots. These abundance levels for adults are very close to those reported for adults and juveniles by Hutcheon *et al* (2001) from a field study conducted at Long Ashton in the UK.

3.2 REVIEW OF PUBLICATIONS RELEVANT FOR UK ABUNDANCE

In 2014 Natural England published a report on the conservation status, population size, range and habitat preferences of earthworms in the UK. This report (Sheppard, 2014) presented the results from citizen science, with volunteer sampling, at 333 sites in 56 areas and in many habitat types, but with sample identification confirmed by the British Museum (Natural History). Due to this varying level of commitment from the volunteers, sampling coverage across the country was unequal, with a majority of sampling sites being in southern or central England.

This report provides a historical perspective regarding British earthworm populations. During the Pleistocene glacial advances, the entire earthworm fauna of the British Isles is thought to have been exterminated (Simms and Gerard, 1999). Today's native fauna, which is a subset of species belonging to the native Lumbricidae fauna of Western Palaearctic, is presumed to have recolonised from continental Europe. This is relevant for evaluation of the Axmann (2019) field study since the earthworms at the German field site can be considered to be genetically similar to those that recolonised Britain after the ice age. As shown in Fig. 14, the UK survey found that the most numerous species sampled was *Allolobophora chlorotica* (34% of identified specimens), followed by *Aporrectodea caliginosa* (19%) and *Lumbricus castaneus* (12%). Twelve species each represented less than one percent of the dataset. The survey concluded that both *L. terrestris* and *A. longa* were common and widespread in the UK with a preference for grasslands and garden lawns where they can become moderately abundant in undisturbed sites. Both these anecic species were described as being usually sparse when they occur in woodland or arable soils. *L. terrestris* was recorded as having a high habitat specificity, only being recorded from one or two habitat types whereas *A. longa* had moderate specificity, being abundant in less than 9 of the 14 habitat types sampled. The relative abundance of both *A. longa* and *L. terrestris* in the UK is indicated in Figs. 14 and 15, taken from (Sheppard, 2014). In all the samples in the UK survey *A. longa* was the 7th most abundant species (with 2.62% of all worms collected) and *L. terrestris* was the 10th most abundant species, being 1.24% of all worms collected in samples. Fig. 15 shows that approximately 30% of the 333 sites sampled contained *A. longa* and 15% of the sites contained *L. terrestris*.

The results from this UK survey indicate that whilst both anecic species have a preference for grassland habitats, *A. longa* is more abundant and occurs in a wider range of habitats than *L. terrestris*. This survey included samples from grassland on acid soil (137), grassland on base-rich or neutral soil (66), set-aside grassland (25) as well as arable field sites (179), hedgerows (61) and field margins (376).

These data for UK abundance of anecic species are surprising to the author of this literature review and possibly indicate a bias against effective sampling of anecic earthworms when digging is the main method of sampling. The standard methodology used in the UK survey (Sheppard, 2014) involved digging 5 soil pits, each 25 cm x 25 cm and 10 cm deep, hand sorting the dug soil and then applying mustard solution to the dug hole (25 mL volume of mustard powder in 0.75 litre tap water) and checking for earthworms for 10 minutes. The physical disturbance

caused by the digging would have resulted in adults of both *A. longa* and *L. terrestris* moving deep into their burrows. The 10 minute observation period may not have been long enough to cause adult worms to leave their burrows. A similar sampling bias would have applied in the Axmann (2019) field study but the digging was to a greater depth (20 cm) and the earthworms were collected over a 30 minute period after applying 2% formalin.



Fig. 14: Ranked abundance of earthworms (percentage of total, n=6309 specimens) extracted from 1503 samples collected from 333 UK sites. From Natural England Survey (Sheppard, 2014).



Figure 15: The percentage of sampling sites (n=333) at which species were recorded. Species are listed on the vertical axis in the same order in which they appear in Figure 14. From Natural England Survey (Sheppard, 2014).

The most comprehensive survey of earthworm numbers from agricultural sites is provided by Boag *et al* (1997). The earthworms of arable and pasture fields from 100 randomly chosen arable farms in Scotland were identified and counted and their relationship with soil factors examined. The most prevalent and numerous species were *Aporrectodea longa*, *Aporrectodea caliginosa* and *Lumbricus terrestris*. All three of these earthworm species had cosmopolitan distributions across Scotland.

Four soil characteristics (% moisture, % sand, % organic matter and pH) shared no relationship with species recorded but tillage may have had a detrimental effect on species composition and size of population. *L. terrestris* (adults and juveniles combined) had a mean abundance in grass fields of 44.8 individuals/m² and in arable 31.2/m². *A. longa* (adults and juveniles combined) had a mean abundance in grass fields of 56.8 individuals/m² and 38.4/m² in arable fields.

Hutcheon *et al* (2001) conducted a multi-year field study at Long Ashton in SW England using formalin sampling in 25 ha conventional (CFS) and integrated farming system (IFS 2 and IFS3) plots. *A. caliginosa* made up 80% of the sampled worms with anecic species comprising 20% by number.

The Integrated Farming Systems (IFS) included both direct drilling and reduced tillage. The authors considered formalin sampling to be the most efficient method for sampling anecic earthworms. Earthworms were sampled in the spring and autumn each year and the results (adults and juvenile combined) are summarized in Fig. 16 below.

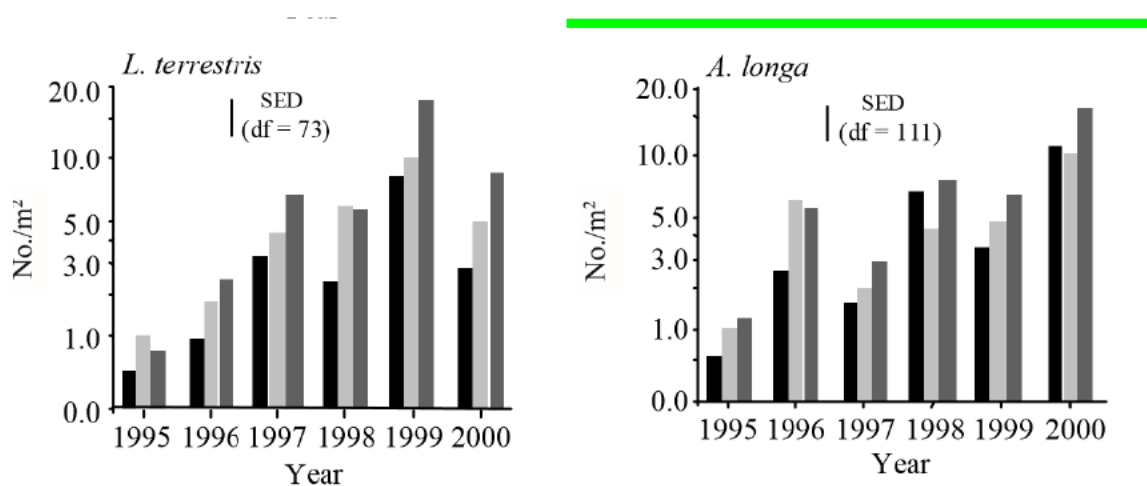


Fig. 16: Mean density of *Lumbricus terrestris* and *Aporrectodea longa* in conventional (black bars) and integrated (pale and dark grey bars) farming systems (1995–2000). From Hutcheon *et al* (2001).

Fig. 16 shows that both anecic species increased in abundance over the five years of this study in both the conventional and the integrated farming systems. In 1995 numbers of both species were found at low levels of 1 individual per m² or less. By the end of the study, in 2000, numbers of *A. longa* had increased to above 10/m² in all three farming systems whereas *L. terrestris* was found at about 3/m² in conventional system and just less than 10/m² in the integrated system with the least tillage.

In a field study conducted at the University of Leeds farm in Yorkshire, England, Holden *et al* (2019) reported that the main anecic species in hedgerows, field margins, arable fields and leys was *Aporrectodea longa* (as approx. 2% of earthworms per land cover type). Land cover had a significant effect on absolute earthworm species density ($p < 0.001$ for all), but not for *A. longa*, where land cover effects were not significant. In general, earthworm species densities were similar in pasture and margin soils, but higher in these compared to the arable (and sometimes hedgerow) soil.

Lumbricus terrestris was more abundant in the field margin compared to hedgerow or arable soils. In terms of biomass per individual, anecic earthworms in margin and pasture soils tended to be larger compared to anecic

earthworms in hedge and arable soils: *A. longa* ($p = 0.007$, pasture > arable), *L. terrestris* ($p < 0.001$, margin > arable) and anecic juveniles ($p = 0.001$, margin > hedge, arable).

In the study of Prendergast-Miller *et al* (2021) *L. terrestris* and *A. longa* adults made up a very small percentage of the total earthworms in arable fields (Fig. 17 below). *A. longa* was more abundant than *L. terrestris* in both years of the study (2015 and 2017) with many more anecic juveniles being found in samples than adults.

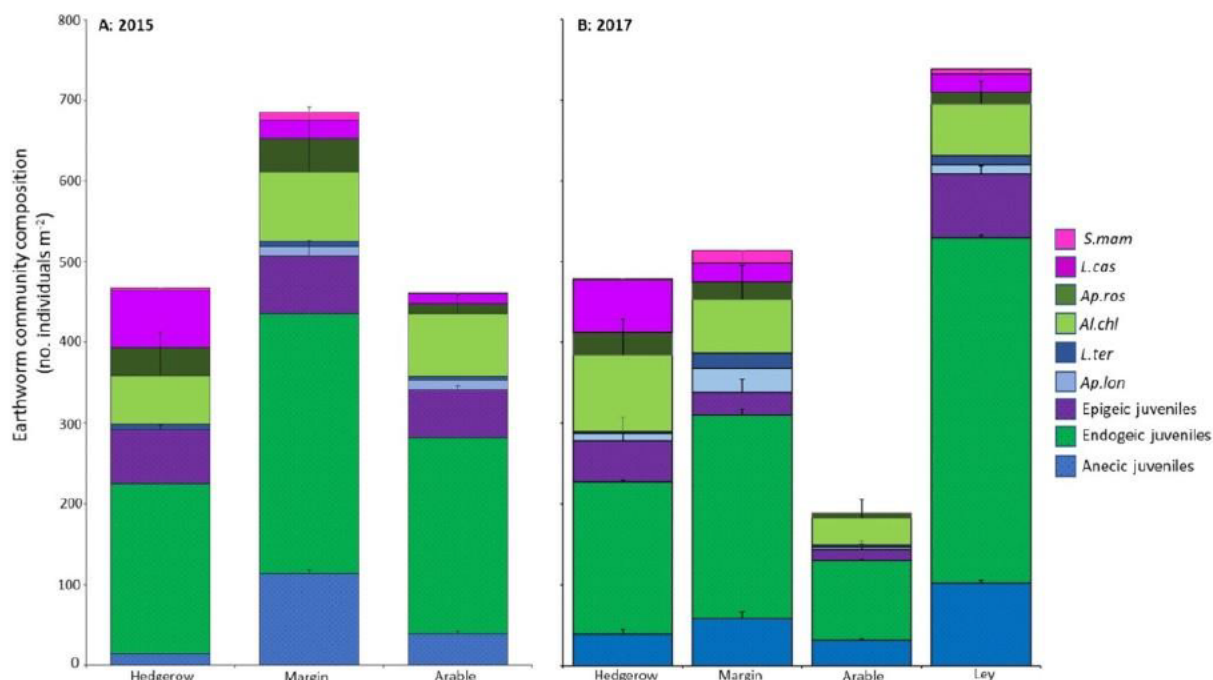


Fig. 17: Earthworm community mean abundance and composition by species and functional group in the hedgerow, field margin, arable in April 2015 (A) (baseline data, before ley strips were established) and in new ley soils in April 2017 (B). Colours indicate earthworm functional group (purple tones are epigeic species and juveniles; green tones are endogeic species and juveniles; blue tones are anecic species and juveniles). $n = 12, 12, 24$ and 48 in hedgerow, margin, arable and ley soils respectively. Error bars show $+1$ standard error. From Prendergast-Miller *et al* (2021).

Key: *S. mammalis* (S.mam; pink); *L. castaneus* (L.cas; dark pink); *A. rosea* (Ap.ros; dark green); *A. chlorotica* (Al.chl; light green); *L. terrestris* (L.ter; dark blue); *A. longa* (Ap.lon; light blue); Epigeic juveniles

Fig. 17 above from Prendergast-Miller *et al* (2021) shows abundance in two separate years by species for adult earthworms and by functional groups for juveniles. Despite the overall high total earthworm numbers, the adults of *A. longa* and *L. terrestris* were found in very low numbers in hedgerows, field margins, arable field and leys. Anecic juveniles were most abundant in field margins and leys but endogeic species juveniles were by far the most numerous in all habitat types.

This is in agreement with the findings of Johnston *et al* (2018) who modelled the mechanisms driving both tillage and climate effects on anecic earthworms. In reduced tillage agriculture, a decline in mechanical disturbance allows for *L. terrestris* proliferation, whilst the activities of *L. terrestris* can replace many of the soil functions provided by tillage. However, anecic earthworm populations are significantly reduced in agricultural fields due to tillage (van Capelle *et al*, 2012). Climatic factors also moderate earthworm populations and can directly influence their response to tillage practices (Briones and Schmidt, 2017).

Sizmur *et al* (2017) sampled earthworms near Rothamsted, UK, in long-term winter wheat plots with different methods for straw incorporation. Sampling was by digging and then applying mustard solution and collecting all worms coming to the surface over a 1 hour period. No *A. longa* or *L. terrestris* adults or juveniles were found in the site where straw and/or farmyard manure had been applied. 6.3 ± 3.6 adult *L. terrestris* and 6.3 juveniles/m² were recorded from the control plots of the long-term straw incorporation experiment whereas 3.1 ± 3.1 adults and 9.4 ± 6.0 juvenile *L. terrestris*/m² were recorded in the plots amended with straw in the long-term straw incorporation experiment.

Many agronomically or tillage-focused studies report total earthworm numbers (and less frequently, biomass) as one of several commonly measured indicators of the biological effects of tillage practices. Meta-analysis by Briones and Schmidt (2017) provides evidence for the significantly different responses to tillage by ecological groups and also individual species of earthworms. *L. terrestris* exhibited the strongest positive response to reduced tillage, increasing average numbers of their populations by about 124% compared to the overall mean.

A common survival strategy exhibited by earthworms during adverse environmental conditions is to move down to deeper layers and enter into a resting stage (by emptying their gut and coiling up into a tight ball inside an aestivation chamber which has been made previously by mixing casts and mucus; (Sims and Gerard, 1999). By disturbing the soil (e.g. by inversion tillage) during the inactive periods of the year (late autumn, early spring) and exposing the earthworms that have entered into this inactive stage to the surface, where they can be easily predated, can cause severe reductions in their populations (Curry *et al*, 2002). The most likely species to be affected by these disturbances are surface dwellers (i.e. belonging to the epigeic ecological group) and the anecic worms that build vertical burrows open to the surface where they feed and cast, whereas the small endogeics living in the mineral horizons exhibit fewer alterations in their populations.

In the case of well-studied anecic species, such as *L. terrestris*, there is evidence suggesting that their burrows are permanent or semipermanent structures that can be inherited by their offspring (Butt *et al*, 2003; Grigoropoulou *et al*, 2008). This low mobility might also explain why this species showed the greatest positive effect from reducing tillage operations.

Pelosi *et al* (2008) developed WORMDYN, a model of *Lumbricus terrestris* population dynamics in agricultural fields. The model simulates the population density of *L. terrestris* in the upper layer (soil depth ≤ 30 cm) and takes into account vertical migration between the lower and upper layers, which is driven by environmental conditions since *L. terrestris* has no obligatory diapause (Gerard, 1967; Avel, 1959 and Lee, 1985 in Pelosi *et al*, 2008). WORMDYN also accounts for an initial stock of cocoons at the beginning of spring; it corresponds to the stock of viable cocoons produced in autumn of the previous year that will not hatch till spring of the following year due to unfavorable conditions.

Lee (1985) explained that incubation times of lumbricids in the field are similarly delayed by the intervention of cold temperatures or drought, and the cocoons hatch when temperatures or soil moistures are more favorable.

Pelosi *et al* (2008) assumed that juvenile *L. terrestris* do not migrate vertically since they are generally found within 8 cm of the soil surface (Edwards and Bohlen, 1996), close to the food source. Moreover, they may not yet have a sufficiently developed muscular system that Bouché (1977) called “digging muscles”. Horizontal migration is not taken into account in the model because the rate at which *L. terrestris* moves is on average four meters a year (Hoogerkamp *et al*, 1983). Although individuals of *L. terrestris* have been reported to migrate up to 20 m during a single night (Mather and Christensen, 1988), they often return to the same burrow (Edwards and Bohlen, 1996). Although this publication does not provide UK abundance data it indicates why juvenile anecic worms might be well represented in samples collected by the digging method. There is no information concerning the sampling efficiency of applying formalin or AITC solutions, either alone or in conjunction with digging.

In summary the abundance of both *L. terrestris* and *A. longa* in UK agricultural soils appears to be highly variable and to depend on historical land use and the extent of cultivation. Typical levels of abundance ranged from 1 to 20 individuals/m² (as seen at Long Ashton by Hutcheon *et al*, 2001) but could be as low as 0 (Sizmur *et al* (2017) at Rothamsted) or as high as 50/m² (Boag *et al* (1997) across Scotland). Populations of both anecic species are consistently higher in grassland and consistently lower in arable systems.

3.3 HOW DOES ABUNDANCE OF *A. LONGA* AND *L. TERRESTRIS* IN THE AXMANN (2019) STUDY COMPARE WITH UK REPORTED LEVELS IN AGRICULTURE?

Numbers of adult anecic earthworms from the Axmann (2019) study are reproduced below in Table 5. However, the literature review (especially Prendergast *et al*, 2021) indicated that juvenile anecic worms were considerably more abundant than adults in samples from grass leys and arable fields. Pelosi *et al* (2008) described how juveniles are found nearer the surface which suggests that they would have been more readily sampled in the digging/formalin combination used in the Axmann study.

Numbers of adult *L. terrestris* ranged from 1.0 to 13.3/m² in the control plots and 3.5 to 12.3/m² in the Final Bite treated plots of the Axmann (2019) study. Similarly, numbers of adult *A. longa* ranged from 3.3 to 13.8/m² in the control plots and 3.8 to 21.0/m² in the Final Bite treated plots of the Axmann (2019) study. These abundance

levels are very close to those reported by Hutcheon *et al* (2001) from the Long Ashton field study, as presented in Fig. 16.

Boag *et al* (1997) reported numbers for combined adults and juveniles of *L. terrestris* and *A. longa*. Axmann (2019) didn't record juvenile *L. terrestris* or *A. longa* numbers separately so they have been classified as "tanylobous" and "epilobous" juveniles, respectively. It seems most likely that the abundance levels reported from Scotland by Boag *et al* (1997) for *L. terrestris* (44.8 individuals/m² in grass and 31.2/m² in arable) and for *A. longa* (56.8 individuals/m² in grass and 38.4/m² in arable fields) were much higher than seen in the Axmann (2019) study. Conversely, Sizmur *et al* (2017) found very low numbers of *A. longa* and *L. terrestris* adults or juveniles in two long-term field studies near Rothamsted. The highest levels of *L. terrestris* recorded at Rothamsted were 6.3 adults/m² and 9.4 juveniles/m², somewhat lower than the levels recorded in the Axmann (2019) field study.

Table 5: Mean number (n/m²) for adults of anecic earthworm species, standard deviation and percentage change of species earthworm abundance in the test item treatment (T) and toxic reference treatment (R) relative to the control (C) from Axmann (2019)

Species / group	Timing	Mean number (n/m ²) ± standard deviation and deviation from control (%) ^{a)}		
		C (untreated)	Final Bite (T)	Carbomax (R)
<i>Aporrectodea longa</i>	7 DBA1	7.5±1.0	8.8±4.6 (16.7)	6.8±1.7 (-10.0)
	34 DAA1	13.3±5.7	9.8±4.7 (-26.4)	2.3±2.1 * (-83.0)
	56 DAA1	7.5±2.6	9.5±4.8 (26.7)	1.0±1.2 * (-86.7)
	204 DAA1	13.8±4.6	21.0±5.8 (52.7)	2.0±2.2 * (-85.5)
	366 DAA1	3.3±1.9	3.8±2.1 (15.4)	0.0±0.0 * (-100.0)
<i>Lumbricus terrestris</i>	7 DBA1	13.3±2.6	12.3±4.7 (-7.5)	11.8±3.5 (-11.3)
	34 DAA1	6.5±4.5	8.3±5.1 (26.9)	2.0±2.2 (-69.2)
	56 DAA1	1.0±0.8	3.5±1.7 (250.0)	0.0±0.0 (-100.0)
	204 DAA1	8.0±3.7	12.0±4.1 (50.0)	1.5±0.6 * (-81.3)
	366 DAA1	7.0±2.2	5.8±3.3 (-17.9)	2.0±1.4 * (-71.4)

^{a)} negative values (-) indicate lower earthworm numbers compared to the control; positive values (+) indicate higher earthworm numbers compared to the control

DBA1 = days before application 1, DAA1 = days after application 1

* significantly different from control (p ≤ 0.05)

From the literature review it is clear that *A. longa* is at least as abundant as *L. terrestris* in UK agricultural fields. Numbers of adult *A. longa* in the Axmann (2019) field study (Table 5) were in accordance with what would generally be expected from the UK and showed no decline due to the Final Bite (elemental iron) treatment at any of the five sampling occasions. In fact, mean *A. longa* numbers were slightly higher in the Final Bite treatment than in the control on all but one sampling occasion.

3.4 REVIEW OF CONTROL DATA FROM ADAMA OWNED EARTHWORM FIELD STUDIES

A search of ADAMA earthworm field studies identified 15 conducted in Germany and one conducted in the UK. The UK study (Forster and Salaun, 2003) in a long-term grass field in South West England was sampled using the formalin method only. In 2003 this was accepted practice with digging being introduced later, initially as an efficiency check for the formalin sampling and then as an additional approach to complement it. *A. longa* was not present in this study in large enough numbers to draw any conclusions. *L. terrestris* numbers ranged from 1.3 adults and 8.5 juveniles/m² in May 2001 to 4 adults and 15 juveniles/m² in July 2002. Of the 15 studies performed in Germany all recorded numbers of *L. terrestris* but seven recorded no *A. longa* adults or juveniles. All of these studies except one were sampled using digging to a 20 cm depth followed by either formalin or more recently AITC to sample anecic worms. These earthworm field studies were conducted in a range of crop systems from grass fields, barley fields and maize fields to bare soil sown with clover after application.

The control numbers for *L. terrestris* and *A. longa* from these studies are presented in Table 6. In many of the studies where sampling took place in June or July numbers of adult *L. terrestris* were very low. There was considerable variability between studies with some having relatively high numbers of anecic worms and others very low numbers. *A. longa* was completely absent from 6 of the 15 German field studies. The octet method for sampling with electrical currents used in the study of Rozencranz (2009; Study 6 in Table 6 below) appears to have been particularly effective when sampling juvenile *L. terrestris* but generated very low numbers of adults.

Table 6: Mean numbers of *L. terrestris* and *A. longa* from control plots in ADAMA owned field studies

	Study Author	Year	Adama Ref	System	Sampling methods	Country	Sampling Date	Control <i>Lumbricus terrestris</i> /m ²			Control <i>Aporrectodea longa</i> /m ²			Control Anecic earthworm total /m ²
								Mean No. Adults	Mean No. Juveniles	Mean total	Mean No. Adults	Mean No. Juveniles	Mean total	
1	Forster, A. and Salaun, F.	2003	000058735	Grass	Formalin	U.K.	23. Mai 01	1.3	8.5	9.8	0.5	0	0.5	10.3
							17-18 July 01	3.3	6.3	9.6	0.5	0.25	0.8	10.4
							14. Aug 01	3.3	8.0	11.3	0.75	0.75	1.5	12.8
							28-29 Nov 01	1.8	3.0	4.8	1.5	0	1.5	6.3
							19-20 Feb. 02	1.5	3.0	4.5	2.75	0	2.8	7.3
							17. Jul 02	4.0	15.1	19.1	0.75	0	0.8	19.9
2	Solga, A.	2009	000062192	Spring barley	Digging, hand sorting then formalin	Germany	24. Apr 08	1.0	0.0	1.0	No <i>A. longa</i> were collected or identified in this study			1.0
							09. Jul 08	1.8	2.8	4.5	0.0	0.0	0.0	4.5
							22 Oct. 08	3.0	4.5	7.5	0.0	0.0	0.0	7.5
							05. Mai 09	2.0	3.3	5.3	0.0	0.0	0.0	5.3
3	Solga, A.	2009	000062193	Spring barley	Digging, hand sorting then formalin	Germany	24. Apr 08	1.0	0.0	1.0	No <i>A. longa</i> were collected or identified in this study			1.0
							09. Jul 08	1.8	2.8	4.5	0.0	0.0	0.0	4.5
							22 Oct. 08	3.0	4.5	7.5	0.0	0.0	0.0	7.5
							05. Mai 09	2.0	3.3	5.3	0.0	0.0	0.0	5.3
4	Kruk, S.	2009	000014987	Grass	Formalin /digging hand sorting	Germany	23. Apr 08	5.5	12.0	17.5	11.0	29.0	40.0	57.5
							30. Jun 08	1.0	2.0	3.0	2.0	17.0	19.0	22.0
							20. Okt 08	3.0	6.5	9.5	23.0	27.5	52.0	61.5
							30. Mrz 09	4.0	7.0	11.0	16.5	11.5	29.0	40.0
5	Klein, O.	2009	000059879	Grass	Formalin then digging 20 cm hand sorting	Germany	10-12 April 07	8.8	Juveniles not recorded separately		2.0	Juveniles not recorded separately		10.8
							5-8 June 07	10.0			2.0			12.0
							22-24 Oct 07	15.3			4.0			19.3
							09-11 April 08	12.5			2.0			14.5

Table 6 (ctd): Mean numbers of *L. terrestris* and *A. longa* from control plots in ADAMA owned field studies

	Study Author	Year	Adama Ref	System	Sampling methods	Country	Sampling Date	Control <i>Lumbricus terrestris</i> /m ²			Control <i>Aporrectodea longa</i> /m ²			Control Aneic earthworm total /m ²
								Mean No. Adults	Mean No. Juveniles	Mean total	Mean No. Adults	Mean No. Juveniles	Mean total	
6	Rozenkranz, B.	2009	000060030	Grass	Electrical (Octet) plus hand sorting	Germany	07. Mai 07	0.5	50.5	51.0	No <i>A. longa</i> were collected or identified in this study			51.0
							27. Jun 07	4.0	68.0	72.0	0.0	0.0	0.0	72.0
							22 Oct. 07	2.5	38.0	40.5	0.0	0.0	0.0	40.5
							19. Mai 08	1.0	49.8	50.8	0.0	0.0	0.0	50.8
7	Henkes, G.	2010	000065962	Grass	Formalin /digging hand sorting	Germany	14. Mai 09	5.5	8.3	13.8	No <i>A. longa</i> were collected or identified in this study			13.8
							06. Jul 09	4.0	9.3	13.3	0.0	0.0	0.0	13.3
							05. Okt 09	10.0	25.8	35.8	0.0	0.0	0.0	35.8
							02. Apr 10	12.5	28.5	41.0	0.0	0.0	0.0	41.0
8	Klein, O.	2012	000071503	Bare soil followed by clover + ryegrass	Digging, hand sorting then formalin	Germany	27-29 April 11	23.0	Juveniles not recorded separately		4.5	Juveniles not recorded separately		27.5
							28 June - 1 July 11	22.0			6.5			28.5
							12 Oct - 14 Oct 11	23.8			13.3			37.1
							7-9 May 12	22.5			10.3			32.8
9	Schulz, L.	2012	000071110	Maize	Digging, hand sorting then formalin	Germany	02. Mai 11	7.5	28.5	36.0	0.5	1.0	1.5	37.5
							14. Jun 11	5.5	15.0	20.5	0.5	2.0	2.5	23.0
							18. Okt 11	15.5	30.0	45.5	0.5	3.0	3.5	49.0
							23. Apr 12	14.0	23.5	37.5	1.0	4.5	5.5	43.0
10	Hamberger, A	2014	000075162	Grass	Digging, hand sorting then formalin	Germany	2-4 April 13	13.5	37.5	51.0	4.8	5.5	10.3	61.3
							7-8 May 13	10.0	38.3	48.3	5.5	7.5	13.0	61.3
							11-12 Jun 13	14.5	38.3	52.8	5.3	8.8	14.1	66.9
							9-10 July 13	6.5	20.6	27.1	4.3	5.3	9.6	36.7
							9-10 Oct 13	25.5	22.6	48.1	11.3	3.3	14.6	62.7
							31 Mar - 2 April 14	26.3	11.8	38.1	8.0	2.3	10.3	48.4

Table 6 (ctd): Mean numbers of *L. terrestris* and *A. longa* from control plots in ADAMA owned field studies

	Study Author	Year	Adama Ref	System	Sampling methods	Country	Sampling Date	Control <i>Lumbricus terrestris</i> /m ²			Control <i>Aporrectodea longa</i> /m ²			Control Anecic earthworm total /m ²
								Mean No. Adults	Mean No. Juveniles	Mean total	Mean No. Adults	Mean No. Juveniles	Mean total	
11	Schulz, L.	2015	000024195	Grass	Digging, hand sorting then formalin	Germany	10. Jun 13	9.5	67.0	76.5	2.5	49.0	51.5	128.0
							24. Jul 13	1.5	13.5	15.0	4.5	16.5	21.0	36.0
							13. Okt 17	12.0	12.5	24.5	10.0	26.5	36.5	61.0
							12. Mai 14	12.5	21.0	33.5	2.5	17.5	20.0	53.5
12	Schulz, L.	2017	000087754	Arable site, bare soil	Digging, hand sorting then formalin	Germany	20 Oct. 16	12.5	64.0	76.5	12.0	38.0	50.0	126.5
							23 Nov. 16	9.5	50.0	59.5	13.0	73.5	86.5	146.0
							11. Apr 17	13.5	30.5	44.0	5.0	30.5	35.5	79.5
13	Axmann, S.	2018	000085169	Bare soil with grass clover sown after application	Digging, hand sorting then formalin	Germany	12-15 April 16	12.8	40.0	52.8	No <i>A. longa</i> were collected or identified in this study			52.8
							15-20 Jun 16	9.3	27.5	36.8	0.0	0.0	0.0	36.8
							24-28 Nov 16	13.5	7.3	20.8	0.0	0.0	0.0	20.8
							7-9 Jun 17	14.5	4.0	18.5	0.0	0.0	0.0	18.5
14	Schulz, L.	2019	000083756	Grass	Digging, hand sorting then formalin	Germany	19 Apr. 17	11.5	14.0	25.5	0.0	0.0	0.0	25.5
							29. Mai 17	6.0	19.0	25.0	0.0	0.0	0.0	25.0
							25. Sep 17	9.5	32.5	42.0	0.0	0.0	0.0	42.0
							23. Okt 17	14.5	30.0	44.5	0.0	0.0	0.0	44.5
							25. Apr 18	13.5	21.5	35.0	0.0	0.0	0.0	35.0
15	Vollmer, T.	2019	000104797	Bare soil followed by clover	Digging, hand sorting then formalin	Germany	23-26 April 18	10.3	12.3	22.5	6.0	13.0	19.0	41.5
							4-7 June 18	5.3	11.5	16.8	10.5	11.8	22.3	39.1
							9-12 Jul 18	9.8	5.0	14.3	13.0	9.3	22.3	36.6
							12-14 Nov. 18	7.3	0.3	7.3	13.3	9.2	22.5	29.8
							9-14 May 19	5.5	0.3	5.5	12.3	3.2	15.5	21.0

Table 6 (ctd): Mean numbers of *L. terrestris* and *A. longa* from control plots in ADAMA owned field studies

	Study Author	Year	Adama Ref	System	Sampling methods	Country	Sampling Date	Control <i>Lumbricus terrestris</i> /m ²			Control <i>Aporrectodea longa</i> /m ²			Control Anecic earthworm total /m ²
								Mean No. Adults	Mean No. Juveniles	Mean total	Mean No. Adults	Mean No. Juveniles	Mean total	
16	Henkes, G.	2021	000108994	Bare soil, homogenised after treatment then sown with grass and clover	Formalin /digging followed by hand sorting	Germany	19-21 Mar 18	10.5	7.8	18.3	No <i>A. longa</i> were collected or identified in this study			18.3
							9-11 April 18	13.3	10.8	24.0	0.0	0.0	0.0	24.0
							23-25 May 18	20.8	5.0	25.8	0.0	0.0	0.0	25.8
							29-31 Oct 18	12.0	1.5	13.5	0.0	0.0	0.0	13.5
							18-20 Mar 19	5.3	9.5	14.8	0.0	0.0	0.0	14.8

4. DO FORAGING ANECIC EARTHWORMS ENCOUNTER AND CONSUME SLUG PELLETS AND ARE THEY AFFECTED BY IRON CONTAINING SLUG PELLETS?

4.1 SUMMARY

The literature search revealed 8 publications containing information on whether anecic earthworms consume slug pellets and whether they are affected by them. Several of these (e.g. Iglesias *et al.*, 2003) referred to metaldehyde treatments only and confirmed that even at very high rates, many times more than the application rate, metaldehyde had no harmful effects on anecic earthworms.

As the closest substance to elemental iron, iron phosphate alone was of low toxicity to earthworms with an LD₅₀ for *Eisenia fetida* of >10000 ppm. Toxicity of slug pellets containing iron phosphate to earthworms was largely due to the presence of the [REDACTED], which has an LD₅₀ for *Eisenia fetida* of 156.46 mg/kg. Formulated together with [REDACTED], iron phosphate about twice as toxic, with an LD₅₀ of 78.16 mg/kg to *Eisenia fetida*. In the ADAMA elemental iron formulation the [REDACTED] and the iron are separate and only form a [REDACTED] in the gut of slugs where the pH is low (<3). The pH values of gut extracts from 47 individual worms collected from six different sites in Germany ranged from 6.8 to 7.1 (Hom *et al.*, 2003), so the [REDACTED] might not be formed in earthworms.

1. *Lumbricus terrestris* has been observed (Gavin *et al.*, 2012) foraging on the soil surface at night and consuming slug pellets containing metaldehyde at 4% and 5% as well as pellets containing iron phosphate. It is more common for the worms to pull the baits into their burrows than to consume them on the surface. Although iron phosphate and pellets with iron phosphate and [REDACTED] were taken less readily than those with metaldehyde, Edwards *et al.* (2009) found that less than 10% of slug pellets remained on the surface after 14 days of exposure to field rate and 5 x field rate.
2. The microcosm experiments of Edwards *et al.* (2009) found no significant mortality of *L. terrestris* in response to any of the pellets containing iron phosphate over a 14 day period. However, at the recommended rate and five times this rate, pellets containing iron phosphate and the chelating agent EDTA (as Sluggo) significantly reduced weight gains by the earthworms ($P < 0.05$) compared to those which took pellets containing iron phosphate alone.
3. Analysis of the weight per individual adult *L. terrestris* and *A. longa* showed no differences between the Final Bite (elemental iron) treatment and the control. Adult worm weights at the end of the Axmann (2019) study were comparable with healthy worm weights from laboratory cultured worms on an optimal diet.

4.2 REVIEW OF RELEVANT PUBLICATIONS

Gavin *et al.* (2012) conducted forty hours of nocturnal observations after earthworms had become accustomed to the light from a 40 W incandescent bulb for two nights. Observations were limited to 2-3 h after sunset when earthworms were most active. Fresh metaldehyde or iron phosphate baits were added to the surface of the soil 2 h before dark. Four observation stations were monitored until the onset of activity, then observations commenced on the most active subjects. Observations on individuals were discontinued when earthworms withdrew into their burrows. Observations were repeated for 10 days (4 h per night) when weather conditions permitted.

Night time foraging by earthworms began several hours after sunset and continued throughout the night while *L. terrestris* foraged for litter and fine roots of decaying plants. Hunting and gathering appeared deliberate, and often involved apparent sampling of substrates that were consumed on the surface or pulled into the burrow. Many substrates were rejected during this sampling process, followed by resumed foraging until successful harvest encounters occurred or external influences caused withdrawal into the burrow.

No behavioral differences were noted when earthworms approached bait pellets relative to that which occurred when natural substrates were approached. Bait pellets were either accepted or rejected. Approximately 20% of the time pellets were engulfed entirely on the surface, while 10% of the time baits were engulfed and then rejected. The most common response involved pulling the bait into the burrow. Individuals removed as many as three bait pellets per hour. In a separate field study, 20% of the bait pellets could be recovered from the upper 25 cm of the burrow.

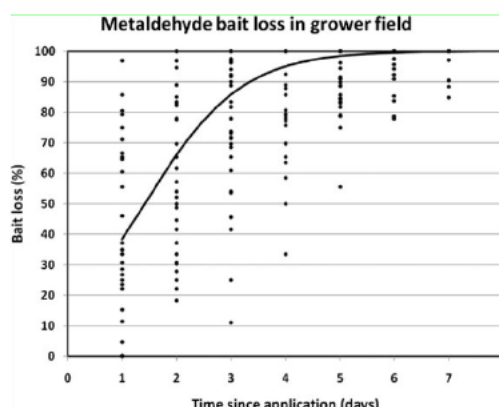


Fig. 18: Loss in the field of 4 and 5% metaldehyde bait products over time at fifteen randomly selected 0.25 m² stations on a 300-m transect visited daily until all bait pellets had been removed. From Gavin *et al* (2012).

In field studies conducted in grass seed fields of Oregon, USA, Gavin *et al* (2012) found that a mean of 17% of molluscicide bait pellets were removed nightly by earthworms. Combining observations from 3 years field studies at 17 locations it typically took between 5.1 and 6.4 days for 100% of applied pellets to be removed. Pellets containing 5% metaldehyde were removed faster than 4% metaldehyde or 1% iron phosphate pellets, possibly due to their smaller pellet size (Fig. 18).

Dörler *et al* (2019) studied the effects of different slug control measures (metaldehyde and iron phosphate pellets as well as nematodes) in microcosm containers in Austria under different watering regimes both with and without earthworms. They used 19 L plastic pots (diameter 29 cm, height 29 cm) as experimental units filled with 14 L of a commercial compost soil mixture. Three adult *L. terrestris*, with a mean fresh mass of 13.1 ± 1.8 g/pot) were introduced to half of the pots.

The three treatments were added to the pots according to the manufacturer's recommendation.

- 1) META: Metaldehyde (Schneckenkom Limex ultra, active component 30.0 g/kg metaldehyde, Scotts Celta flor, Salzburg, Austria): 4 pellets/pot added on two occasions
- 2) FE3P: Iron-III-phosphate (Ferramol Schneckenkom, active component 9.9 g/kg iron phosphate; W. Neudorff, Emmerthal, Germany): 15 pellets/pot. After the application the pots were irrigated as recommended.
- 3) NEMA: Parasitic nematode *P. hermaphrodita* (Nemaslug40 m pack, Save the Plants/Birds, UK). As recommended, applied at 0.1 L/pot of a nematode-water solution. Prior to nematode introductions pots were watered with 0.1 L/pot tap water as recommended. In total approximately 62.500 infective juvenile nematodes/pot or 1 million/m² were applied.
- 4) Control pots did not receive a slug control measure.

Neither of the molluscicide treatments resulted in any effect on earthworm activity or body weight after 9 weeks. Earthworm activity was significantly decreased ($p < 0.001$) in pots with watering every three days compared to pots with daily watering. There were no adverse effects of earthworm presence on the effectiveness of metaldehyde or iron-III-phosphate to control slugs. The authors suggest that in this study the food provided in form of hay may have been more attractive for earthworms than slug pellets or apple leaves as in other studies.

Edwards *et al* (2009) studied the relative toxicity of metaldehyde and iron phosphate-based molluscicides to earthworms in a standard laboratory assay with *Eisenia fetida* and in a microcosm study with *L. terrestris* where they looked at rates of pellet removal.

In a standard OECD artificial soil test with *Eisenia fetida* the worms were not killed by concentrations of iron phosphate as high as 10,000 ppm. By contrast, when they were exposed to chelating agents EDTA (ethylene

diamine tetracetic acid also called edetic acid) or EDDS (ethylene diamine succinic acid) they were affected by concentrations as low as 100 ppm with LD₅₀ values of 156.46 mg/kg for EDTA and 145.57 mg/kg for EDDS. When they were exposed to iron phosphate chelated with EDTA and EDDS the toxicity was even greater (LD₅₀ of 78.16 mg/kg for iron phosphate and EDTA and 82.98 mg/kg for iron phosphate and EDDS). Clearly, both the chelating agents and their complex with iron phosphate increased the toxicity of earthworms from this form of exposure considerably.

In their microcosm experiment with adult *L. terrestris* Edwards *et al* (2009) assessed the rate of disappearance of pellets from the surface of the microcosms at the equivalent of the recommended application rates and five times that rate, for a control, metaldehyde, iron phosphate, Sluggo (1% chelated iron phosphate), EDTA and EDDS (see Fig. 19 below).

In all treatments less than 10% of the pellets remained on the surface of the microcosms after 14 days of exposure and the authors confirmed that adult *L. terrestris* will consume slug pellets when exposed in microcosms.

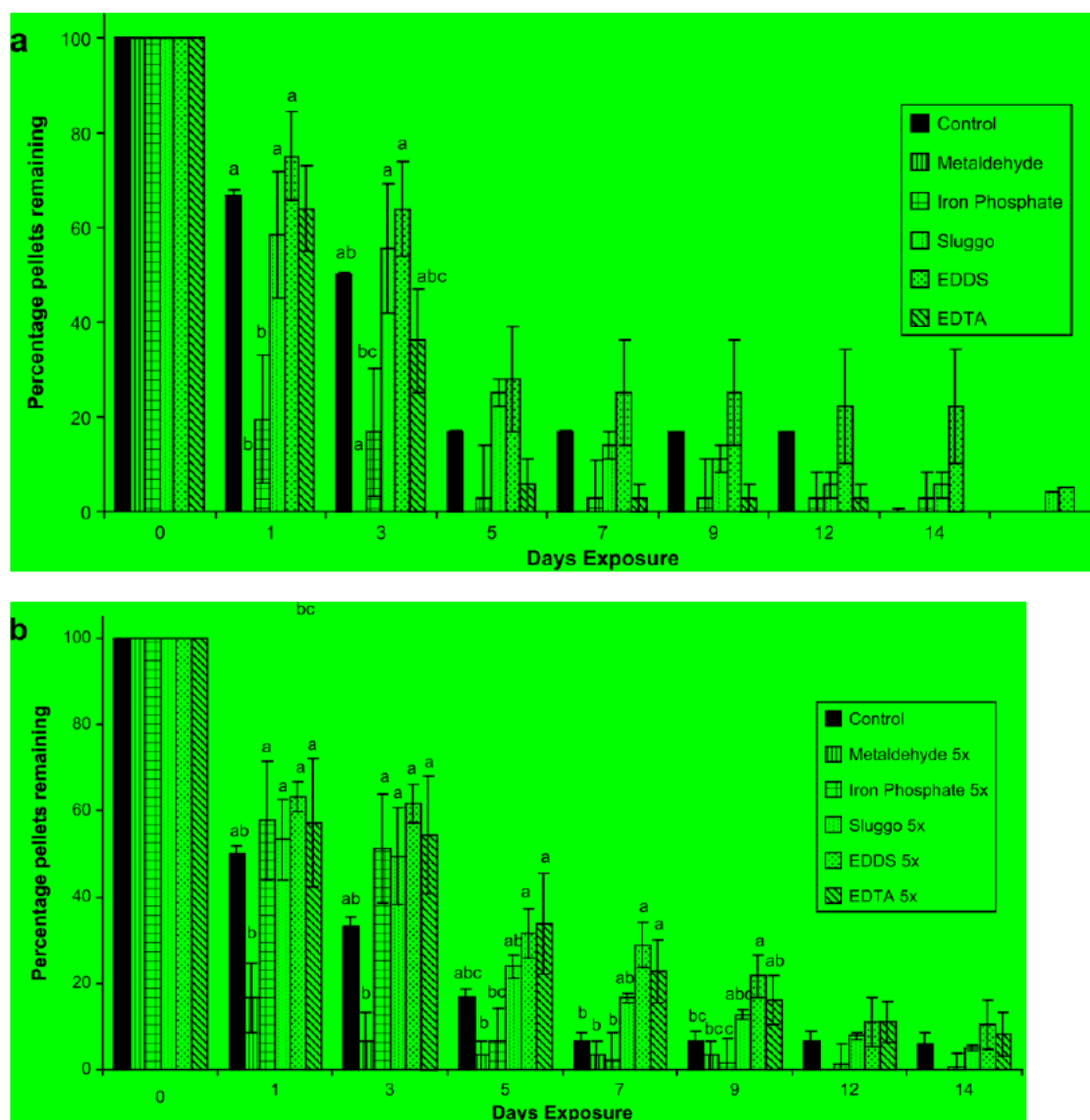


Fig. 19: (a) Percentage of pellets remaining per microcosm with molluscicides at their recommended application rate. For each date, significant differences ($P < 0.05$) between treatments are indicated by suffixes a, b, c, and; those with different letters are significantly different. Those with no letters are not significantly different.

(b) Percentage of pellets remaining per microcosm, recommended application rate x 5. For each date, significant differences ($P < 0.05$) between treatments are indicated by suffixes a, b, c, and; those with different letters are significantly different. Those with no letters are not significantly different. Reproduced from Edwards *et al* (2009).

At both the recommended and five times the recommended application rates the control pellets and metaldehyde pellets both disappeared most rapidly. Earthworms that were exposed to Sluggo (at the recommended application rate) gained significantly less weight ($P < 0.05$) than those exposed to iron phosphate only, as did those exposed to five times the recommended application rate of Sluggo. This indicates that for the Axmann (2019) field study a comparison of the adult weights between treatments per individual worm might be an appropriate way to investigate potential sub-lethal treatment related effects.

Langan and Shaw (2006) also studied the responses of *Lumbricus terrestris* (L.) to iron phosphate and metaldehyde slug pellet formulations. Juvenile *L. terrestris* were held individually in artificial burrows using the Daniel funnel method. The experimental chambers consisted of a plastic funnel (upper diameter 140 mm), covered by black tape to minimize the amount of light able to penetrate the soil used to fill the funnel. Artificial burrows (ca. 8 mm diameter) were created in the compacted soils linking the tubing with the soil surface, providing a total artificial burrow length (comprising tube and soil) of ca. 400 mm. After 5 days of starvation juvenile commercially supplied *L. terrestris* individuals were weighed and placed into the artificial burrows constructed in the funnels. Twenty slug pellets of either iron phosphate (Sluggo, Lawn and Garden Products, CA), metaldehyde (Lonza, Switzerland) or a control (a placebo containing only the bran-based pellet matrix of the metaldehyde formulation; also supplied by Lonza, Switzerland) were placed on the soil surface of each funnel which was ca. 0.015 m². Apple leaves (*Malus* sp.) collected from an organic orchard were cut into five approximately equal sized pieces (ca. 20 mm²) and also placed on the surface of the soil in funnels. Treatments were replicated 20 times, with 60 funnels in a stratified random design. Funnels were held in a wooden grid and rotated every ten days to reduce effects of location within the test area in a cold store facility kept at 15 °C under 14 hours light:10 hours dark. Funnels were monitored daily to record the number of leaves and pellets remaining. On each occasion the soil surface was lightly misted with water. After 10, 20 and 30 days all pellets and leaves were removed and replaced with 20 new pellets and five new leaf pieces. Monitoring was carried out for 33 days. At the end of the study, surviving earthworms were removed and their body mass was measured by washing them in water at room temperature and lightly drying surface water with a paper tissue.

During the first 10 days there was little activity in any treatment, with a mean 18.77 (0.25) pellets and 4.7 (0.11) leaves remaining overall. At this early stage, the number of leaves remaining did not differ significantly between treatments with the majority of earthworms in each treatment still having five leaves remaining. However, more iron phosphate pellets remained after the first 10 days than metaldehyde or control pellets, with earthworms in the iron phosphate treatment removing only one or two pellets. Earthworms exposed to iron phosphate pellets showed reduced surface activity, with lower numbers of pellets (Fig. 20i) and leaves (Fig. 20ii) being removed from the surface throughout the study.

After the first replacement of pellets and leaves (at 10 days) activity increased and earthworms exposed to metaldehyde and control (and to a lesser extent iron phosphate) had pellets in their burrows. At this time several individuals began to create additional burrows in the soil in the funnel, resulting in a churning of the soil surface, a behaviour confined almost entirely to control and metaldehyde treated funnels. Consequently, some pellets were buried rather than taken into the burrow. After the second replacement of pellets and leaves (after 20 days), surface activity further increased, with a mean of 14.79 (0.67) leaves and 2.96 (0.25) pellets remaining. At this time, fewer iron phosphate pellets were removed than control or metaldehyde pellets. The number of leaves remaining was also higher in iron phosphate funnels but, again, not significantly different between treatments.

This pattern of pellet removal was repeated from days 20–30, whereas the number of leaves removed from the surface further increased. When compared after 30 days, fewest iron phosphate pellets had been removed. At this stage of the experiment, earthworms exposed to control and metaldehyde pellets had taken significantly more leaves into the burrows, with most of the control group removing more than three leaves in this final experimental period (Fig. 20ii). There was no evidence that the mass of earthworms correlated with removal of pellets for any treatment at the end of the three observation periods.

Interpreting the results from this study with respect to potential field effects of iron phosphate slug pellets on earthworms requires some caution. The application rate used in this study for the iron phosphate treatment was 8 x higher than that of the proposed UK GAP. The authors do not state the size of the respective pellets. Gavin *et al*

(2012) stated that metaldehyde pellets were smaller than iron phosphate pellets, so these might be much easier for earthworms to remove. There was a large range in individual weights of the worms added to the funnels, from 0.97 g to 2.34 g in the iron phosphate treatment. The smaller worms may have been unable to consume whole pellets or even the apple leaf segments provided. The earthworms in the iron phosphate treatment were almost 20% smaller than control worms (Table 7). These were juvenile worms, so smaller worms would have been less able to take pellets than larger worms.

Overall, there were detectable, negative impacts of iron phosphate at 8 times the proposed application rate on the survival and growth of *L. terrestris* in artificial burrows compared with earthworms exposed to the same number of metaldehyde and control pellets.

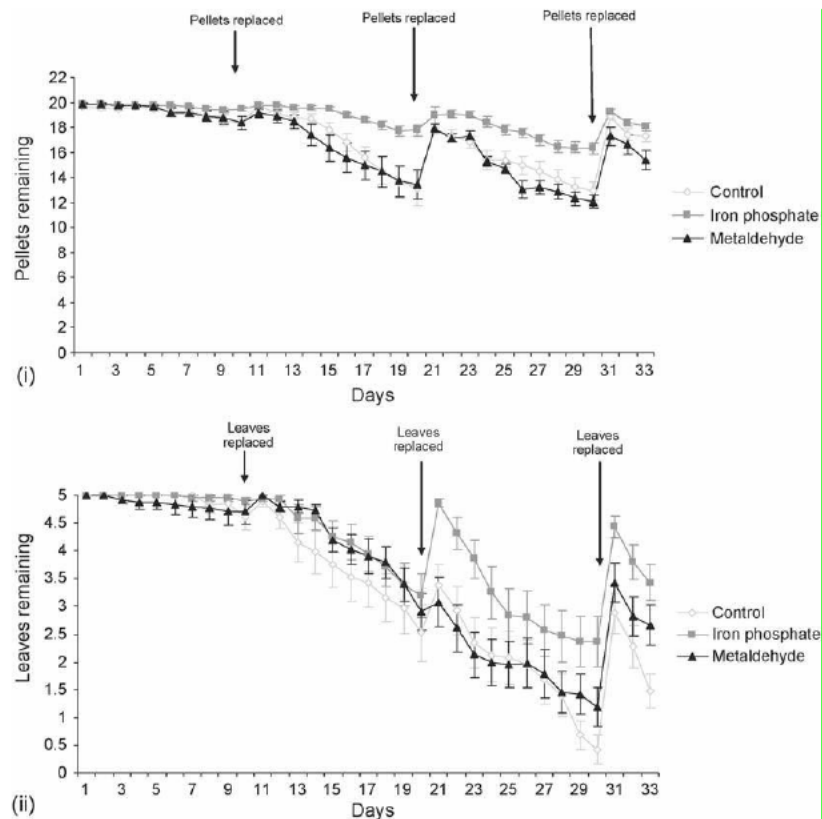


Fig. 20: Number of (i) pellets and (ii) apple leaves remaining on the soil surface, for earthworms (*L. terrestris*; n = 20 per treatment) exposed to metaldehyde, iron phosphate and control slug pellets over time. Removal of surface items indicates surface activity of earthworms in Daniel funnels. Pellets and leaves were replaced at 10, 20 and 30 days. Means (S.E.) are presented. From Langan and Shaw (2006).

Table 7: Mean earthworm masses (S.E.) after retention in Daniel funnels for 33 days with five leaves and twenty pellets on the soil surface; two molluscicides and an untreated control pellet. Taken from Gavin *et al* (2012)

Treatment	Mean initial mass (S.E.)	Initial mass range (g)	Mean final mass (S.E.)	Final mass range (g)	Mean final mass gain ^a (S.E.)
Control	2.03 g 0.15 (n = 20)	0.97–3.21	2.91 g 0.22 (n = 19)	1.23–4.81	0.86 g 0.09 (n = 19)
Iron phosphate	1.68 g 0.09 (n = 20)	0.97–2.34	2.19 g 0.11 (n = 16)	1.65–3.01	0.40 g 0.08 (n = 16)
Metaldehyde	1.81 g 0.11 (n = 20)	1.05–3.08	2.30 g 0.14 (n = 19)	1.43–3.94	0.49 g 0.08 (n = 19)

^a Final mass gains are the mean mass change of individuals that survived the full experimental period only. Pellet and leaf removal are shown for the final ten day period when differences were most pronounced.

4.3 DO THE RESULTS OF THE AXMANN (2019) FIELD STUDY SHOW ANY POTENTIAL EFFECTS OF THE IRON CONTAINING SLUG PELLETS ON *A. LONGA* AND *L. TERRESTRIS*?

4.3.1 Mean body weight per adult earthworm

Several of the publications in the literature review (notably Edwards *et al*, 2009; Langan and Shaw, 2006) reported weight loss or slower weight gain as a response of earthworm exposure to iron phosphate slug pellets with usually EDTA as a chelating agent. In the ADAMA elemental iron formulation the [REDACTED] and the iron are separate and only form a [REDACTED] in the gut of slugs, where the pH is low (<3). Earthworm guts do not have such a low pH so the [REDACTED] might not be formed.

Although the findings of Edwards *et al* (2009) were not with the same product, the mean weights per adult earthworm from the Axmann (2009) study can be calculated using the existing abundance and weight data. Juvenile data was not generated at the species level so weights per worm for juveniles cannot be combined in this way. Mean weights per adult earthworm were calculated for *A. longa* and *L. terrestris* at each sampling occasion and are tabulated together with their standard deviations and the total number of worms weighed per species at each date (n) in Tables 8 and 9.

Table 8: Mean weight of individual adult *Lumbricus terrestris* (g) in Axmann (2019) field study samples

Sampling Date	Days after A1	Control			Final Bite			Reference item		
		Mean weight per adult worm (g)	(n)	ST Dev	Mean weight per adult worm	(n)	ST Dev	Mean weight per adult worm (g)	(n)	ST Dev
18-20 April 2018	-7	3,11	53	0,61	3,32	49	0,18	3,59	47,00	0,41
28-29 May 2018	34	3,16	26	0,65	3,32	33	0,46	2,34*	8,00	0,30
19-26 June 2018	56	3,08	4	1,77	3,26	14	0,56	N/A	0,00	N/A
14-19 Nov 2018	204	5,14	32	0,18	4,96	48	0,34	4,34	6,00	1,27
25-29 Apr 2019	366	6,34	28	0,80	6,73	23	0,43	5,79	8,00	0,86

N/A = not able to be calculated. n= the total number of worms of each taxon * = sig. diff. (P<0.05) t-test

Table 9: Mean weight of individual adult *Aporrectodea longa* (g) in Axmann (2019) field study samples

Sampling Date	Days after A1	Control			Final Bite			Reference item		
		Mean weight per adult worm (g)	(n)	ST Dev	Mean weight per adult worm	(n)	ST Dev	Mean weight per adult worm (g)	(n)	ST Dev
18-20 April 2018	-7	1,30	30	0,07	1,52	35,00	0,20	1,36	27	0,20
28-29 May 2018	34	1,09	53	0,09	1,48	29,00	1,59	0,91	9	0,09
19-26 June 2018	56	1,09	30	0,18	1,17	38,00	0,14	0,97*	4	0,01
14-19 Nov 2018	204	2,15	55	0,21	2,05	84,00	0,05	1,56*	8	0,31
25-29 Apr 2019	366	2,37	13	0,57	1,90	15,00	0,22	N/A	0	N/A

N/A = not able to be calculated. n= the total number of worms of each taxon * = sig. diff. (P<0.05) t-test

For *L. terrestris* the mean weight per individual adult worm after treatment was almost identical on every sampling occasion in the control and elemental iron treatments throughout the Axmann (2019) field study. There was no indication of any treatment related weight loss due to Final Bite in adult *L. terrestris* over the duration of the study and no statistically significant differences from the control at any time point at any time point. Individual *L. terrestris* mean weights increased from 3.11 g per individual to 6.34 g in the control and from 3.32 g to 6.73 g in the worms from the elemental iron treated plots. These final mean weights of individual adult *L. terrestris* compare favourably with those of Butt (2011), see Fig. 3, for worms fed an optimal diet including horse manure. A significant reduction in mean adult *L. terrestris* weights occurred in the reference item treatment 34 days after the first application of treatments.

For *A. longa* the mean weight per adult worm was almost identical on all except the final sampling occasion in the control and elemental iron treatments and no statistically significant differences from the control occurred at any time point. There was no indication of any treatment related weight loss in adult *A. longa* over the duration of the study. Individual *A. longa* mean weights increased from 1.30 g per individual to 2.37 g in the control and from 1.52 g to 1.90 g in the worms from the elemental iron treated plots. On the final sampling occasion, the number of adults in control and test item treated plots was relatively low (means of 3.3 and 3.8/m² respectively) so the weights per worm at this time point must be considered with caution. Significant reductions in mean adult *A. longa* weights occurred in the reference item treatment 56 and 204 days after the first application of treatments.

When the weights per adult *A. longa* from control plots in the ADAMA owned field studies (shown in Table 6) are considered together, the individual weight in samples taken in spring-early summer was 1.48 g/worm (Standard Deviation 0.35 g). The mean control autumn weight was 1.98 g/worm (Standard deviation 0.35 g) and the mean control weight at study end (spring or early summer) was 1.88 g/worm (Standard deviation 0.61 g). The weight of adult *A. longa* in the Final Bite treatment at the study end (1.87 g/worm) almost exactly matches the mean end of study spring weight over many studies (1.88 g/worm).

4.3.2 Other studies with iron formulations

The final addendum to the Renewal Assessment Report for Ferric Phosphate (EFSA, 2014) contains the results from two field studies in Germany, Luhrs, (2009) and Luhrs (2010) conducted in accordance with the same Guideline as the Axmann (2019) study with elemental iron. Although reported separately it is clear from the identical control and reference item data that these are in fact from the same study. The results for the two tested ferric phosphate +EDTA formulations are reproduced below in Table 10.

Table 10: Abundance of *L. terrestris* adults (n/m²) in Luhrs (2009) and Luhrs (2010) field study with Ferric phosphate (from RAR, 2014)

Treatments	Pre-treatment	4 weeks after Application 1	7 months after Application 1	12 months after Application 1
Control	13.0	36.0	17.3	16.0
NEU 1166 M 200 kg*/ha	18.0	42.0	22.5	22.5

NEU 1181 M 28** kg/ha	26.0	46.5	36.8	18.0
Reference item	19.5	7.0	5.8	0.5

* Ferric phosphate as 9 g as/kg analysed (containing EDTA as a chelating agent) applied at an equivalent to 2.0 kg a.s./ha.

** Ferric phosphate as 30.61 g as/kg analysed, containing EDTA as a chelating agent applied at an equivalent to 0.84 kg a.s./ha.

Both formulations of ferric phosphate+EDTA shown in Table 10 had no adverse effects on adult *L. terrestris* and numbers in the test item treated plots exceeded those in the control plots on every sampling occasion up to one year after treatment.

5. OVERALL CONCLUSIONS

CLIMATIC CONDITIONS

The optimum soil moisture content for anecic earthworm species from the literature was 25 -30%, both *L. terrestris* and *A. longa* prefer a soil moisture content above 14%. The optimum temperature is considered to be 15 °C for *A. longa* and 15-20 °C for *L. terrestris*, with incubation of cocoons occurring best at 20 °C (Butt *et al* 1992). The soil moisture in the Axmann (2019) field study over the 53 days after the first application was favourable for anecic earthworms.

The soil temperature at 5 cm depth was optimal for both anecic species during the first 28 days after the first application of slug pellets, with only a few daily maxima above 20 °C and none above 25 °C. For the period from 28 days to 50 days after the first application the daily maximum soil temperature at 5 cm was consistently higher than 25 °C and this would have been sub-optimal for both species. However, both *A. longa* and *L. terrestris* adults would have been present in deep vertical burrows and able to avoid exposure to these conditions.

The soil temperature at 20 cm depth for the first four weeks of the study, when exposure to the slug pellets would have been greatest were close to optimal for anecic earthworms. Over a two week period (from 34 to 48 days after the first application) the mean soil temperature at 20 cm was slightly higher than 20 °C but never reached the lethal thresholds of 25.7 °C for *A. longa* and 28 °C for *L. terrestris*.

Air temperatures over the 53 nights after the first application of treatments appear to have been favourable for foraging by anecic worms. The maximum record daily air temperatures would have occurred during the day when all earthworms would have been found in the soil. The mean air temperature only rose above 20 °C on three occasions and the minimum air temperature, presumably recorded after many hours of darkness on clear nights, was mostly between 5 and 10 °C for the first month and then between 10 and 15 °C for the second month of the study.

ABUNDANCE OF *A. LONGA* AND *L. TERRESTRIS* IN THE UK AND IN THE AXMANN (2019) FIELD STUDY

The abundance of both *L. terrestris* and *A. longa* in UK agricultural soils appears to be highly variable and depends largely on historical land use and the extent of cultivation. *A. longa* is more widely distributed and common in agricultural soils than *L. terrestris*. Typical levels of abundance for both anecic species ranged from 1 to 20 individuals/m² but could be as low as 0 or as high as 50/m². Populations of both anecic species are consistently higher in grassland and consistently lower in arable systems.

Numbers of adult *L. terrestris* from the Axmann (2019) study ranged from 1.0 to 13.3/m² in the control plots and 3.5 to 12.3/m² in the Final Bite treated plots. Similarly, numbers of adult *A. longa* ranged from 3.3 to 13.8/m² in the control plots and 3.8 to 21.0/m² in the Final Bite (elemental iron) treated plots. These abundance levels for adults are very close to those reported for adults and juveniles by Hutcheon *et al* (2001) from a field study conducted at Long Ashton in the UK.

DO FORAGING ANECIC EARTHWORMS ENCOUNTER AND CONSUME SLUG PELLETS AND ARE THEY AFFECTED BY IRON CONTAINING SLUG PELLETS?

In microcosm studies *L. terrestris* were observed at night consuming slug pellets and can therefore be exposed to iron phosphate. The microcosm experiments of Edwards *et al* (2009) found no significant mortality of *L. terrestris* in response to any of the pellets containing iron phosphate over the 14 days of the experiment. However, at the recommended rate and five times this rate, pellets containing iron phosphate and the chelating agent EDTA (as Sluggo) significantly reduced weight gains by the earthworms ($P < 0.05$) compared to those which took pellets containing iron phosphate alone.

When individual adult earthworm weights from the Axmann (2019) study were evaluated, there was no difference between the control and the Final Bite (elemental iron) treatment on any sampling occasion for either *A. longa* or *L. terrestris*.

In the ADAMA elemental iron formulation the [REDACTED] and the iron are separate and only form a [REDACTED] in the gut of slugs where the pH is low (< 3). Earthworm guts have a typical pH of 7.0 so the [REDACTED] might not be formed when the pellets are consumed by earthworms.

6. REFERENCES

- Baker, G.H., Carter, P.J., Barrett, V.J. (1999) Survival and biomass of exotic earthworms, *Aporrectodea* spp. (Lumbricidae), when introduced to pastures in south-eastern Australia. *Australian Journal of Agricultural Research* 50, 1233–1245.
- Baker, G. H.; Whitby, W. A., (2003) Soil pH preferences and the influences of soil type and temperature on the survival and growth of *Aporrectodea longa* (Lumbricidae). *Pedobiologia*, 47, 754–753.
- Bastardie, F.; Capowiez, Y.; de Dreuz, J. R.; Cluzeau, D. (2003) X-ray tomographic and hydraulic characterization of burrowing by three earthworm species in repacked soil cores. *Applied Soil Ecology*, 24, 3–16.
- Berry, E. C., & Jordan, D. (2001). Temperature and soil moisture content effects on the growth of *Lumbricus terrestris* (Oligochaeta: Lumbricidae) under laboratory conditions. *Soil Biology and Biochemistry*, 33, 133–136.
- Boag, B.; Palmer, L. F.; Neilson, R.; Legg, R.; Chambers, S. J. (1997) Distribution, prevalence and intensity of earthworm populations in arable land and grassland in Scotland. *Annals of Applied Biology*, 130, 153–165.
- Bouché, M.B., (1972). *Lombriciens de France: Ecologie et Systématique*. INRA Ann. Zool. Ecol. Anim. Publication, France, 671 pp.
- Bouché, M.B., (1977) Stratégies lombriciennes. In: Lohm, U., Persson, T. (Eds.), *Soil Organism as Components of Ecosystems*. Biol. Bull. (Stockholm), pp. 122–132.
- Briones, M. J. & Schmidt, O. (2017) Conventional tillage decreases the abundance and biomass of earthworms and alters their community structure in a global meta-analysis. *Glob Change Biol*. 2017;23:4396–4419
- Butt, K.R. (1991) The effects of temperature on the intensive production of *Lumbricus terrestris* (Oligochaeta: Lumbricidae). *Pedobiologia*, 35, 257–264.
- Butt, K. R.; Frederickson, J.; Morris, R. M. (1992) The intensive production of *Lumbricus terrestris* L. for soil amelioration. *Soil Biology and Biochemistry*, 24, 1321–1325.
- Butt, K. R. (1993). Utilisation of solid paper-mill sludge and spent brewery yeast as a feed for soil dwelling earthworms. *Bioresource Technology*, 44, 105–107.
- Butt, K. R., Nuutinen, V., & Siren, T. (2003). Resource distribution and surface activity of adult *Lumbricus terrestris* L. in an experimental system. *Pedobiologia*, 47, 548–553.
- Butt, K. R. (2011). Food quality affects production of *Lumbricus terrestris* (L.) under controlled environmental conditions. *Soil Biology and Biochemistry*, 43, 2169–2175. <https://doi.org/10.1016/j.soilbio.2011.06.021>
- Curry, J. P., Byrne, D., & Schmidt, O. (2002). Intensive cultivation can drastically reduce earthworm populations in arable land. *European Journal of Soil Biology*, 38, 127–130.
- Daniel, O., Kohli, L., & Bieri, M. (1996). Weight gain and weight loss of the earthworm *Lumbricus terrestris* L. at different temperatures and body weights. *Soil Biology and Biochemistry*, 28, 1235–1240. [https://doi.org/10.1016/0038-0717\(96\)00121-6](https://doi.org/10.1016/0038-0717(96)00121-6)
- Daughbjerg, P. (1988) Temperature and moisture preferences of three earthworm species. *Pedobiologia*, 32, 57–64.
- Dörler, D.; Scheucher, A.; Zaller, J. G. (2019) Efficacy of chemical and biological slug control measures in response to watering and earthworms. *Scientific Reports*, 9, 1 <https://doi.org/10.1038/s41598-019-39585-5>
- Edwards, C. A.; Arancon, N. Q.; Vasko-Bennett, M.; Little, B.; Askar, A. (2009) The relative toxicity of metaldehyde and iron phosphate-based molluscicides to earthworms. *Crop Protection*, 28, 289–294.
- Edwards, C.A., Bohlen, P.J., (1996). *Biology and Ecology of Earthworms*, 3rd ed. Chapman and Hall, London, p.89–111

Euteneuer, P.; Wagentristsl, H.; Steinkellner, S.; Fuchs, M.; Zaller, J. G.; Piepho, H. P.; Butt, K. R. (2021) Contrasting effects of cover crops on earthworms: Results from field monitoring and laboratory experiments on growth, reproduction and food choice. *European Journal of Soil Biology*, 100, 103225. <https://doi.org/10.1016/j.ejsobi.2020.103225>

Evans, A.C., Guild, W.J. Mc. L., (1948) Studies on the relationships between earthworms and soil fertility IV. On the life cycles of some British Lumbricidae. *Annals of Applied Biology* 35, 471-484.

Forster, A. and Salaun, F. (2003) Field study to evaluate the effects of Endosulfan 35 EC (AE F002671 EC33 C702 and AE F002671 EC33 C70) on earthworms in a grass field in Cornwall, U.K. Ecotox Ltd. Tavistock, Devon, U.K. Report ER01-KCB146, Bayer Crop Science, Unpublished report, Adama Ref.: 000058735

Gavin, W. E.; Mueller-Warrant, G. W.; Griffith, S. M.; Banowetz, G. M. (2012). Removal of molluscicidal bait pellets by earthworms and its impact on control of the gray field slug (*Derocerus reticulatum* Mueller) in western Oregon grass seed fields. *Crop Protection*, 42, 94-101.

Gerard, B.M., (1967) Factors affecting earthworms in pastures. *Journal of Animal Ecology* 36, 235-252.

Grigoropoulou, N., Butt, K. R., & Lowe, C. N. (2008). Effects of adult *Lumbricus terrestris* on cocoons and hatchlings in Evans' boxes. *Pedobiologia*, 51, 343–349.

Holden, J.; Grayson, R. P.; Berdeni, D.; Bird, S.; Chapman, P.J.; Edmondson, J. L.; Firbank, L. G.; Helgason, T.; Hodson, M. E.; Hunt, S. F. P.; Jones, D. T.; Lappage, M. G.; Marshall-Harries, E.; Nelson, M.; Prendergast-Miller, M.; Shaw, H.; Wade, R. N.; Leake, J. R. (2019) The role of hedgerows in soil functioning within agricultural landscapes. *Agriculture Ecosystems & Environment*, 273, 1-12

Holmstrup, M., Estergaard, I.K., Nielsen, A., Hansen, B.T., (1996). Note on the incubation of earthworm cocoons at three constant temperatures. *Pedobiologia* 40, 477-478.

Holmstrup, M. (1999) Cocoon production of *Aporrectodea longa* and *Aporrectodea rosea* (Oligochaeta; Lumbricidae) in a Danish grass field. *Soil Biology and Biochemistry*, 31, 957-964.

Hoogerkamp, M., Rogaar, H., Eijssackers, H.J.P., (1983). Effect of earthworms on grassland on recently reclaimed polder soils in The Netherlands. In: Satchell, J.E. (Ed.), *Earthworm Ecology—From Darwin to Vermiculture*. Chapman and Hall, London, pp. 85–105.

Horn, M. Schramm, A. and Drake, H. (2003) The earthworm gut: an Ideal Habitat for Ingested N₂O-Producing Microorganisms. *Appl Environ Microbiol* 2003 Mar; 69(3): 1662–1669. doi: 10.1128/AEM.69.3.1662-1669.2003

Hutcheon, J. A.; Iles, D. R.; Kendall, D. A. (2001) Earthworm populations in conventional and integrated farming systems in the LIFE Project (SW England) in 1990-2000. *Annals of Applied Biology* 139, 361-372

Iglesias, J.; Castillejo, J.; Castro, R., (2003) The effects of repeated applications of the molluscicide metaldehyde and the biocontrol nematode *Phasmarhabditis hermaphrodita* on molluscs, earthworms, nematodes, acarids and collembolans: a two-year study in north-west Spain. *Pest Management Science*, 59, 1217-1224.

Jefferson, P. (1959) Earthworms and turf culture. *The Journal of the Sports Turf Research Institute* 10, 276–289.

Johnston, A., Sibly, R. and Thorbeck, P. (2018) Forecasting tillage and soil warming effects on earthworms. *Journal of Applied Ecology*, 55, 1498-1509

Kanianska, R.; Jad'ud'ova, J.; Makovnikova, J.; Kizekova, M. (2016) Assessment of Relationships between Earthworms and Soil Abiotic and Biotic Factors as a Tool in Sustainable Agricultural. *Sustainability*, 8, 906.

Khan, M. A.; Ahmed, S. A.; Salazar, A.; Gurumendi, J.; Khan, A.; Vargas, M.; von Catalin, B., (2007)

Effect of temperature on heavy metal toxicity to earthworm *Lumbricus terrestris* (Annelida: Oligochaeta). *Environmental toxicology*, 22, 487-494.

Khan, M. A. Q.; Khan, M. A.; Hurlock, P.; Ahmed, S. A. (2012), Physiological responses to temperature and haeme synthesis modifiers in earthworm *Lumbricus terrestris* (Annelida: Oligochaeta). *Environmental Toxicology*, 27, 1-10.

Langan, A. M.; Shaw, E. M. (2006) Responses of the earthworm *Lumbricus terrestris* (L.) to iron phosphate and metaldehyde slug pellet formulations. *Applied Soil Ecology*, 34, 184-189

Laverack, M.S. (1961) Tactile and chemical perception in earthworms – II. Responses to acid pH solutions. *Comparative Biochemistry and Physiology* 2, 22–34.

Lee, K.E. (1985) Earthworms. Their ecology and relationships with soils and land use. Academic Press, Sydney. 36-48.

Lowe, C. N.; Butt, K. R (2005) Culture techniques for soil dwelling earthworms: A review. *Pedobiologia*, 49, 401-413.

Mather, J.G., Christensen, O., (1988). Surface movements of earthworms in agricultural land. *Pedobiologia* 32, 399–405.

Meller, M., 2022. Report: Systematic literature search with respect to specific aspects of earthworm ecology & ecotoxicity of slug baits in the UK (ADAMA reference number 000110089)

Miles, H.B. (1963) Heat-death temperature in *Allolobophora terrestris* (Sav.) forma *longa* (Ude) and *Eisenia foetida* (Sav.). *Nature* 199, 826.

Moreau-Valancogne, P.; Bertrand, M.; Holmstrup, M.; Roger-Estrade, J., (2013), Integration of thermal time and hydrotime models to describe the development and growth of temperate earthworms. *Soil Biology & Biochemistry*, 72, 66-74.

Nordstrum, S. (1975) Seasonal activity of lumbricids (Lumbricidae) in southern Sweden. In: Burges, A., Raw, F. and Satchell, J.E. (1967) *Oikos* 26, 307–315. (eds) *Soil Biology*. Academic Press, London, pp. 259–322.

Pelosi, C., Bertrand, M., Makowski, D. and Roger-Estrade, J., (2008) WORMDYN: A model of *Lumbricus terrestris* population dynamics in agricultural fields. *Ecological Modelling* 218 (2008) 219–234

Perreault, J. M.; Whalen, J. K., (2006) Earthworm burrowing in laboratory microcosms as influenced by soil temperature and moisture. *Pedobiologia*, 50, 397-403.

Prendergast-Miller, M. T.; Jones, D. T.; Berdeni, D.; Bird, S.; Chapman, P. J.; Firbank, L.; Grayson, R.; Helgason, T.; Holden, J.; Lappage, M.; Leake, J.; Hodson, M. E. (2021) Arable fields as potential reservoirs of biodiversity: Earthworm populations increase in new leys. *The Science of the total environment*, 789, 147880. <https://doi.org/10.1016/j.scitotenv.2021.147880>

Rozencranz, B. and Meinerling, M. (2009) Field study to evaluate the effects of chlorpyrifos plus betacyfluthrin granules on earthworms. Ibacon Report No.: 34366023 Irvita Plant Protection unpublished report. Ref. No. 000060030

Sheppard, D. (2014) Earthworms in England: distribution, abundance and habitats. Natural England Commissioned Report NECR145

Sims, R.W. & Gerard, B.M. (1999). Earthworms. Synopsis of the British fauna (New Series), No. 31 (revised). Field Studies Council, Shrewsbury. P.32, P.106-108.

Sizmur, T.; Martin, E.; Wagner, K.; Parmentier, E.; Watts, C.; Whitmore, A. P., (2017) Milled cereal straw accelerates earthworm (*Lumbricus terrestris*) growth more than selected organic amendments. *Applied soil ecology: a section of Agriculture, Ecosystems & Environment*, 113, 166-177.

Tsukamoto J. and Watanabe H. (1977) Influence of temperature on hatching and growth of *Eisenia foetida* (Oligochaeta Lumbricidae). *Pedobiologia* 17, 338—342.

van Capelle, C., Schrader, S., & Brunotte, J. (2012). Tillage-induced changes in the functional diversity of soil biota—a review with a focus on German data. *European Journal of Soil Biology*, 50, 165–181.