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Toxicity of fungicides to terrestrial non-target fauna – Formulated products versus active ingredients (azoxystrobin, cyproconazole, prothioconazole, tebuconazole) – A case study with Enchytraeus crypticus (Oligochaeta)

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20 November 2026

Toxicity of fungicides to terrestrial non-target fauna – formulated products versus active ingredients (azoxystrobin, cyproconazole, prothioconazole, tebuconazole) – a case study with *Enchytraeus crypticus* (Oligochaeta)

Susana I.L. Gomes [a1], Anna Ammendola [a1], Silvia Casini [b] and Mónica J.B. Amorim [a]*

[a] Department of Biology & CESAM, University of Aveiro, 3810-193 Aveiro, Portugal

[b] Department of Physical, Earth and Environmental Sciences, University of Siena, via Mattioli, 4, 53100 Siena, Italy

[1] Both first authors

* corresponding author at: Department of Biology & CESAM, University of Aveiro, 3810-193 Aveiro, Portugal. E-mail address: mjamorim@ua.pt (MJB Amorim)

***Responses to Reviewers Comments**

Answers to reviewers' comments

Reviewers/Editor comments:

Reviewer #1: This manuscript reports on the toxicity of Amistar® XTRA and Prosaro® 250 EC and pure active ingredients (a.i.s): azoxystrobin, cyproconazole, prothioconazole, and ebuconazole on Enchytraeus crypticus. Endpoints were the LC and EC for toxicity. The authors conclude that compared with Amistar, Prosaro exhibited a higher toxicity. And compared with formulations, a.i.s exhibited a higher toxicity. The work appears to have been carefully performed and appropriately analyzed on evaluating the toxicity of formulations and a.i.s respectively, while for whole study it is better to add other biomarkers to explain the difference of toxicity between formulation and a.i.s. I suggested a major revision before publication.

Other comments as follow:

1. Abstract: in this part the indictors of reproductive effects were LC50 and EC50, while in Discussion, you focused on the LC50 and EC50.

Answer: Done. We think it is meant EC10 is mentioned in the abstract while the discussion focus on EC50. We have updated the abstract to EC50s instead of EC10 values.

2. Line 42-44, there is no logical connection between this sentence and the previous one. Please delete or rewrite this sentence.

Answer: Done. The sentence was reorganised. Please see the alterations in the manuscript.

3. ca?

Answer: Done. It was corrected to "ca." which is the commonly used abbreviation of *circa* meaning approximately.

4. Line 77-78, why you chose Amistar and Prosaro? Are these two formulation top 2 best selling products in cereal culture productive market? Please provide detail data.

Answer: we have no information on the top sell, but they are widely used across the world to protect cereal cultures, among other possible applications, while cotoxicity information is limited.

5. Line 125-130: it is strange for 0-9.6-29.9-96-299-960, please use 0, 9.6, 29.6, 96, 299, 960, the same with others.

Answer: Done. We have updated the format.

6. Results: Please provide information on growth in the controls. Please provide a graph of the fitted curves to allow assessing whether the derived EC50 values originate from a good fit.

Answer: Growth is not an endpoint assessed in the Enchytraeid Reproduction Test. The lines in the graphs provided (Fig 1) are the fitted curves, models are mentioned in data analysis, in the caption we say: "Lines represent the model fit to data".

7. Line 239: Please explain LC50 at it first appeared position in your manuscript.

Answer: Done. The explanation was added at its first appearance in the Introduction.

8. Line 281-283, you said "adjuvants effects", please use specific analyze method or equation to define the effect, the same with line 258 low antagonism effect.

Answer: We are not clear about the requested: we say that the toxicity of Prosaro, which is not totally explained by any of its active ingredients "can be due to toxic effects caused by the adjuvants (often called inert ingredients)", that is, the adjuvants are the other ingredients present in the formulation in addition to the active ingredients. The meaning/definition of antagonistic effect is also provided "(i.e., the combined effect of two or more chemicals is less than the sum of the effects of each individually)". We have not designed the experiment as a mixture toxicity so we did not engage onto mixture toxicity models.

Reviewer #2: This paper deals with a study into the toxicity of two commercial pesticide products as compared to their active ingredients to Enchytraeus crypticus. The paper is within the scope of STOTEN and overall reads well. Besides a number of minor issues, my main comment(s) are related

with the interpretation of the results (see below). However, I consider that after minor revisions, the MS will be acceptable for publication in STOTEN.

Graphical abstract: Rather unclear without having read the MS. Maybe simplifying it may aid
Answer: Done. We have tried to improve, added reproduction effect curves title and updated the symbols between graphs.
Title: I suggest changing "fungicide in" to "fungicides to"
Answer: Done.
Abstract
Ln 15: Maybe use "soil dwelling organisms"
Answer: Done.
Ln 16: I suggest changing "however the ecotoxicity knowledge is" to "however, their risks are"
Answer: Done.
Ln 19 and throughout the MS: "formulations" may lead to the misinterpretation of the readers that you are only evaluating the formulations (i.e. without the a.i.). "Pesticide products" or "formulated products" (eventually using FP as acronym after first mention) would be more correct in my opinion. This thus also needs to be amended in the title.
Answer: Done. We used formulated products (FPs) and changed throughout.
Ln 24 ff: Please check spaces throughout the abstract (and probably MS), e.g. "EC10 = 38 mg" instead of "EC10=38mg"
Answer: Done. It was corrected throughout the manuscript.
Introduction
Ln 46: Please check brackets at citations throughout the MS. This is probably the result of using Mendeley. See also Ln 54, which has a bracket too many.
Answer: Done. It was corrected throughout the manuscript.
Ln 57: Instead of having this long (and highly relevant) text in brackets, I suggest starting a new sentence here (at Lumbricidae) Lns 65-67: In the EU, the prospective ERA of PPPs requires the evaluation of both the a.i. and the formulated product separately already for several years. This should be discussed here and could be used to show the importance of your study, e.g. the need of this.
Answer: Done. Thank you for the note, it was incorporated in the manuscript.
Ln 72: Add "the" before "two"
Answer: Done.
Ln 77: "fungicide formulations" (no need for the "s" in fungicide to indicate that this is plural)
Answer: Done.
Ln 89: Change "in" to "to"
Answer: Done.
Ln 91: "absence of data" instead of "absent data"
Answer: Done.
Ln 98: Maybe use "imperative" instead of "very high"
Answer: Done.
Material and Methods
Ln 111: delete comma before "organic"
Answer: Done.
Ln 116 ff: Please use a dot (.) for decimals instead of a comma
Answer: Done.
Ln 119 (and throughout MS): Names of the active ingredients should not start with a capital letter (only the names of the formulated products).
Answer: Done.
Ln 136: "their" instead of "its"
Answer: Done.

Lns 135-138: Can you assure that the difference in the way the formulated products and a.i. were added to the soil did not influence the exposure levels? Given that the main aim of this study was to compare these, it is a pity that no chemical analyses of the test compounds were made.
Answer: yes, that is a good point and we cannot ensure the same exposure levels to the animals. We followed the OECD guidelines (OECD 220, 2016) recommendations for spiking soil with compounds that are not water soluble, including the appropriate solvent (acetone) controls to assess effects on organisms (even though several previous studies showed acetone caused no effects on *E. crypticus* survival or reproduction). Hence we believe this is the way comparison can be done and should not be the source for total concentration variation. Exposure concentration is a different aspect and can differ because of differences in viscosity, water solubility and bioavailability issues, etc.. But we don't think there is an easy solution for this and an available method.
Ln 165: delete comma before "for"
Answer: Done.
Results
Figure 1 (1): Why are the (pooled) control data not presented in the Figure? For reproduction, the data are presented as % of control, whereas absolute numbers are presented for adult mortality. What is the rationale for presenting these results differently (i.e. why not both % of control or absolute values)? I would prefer to see the actual absolute numbers as also to understand the absolute control values (see also comment below)
Answer: The pooled control data are presented in the figure (concentration 0 mg/kg), the symbols might be difficult to differentiate at the lower concentrations because of the higher density of data. The reproduction data as % of control was needed for a comparison between all (not data as in survival, that is always 10 out of 10). We can make all absolute values (or all %) but that would disturb the comparison across materials in the endpoint reproduction. This does not change the ECs in any of the options of course. We can provide a table with the absolute values in SI if of interest.
Figure 1 (2): Looking at the data presented, there is hardly any difference between the a.i. and formulated products in my opinion. This difference appears larger in terms of generated LC50 and EC50 since there were hardly any concentrations that did not yield an effect between 0% and 100% (i.e. almost all are either 0% or 100% effects). This means that small differences will have a huge effect on the curves and hence the generated toxicity data. For Amistar, the lethal effects are simply driven by azoxystrobin and the difference between 299 Amistar and 55 azoxystrobin could be related with natural variation/chance, for which it would be good to show the control data. The reproduction effects between Amistar and azoxystrobin are exactly the same. For Prosaro, the 2 out of 10 (!) survival (with 0/10 survival for tebuconazole) at the one but highest concentration treatment is the only difference between Prosaro and tebuconazole, but has a huge effect on the toxicity value (and hence apparent but inexistent difference between the f.p. and a.i.). It should also be considered here that this small difference could be due to differences in the application method of the f.p. and a.i. For reproduction, the effects of Prosaro is completely due to teuconazole. All in all, my conclusion here would be that there is no significant (relevant) difference at all between the a.i. and f.p.
Answer: We do tend to agree. In terms of reproductive effects, for both the formulated products, they seem explained by one of the a.i.s, as discussed in the manuscript.
Regarding the effects on survival, for Amistar, despite the differences in the LC50 for azoxystrobin, we cannot rule out natural variation (and that was added in the manuscript), but it can as well be a reduction in toxicity in the FP, as discussed and supported by the literature for other pesticides. For tebuconazole, unfortunately we did not catch a concentration causing intermediate effects on survival (NOEC of 78 mg/kg and LC100 of 250 mg/kg). However for Prosaro, the concentration 615 mg/kg – corresponding to 78 mg tebuconazole/kg – caused 80% mortality (note that 78 mg/kg tebuconazole alone did not cause any effect), thus indicating the higher toxicity of the FP – so we do think this is a case to consider.
Discussion

General: Please check the use of spaces and brackets. I think the authors did a great job in comparing their results with previous studies, despite the few data that are available and compatibility issues due to differences in experimental design.
Answer: Thank you for your comment. The use of spaces and brackets was checked.
Ln 255 ff: See my long comment above for Figure 1. The authors acknowledge that the differences between the a.i. and f.p are small, but I think the issues that I raise above could be incorporated here.
Answer: Done. Thank you for your comment. We have incorporated some of the notes in the manuscript.
Lns 265-267 (and elsewhere): The comparisons in toxicity made between compounds is valid. However, I would have liked to have seen some text on what this means in terms of ERA. For example, a less toxic compound, but applied at higher doses is evidently a greater (risk) problem that a highly toxic compound applied at a dose that is not likely to exert side effects on non-target organisms.
Answer: Yes, we added one line following the acute toxicity differences. We don't think we have the data to support the mentioned discussion in terms of application dosages and other non-target species.
Ln 281: Same here.
Answer: As above.
Ln 301: See my comment made in the introduction section. Please note that in EU both a.i. and f.p. need to be evaluated in the prospective ERA.
Answer: Done. Yes, thank you. The suggestions were incorporated in the manuscript.

Reviewer #4: The study investigates the toxicity of formulations versus active ingredients of fungicides, toward a highly abundant soil organism. The study is clearly presented, easily understood, and a highly valuable input to the ongoing discussion on how to evaluate PPP on non-target organisms. It is well fitted for the STOTEN journal.

Please find following suggestions:
General:
In the introduction and discussion it would likely be relevant to refer to Frampton et al 2006 review - in which the differences between various PPP are evaluated and where the importance of getting to a situation (i.e. more species) where the species sensitivity distribution on PPP can be used.
Throughout - check a.i.s. and a.i.s, a more understandable way would be to use only a.i.(s) where relevant.
Answer: Done. Thank you for the suggestions, they were incorporated.
Abstract:
Update this after the below revisions.
Answer: Done.
Introduction:
Line 45-47: Although E. fetida is the most studied this does not make it well studied in this context. Please just refer that it is the most studied, also because it is the organism required by e.g. EU regulation.
Answer: Done. The sentence was rephrased. Please see the alterations in the manuscript.
Line 55-60: A general sensitivity difference between species does probably not exist, hence please delete this section. The abundance, difference in life style and in testing regime is fully adequate arguments for testing of enchytraeids.
Answer: Done.
Line 63: Insert "often" before the word "lacking" - it is not always lacking but often so, although in certain study types may be conducted with the formulation.
Answer: Done.

Line 80: Delete "low" - low offers no insight and the concentration can from another viewpoint be seen as high.
Answer: Done.
Materials and Methods:
No comments - they are clear.
Results:
Line 176: Figure B No. adults - it may be beneficial to show the "Tebuconazole" values on the figure. It is well taken that they are there but covered by the "Prothioconzole" dots. Perhaps this could be solved technically by moving the "Tebuconazole" values to 10,1 for the values where they are 10 (this obviously should just be commented in the legend).
Answer: Done. Thank you for the note, symbols for Prothioconazole (and Cyproconazole) were changed to triangles, so it is now possible to see the over position.
Line 195: Table 1, for the n.e. and n.d. would it be possible to ">250" for the "Tebuconazole". Just so that it is clear in the table that there was no effect to the highest tested concentration.
Answer: Done.
Discussion and conclusion:
Line 239-243. Reverse the sentence - these values are as mentioned last not comparable, so the argument that E. crypticus is more sensitive is too uncertain. To clear doubt - the reviewer also this that E. crypticus is more sensitive for some parameters - but this is not justified here.
Answer: Done. The sentence was rephrased. Please see the alterations in the manuscript.
Line 251: "…although this is a rather weak evidence being NOEC based." - reformulate to something like "… with the flaws present when comparing NOEC values".
Answer: Done. The sentence was rephrased as suggested.
Line 277: "However, a chronic….." avoid the comparison in sensitivity, as also explained above. Just delete the sentence.
Answer: Done.
Line 281: "However, in terms of survival ….." would it be more correct to insert "adult" before "toxic than any….". Unless juveniles survival is included here.
Answer: Done. No, juveniles' survival is not included. Text was changed as suggested.
Line 320: It may be reiterated that the advantage of also testing E crypticus - without refereeing to the species being more or less sensitive - is that a testing of this species in combination with information on E. fetida - gives a much clearer picture of toxicity. This couples with this difference in test design. If anyone missed it - protecting soil biodiversity is extremely important. Somewhere in the text (probably introduction) it should be stated that testing more species will support a distribution based approach for pesticide testing (see Frampton et al 2006) which offers a better estimation of effect on other nano-target organisms (especially since the fungicides are assumed non-specific on invertebrates.
Perhaps, In line with this, it is also worth noticing that if this was to be evaluate as a chemical, the fact that only 1(or two species) were present would indicate a safety factor of 100 or 10 (depending in further evidence), making in close to the PECs reported. Also considering the uncertainty of the ECx estimates.
Answer: Done. Thank you for the suggestion, we have incorporated it in the manuscript.

*Revised manuscript with changes marked
Click here to view linked References

Toxicity of fungicides ~~in~~to terrestrial non-target fauna – ~~formulations~~formulated products versus active ingredients (azoxystrobin, cyproconazole, prothioconazole, tebuconazole) – a case study with *Enchytraeus crypticus* (Oligochaeta)

Susana I.L. Gomes [a1], Anna Ammendola [a1], Silvia Casini [b] and Mónica J.B. Amorim [a]*

[a] Department of Biology & CESAM, University of Aveiro, 3810-193 Aveiro, Portugal

[b] Department of Physical, Earth and Environmental Sciences, University of Siena, via Mattioli, 4, 53100 Siena, Italy

[1] Both first authors

* corresponding author at: Department of Biology & CESAM, University of Aveiro, 3810-193 Aveiro, Portugal. E-mail address: mjamorim@ua.pt (MJB Amorim)

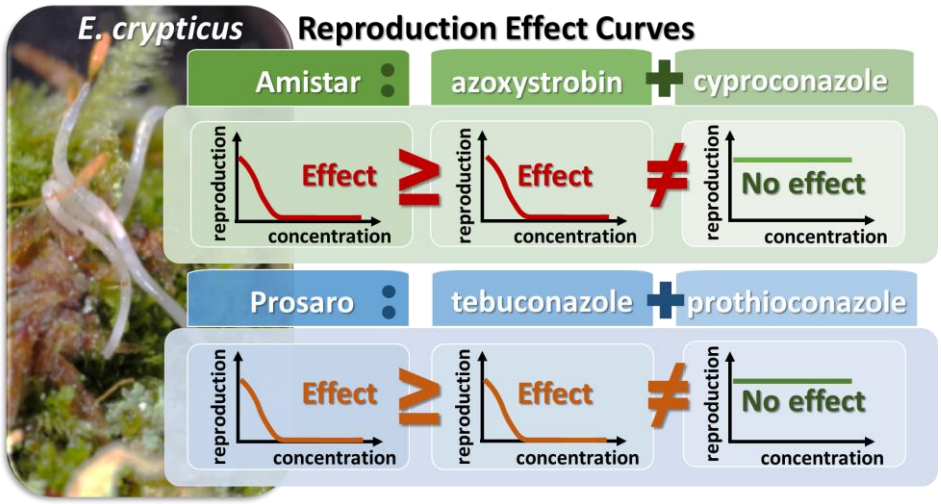
Abstract

Despite the high usage of pesticides in current agricultural practices, its effects to humans and to the environment (non-target species) are a continuous concern. Soil dwelling~~living~~ organisms are among the first in line of exposure to pesticides, however their risks are ~~ecotoxicity knowledge is~~ often based on the pure active ingredient~~s~~ (a.i.) and not on the commercial ~~formulations~~ formulated products (FPs) actually applied in the fields. In the present study, we investigated the effects of two fungicide ~~formulations~~ FPs versus its a.i.(s): Amistar® XTRA and the respective a.i.(s) azoxystrobin and cyproconazole, and Prosaro® 250 EC and the respective a.i.(s) prothioconazole and tebuconazole, to the non-target soil oligochaete *Enchytraeus crypticus*. The standard Enchytraeid Reproduction Test was used to assess effects on survival and reproduction. Results showed that A~~a~~mistar was more toxic

than Ppprosaro, particularly for reproduction ($EC_{5\cancel{1}0}$ = ~~38mg~~ 161 mg Aa~~a~~mistar/kg soil, $EC_{5\cancel{1}0}$ = ~~140mg~~ 350 mg Ppprosaro/kg soil). For both ~~formulations~~FPs, reproductive effects were mainly related to one of its a.i.(s) (azoxystrobin [$EC_{5\cancel{1}0}$ = 3~~1~~7 mg azosxystrobin/kg soil] for Aa~~a~~mistar, and tebuconazole [$EC_{5\cancel{1}0}$ = ~~12mg~~ 41 mg tebuconazole/kg soil] for Pp~~p~~rosaro), while lethal effects were not predicted by the toxicity of its a.i.(s)~~.~~ (particularly in the case of Ppprosaro, which was more toxic than its a.i.(s)). These findings highlight the need to further explore the toxicity data of the ~~formulations~~ FPs compared to the a.i.(s), aiming to predict a more realistic environmental hazard of pesticides.

Keywords: pesticides; Amistar®; Prosaro®; survival; reproduction; ~~formulations~~ formulated products

TOC



1. Introduction

Plant protection products (PPPs) have a very important role in modern agriculture to reduce plant pests and diseases to improve crop yields. However, most of the applied PPPs affect non-target species. For example, it is estimated that less than 0.1% of the applied insecticides reach their target (Pimentel, 1995), and for fungicides this percentage can be even lower (Duke, 2017). Fungicides/bactericides represent 44% of the pesticides sold in the European Union (EU), i~~I~~n 2018~~, almost 44% of the pesticides sold in the EU were fungicides or bactericides~~ (followed by herbicides, haulm destructors and moss killers, with around 34 % of sales ~~)~~ (Eurostat dataset)).

The toxic effects of pesticides, including fungicides, are~~~~ far more studied ~~relatively well studied in soil oligochaetes, particularly~~ in earthworms (e.g. (Rico et al., 2016; Velki et al., 2019; Wang et al., 2012) ~~(Wang et al., 2012)(Rico et al., 2016)(Velki et al., 2019))~~ [also because the assessment of ecotoxicological effects to the earthworm *Eisenia fetida* is required within e.g. EU regulation for PPPs] ~~, and also but to a lesser extent~~than in enchytraeids (~~reviewed in~~e.g. (Römbke et al., 2017)). Even though earthworms (Lumbricidae) are probably the most used bioindicators of soil quality, enchytraeids, which fulfil the similar criteria for good indicators of the biological quality of soils, are particularly relevant in soils with lower abundance of earthworms, being widely distributed and abundant (for review see Pelosi and Römbke (2016)). A clear advantage in the use of enchytraeids is the shorter generation time, which allows the performance of standard chronic (reproductive) tests in 4-6 weeks or less (OECD 220, 2016), ca. half the time used for earthworms (8 weeks) (OECD 222, 2016)~~)~~. ~~In any case, the assessment of differences in sensitivity between the two families (Enchytraeidae for enchytraeids and Lumbricidae for earthworms) did not lead to conclusive results (Lumbricidae appear more sensitive based on acute toxicity whereas the families are similar in sensitivity when the endpoint is reproduction (Jarratt and Thompson, 2009)) with~~

~~no indication of one preferred family in detriment of the other.~~ Hence all good arguments for performing also tests with enchytraeids. Further, the testing of more species will contribute to support a distribution-based approach for pesticide testing (Frampton et al., 2006) which offers a better estimation of effect to other non-target organisms.

Even though the approval of PPP in the EU requires i) the approval of the a.i. at the EU level, and ii) the authorization of the FP at the Member State level, and d~~D~~espite the overall knowledge on pesticides toxicity to soil oligochaetes, the inspection between ~~formulation~~formulated products (FPs) versus active ingredients (a.i.(s)) is often lacking in the literature. ~~-~~However, a study by Mesnage et al. (2014) on three human cell lines showed that 8 out of 9 ~~formulations~~ FPs tested (3 from each category: fungicides, herbicides, and insecticides) were more toxic than the reported a.i. alone, highlighting the importance of the direct study of the commercial ~~formulation~~FP (which includes also the so called adjuvants, additives or inert substances) in regulatory tests. The need for a careful assessment of the impacts of formulated products to soil invertebrates has been highlighted before, e.g. Marques et al. (2009) who tested the effects of two commercial herbicides and the respective a.i.(s) in the earthworm *Eisenia andrei* using avoidance response as endpoint. Still, toxicity studies including both ~~formulations~~FPs and a.i.(s) are rare.

In this study we assessed the effects of the two commercial fungicides Amistar® XTRA and Prosaro® 250 EC (hereinafter referred to as A~~a~~mistar and P~~p~~rosaro), in comparison to its respective active ingredients azoxystrobin and cyproconazole (for Amistar), and prothioconazole and tebuconazole (for Prosaro) on the soil model invertebrate *Enchytraeus crypticus*, based on the standard Enchytraeid Reproduction Test - ERT (OECD 220, 2016). Both fungicide~~s~~ ~~formulations~~FPs are widely used across the world to protect (mainly) cereal cultures. Although the current reported PECs (predicted environmental concentrations) in soils are ~~low,~~ ca. 0.3 mg azoxystrobin/kg and 0.1 mg cyproconazole/kg (Germany National

Assessment, 2018), and ca. 0.08-0.16 mg tebuconazole/kg and 0.08 mg prothioconazole/kg (Netherlands National Assessment, 2017), there is little information to non-~~-~~target species. In terms of toxicity, to the best of our knowledge, only the a.i.(s) were tested on soil oligochaetes. In a study by Wang et al. (2012), azoxystrobin and cyproconazole were classified as extremely toxic to *Eisenia fetida* (48h LC_{50} (50% lethal concentration) = 2.72 and 8.48 µg/cm^2, respectively), based on a filter paper contact test; using the same method, tebuconazole was classified as very toxic (48h LC_{50} = 31.57 µg/cm^2); in OECD soil, the same compounds were acutely toxic to *E. fetida* (14d LC_{50}) at 327.4, 211.8, and 895.2 mg/kg of azoxystrobin, cyproconazole, and tebuconazole, respectively (Wang et al., 2012). Other studies reported higher toxicity for tebuconazole ~~in~~ to *E. fetida*, with 48h LC_{50} [filter paper contact test] of 5.7 µg/cm^2 (Chen et al., 2018), and 14d LC_{50} [OECD soil] of 287 mg/kg (Chen et al., 2018) and 180 mg/kg (Rico et al., 2016). In addition to the complete~~ly absent~~ absence of data on the ~~formulation~~FP's toxicity, data on chronic exposure (reproductive effects) is also very scarce. Among the test substances, only azoxystrobin was tested in *E. crypticus*, causing 50% effects on reproduction (EC_{50}) at 99.2 mg/kg (in a natural Mediterranean soil) (Leitão et al., 2014). In comparison to acute data in soil, results from Leitão et al. (2014) indicate that reproduction is more sensitive, with an EC_{50} of 42 mg/kg for *E. andrei*. Therefore, the relevance to produce toxicity data for pesticides ~~commercial formulations (~~FPs~~ of) pesticides,~~ ~~and~~ in ~~in~~ comparison to its a.i.(s), based on chronic (reproductive) effects to non-target soil species, is still ~~very high~~imperative.

2. Material and methods

2.1. Test organism

The test species *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae) was used. The cultures were kept in agar, consisting of Bacti-Agar medium (Oxoid, Agar No. 1) and a sterilized mixture of four different salt solutions at the final concentrations of 2 mM CaCl2·2H2O, 1 mM MgSO4, 0.08 mM KCl, and 0.75 mM NaHCO3, under controlled conditions of temperature (19 ± 1 °C) and photoperiod (16:8 hours light:dark). The cultures were fed with ground autoclaved oats twice per week.

2.2. Test soil

The standard LUFA 2.2 natural soil (Speyer, Germany) was used. The main characteristics are pH (0.01 M CaCl2) of 5.5, 1.77%~~,~~ organic matter, 10.1 meq/100 g CEC (cation exchange capacity), 44.8% WHC (water holding capacity), 7.3% clay, 13.8% silt, and 78.9% sand regarding grain size distribution.

2.3. Test materials and spiking

The commercial fungicides Amistar® XTRA (Syngenta, 18.~~,~~2% azoxystrobin and 7.~~,~~3% cyproconazole) was purchased from Syngenta Italia (S.p.A, Milan, Italy) and Prosaro® 250 EC (Bayer, 12.~~,~~5% prothioconazole and 12.~~,~~5% tebuconazole) was purchased from Bayer CropScience (S.r.l., Milan, Italy). The pure active ingredients a~~A~~zoxystrobin (Pestanal ®, analytical standard, ≥ 98.0%), c~~C~~yproconazole (Pestanal ®, analytical standard, ≥ 98.0%), p~~P~~rothioconazole (Pestanal ®, analytical standard, ≥ 98.0%), and t~~T~~ebuconazole (Pestanal ®, analytical standard, ≥ 98.0%), were purchased from Merk. The recommended application doses in the field are 0.6-1 L Amistar /ha and 1 L Prosaro /ha, i.e. ca. 1.5 to 7.3 mg/kg if assuming a 1 to 5 cm mixing depth in the soil, or 73.3 mg/kg in the soil surface (1 mm).

For the commercial fungicides the tested concentrations were 0, 9.6, 29.9, 96, 299, and 960 mg Aamistar/kg soil and 0, 19.7, 61.5, 197, 615, and 1970 mg Pprosaro/kg soil. The pure a.i.(s) were tested at the same concentrations, i.e. as present each of the formulationsFPs, resulting in the tested concentration of 0, 0.55, 1.75, 5.5, 17.5, 55, and 175 mg azoxystrobin/kg soil, 0, 0.22, 0.7, 2.2, 7, 22, and 70 mg cyproconazole/kg soil, and 0, 0.78, 2.5, 7.8, 25, 78, and 250 mg/kg soil for prothioconazole and for tebuconazole. The fungicides Aamistar and Pprosaro are water soluble, so they were serially diluted and added to the pre-moistened soil (batches of soil per concentration). The soil was homogeneously mixed and deionised water was added until 50% of soil's maximum water holding capacity (maxWHC). The soil was mixed again, divided into each test vessel, and was allowed to equilibrate for 1 day prior to the start of the tests. The a.i.(s) azoxystrobin, cyproconazole, prothioconazole and tebuconazole were dissolved in acetone, due to its their low solubility in water, and serially diluted to the desired test concentrations (as stated above), homogeneously mixed into the batches of soil (per concentration), and left to evaporate in a fume hood for 24 h. Solvent (acetone) controls were prepared in parallel, adding acetone alone to the soil, in the equivalent volume as that used for the concentration ranges. After 24 h, the soil was moistened (with deionised water) until 50% of soil's maxWHC, and introduced in each test vessel. The exposure started immediately thereafter.

2.4. Test procedures

The standard guideline for the Enchytraeid Reproduction Test (ERT) (OECD 220, 2016) was followed. In short, 10 adults with well-developed clitellum were placed in each test vessel containing 20 g of moist soil and food supply (24 ± 1 mg, autoclaved rolled oats). Test ran at

20 ± 1 ºC and 16:8 h photoperiod during a period of 21 days. Food (12 ± 1 mg) and water were replenished every week, based on weight loss. Four replicates per treatment were done. At the test end, the organisms were fixed with ethanol and stained with Bengal rose (1% in ethanol). After 24 h, soil samples were sieved through meshes with decreasing pore size (1.6, 0.5, and 0.3 mm) to separate the enchytraeids from most of the soil and facilitate counting. Adult and juvenile organisms were counted using a stereo microscope and survival and reproduction assessed.

2.5. Data analysis

To assess differences between treatments and controls, one-way analysis of variance (ANOVA) was performed, followed by the post hoc Dunnett's method for multiple comparisons at a significance level of 0.05 (SigmaPlot 11.0). For the a.i.(s) tests, water and solvent controls were compared using the t-test, at a significance level of 0.05. As there were no significant differences between controls, they were pooled prior to the performance of ANOVA, as described above. Effect concentrations (ECx) were calculated modelling data to logistic or threshold sigmoid 2 parameters regression models, as indicated in Table 1, using the Toxicity Relationship Analysis Program (TRAP 1.30) software. To improve model fitting~~,~~ for azoxystrobin and P~~p~~rosaro survival data, the variable concentration was log transformed.

3. Results

All the tests fulfilled the validity criteria as described in OECD 220 (2016) with adult mortality below 20% and the number of juveniles higher than 50, with a coefficient of variation lower than 50%, in controls. There were no significant differences between the

controls and solvent controls (acetone), hence they were pooled in the graphs (and statistical analysis). The results on adults' survival and reproduction are shown in Fig. 1 and the ECx calculated are shown in Table 1.

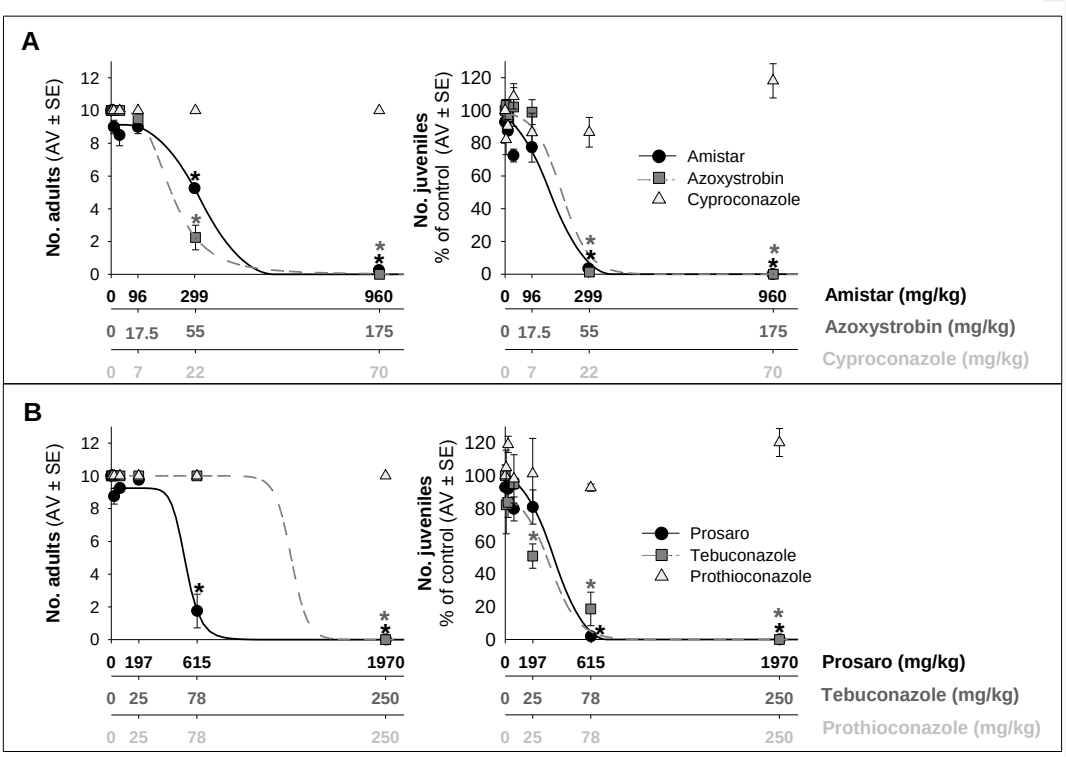


Fig.1. Results of the standard Enchytraeid Reproduction Test (ERT) in terms of survival and reproduction of *Enchytraeus crypticus* exposed in LUFA 2.2 soil to A) the pesticide ~~formulation~~formulated product (FP) Amistar, and its active ingredients (a.i.(s)) Azoxystrobin and Cyproconazole; B) the pesticide ~~formulation~~ FP Prosaro, and its a.i.(s) Tebuconalzole and Prothioconazole. The results are presented as average ± standard error (n = 4). *p < 0.05 (Dunnett's method). The additional x axis shows the correspondence between a.i.(s) and ~~formulation~~ FP concentrations. Lines represent the model fit to data.

Amistar induced a decrease in the number of adults and juveniles at 299, and 960 mg/kg, with higher effects on reproduction than on survival (EC_{50} = 161 and LC_{50} = 320 mg/kg). Concerning Aamistar' a.i.(s), azoxystrobin decreased the number of adults and juveniles at 55 and 175 mg/kg (with similar effects on survival and reproduction LC_{50} = 39 and EC_{50} = 37 mg/kg); while cyproconazole caused no effects. Prosaro induced a decrease in the number of adults and juveniles at 615, and 1970 mg/kg, with higher effects on reproduction than on survival (EC_{50} = 350 and LC_{50} = 533 mg/kg). Concerning Ppprosaro' a.i.(s), tebuconazole decreased the number of adults at 250 mg/mg and the number of juveniles at 25, 78 and 250 mg/kg, showing higher toxicity in terms of reproduction (EC_{50} = 41 and LC_{50} = 164 mg/kg); while prothioconazole caused no effects.

Table 1 Summary of the effect concentrations (ECx with 95% confidence intervals – CI), expressed as mg test substance per kg soil (dry weight), for *Enchytraeus crypticus* exposed to the pesticides formulations FPs Amistar and Prosaro (square brackets show the equivalent ECx based on the active ingredients concentrations present in the formulations FPs – Az: Azoxystrobin, Cy: Cyproconazole, Te: Tebuconazole, and Pr: Prothioconazole), and the respective active ingredients (a.i.(s)) Azoxystrobin and Cyproconazole (Amistar' a.i.(s)) in LUFA 2.2 soil. The models used are Threshold sigmoid 2 or 3 parameters (Thres2P or 3P) or Logistic 2 parameters (Log2P). S: slope; y0: top point; n.e.: no effect; n.d.: not determined.

Endpoint	Test material	EC10 (95% CI)	EC50 (95% CI)	EC90 (95% CI)	Model & parameters
Survival	Amistar [Az + Cy]	172 (74 - 269) [31 Az + 13 Cy]	320 (286 - 355) [58 Az + 23 Cy]	412 (316 - 508) [75 Az + 30 Cy]	ThreSig2P S:3.7E-3; y0:9.1; r^2:1
	Azoxystrobin	21 (15 - 30)	39 (34 - 45)	71 (62 - 83)	Log2P S:2.1; y0:10; r^2: 1
	Cyproconazole	> 70n.e.	> 70n.e.	> 70n.e.	
Reproduction	Amistar	38 (6 - 70)	161 (130 - 193)	238 (154 - 322)	ThreSig2P

	[Az + Cy]	[6.9 Az + 2.8 Cy]	[29 Az + 12 Cy]	[43 Az + 17 Cy]	S:4.5E-3; y0:100; r^2:0.9
	Azoxystrobin	17 (2 - 33)	37 (25 - 49)	57 (40 - 74)	Log2P
	Cyproconazole	> 70n.e.	> 70n.e.	> 70n.e.	S:2.8E-2; y0:100; r^2:0.8
Survival	Prosaro [Te + Pr]	429 (n.d.) [55 Te + 55 Pr]	533 (n.d.) [68 Te + 68 Pr]	662 (n.d.) [84 Te + 84 Pr]	Log2P S:5.8; y0:9.3; r^2:1
	Tebuconazole	148 (145 - 150)	164 (162 - 166)	180 (178 - 183)	Log2P
	Prothioconazole	> 250n.e.	> 250n.e.	> 250n.e.	S:3.4E-2; y0:10; r^2:1
Reproduction	Prosaro [Te + Pr]	139 (48 - 229) [18 Te + 18 Pr]	350 (262 - 438) [45 Te + 45 Pr]	480 (178 - 783) [61 Te + 61 Pr]	ThreSig2P S:2.6E-3; y0:99.5; r^2:0.9
	Tebuconazole	12 (-6 - 31)	41 (16 - 66)	69 (21 - 118)	Log2P
	Prothioconazole	> 250n.e.	> 250n.e.	> 250n.e.	S:1.9E-2; y0:90.3; r^2:0.7

4. Discussion

The toxicity of Aamistar and pProsaro formulations FPs seemed mostly associated to one of their a.i.(s), azoxystrobin (Az) and tebuconazole (Te) respectively. Reproduction confirmed to be a more sensitive endpoint than survival. This is often the case shown in literature for exposure to various classes of chemicals, pesticides included, not only for enchytraeids (Amorim et al., 2005; Castro-Ferreira et al., 2012; Novais et al., 2011, 2010), but also for earthworms (e.g. (Leitão et al., 2014; Römbke et al., 2007)). Regarding the endpoint survival, Aamistar was more toxic than Pprosaro at lower concentrations (Aamistar EC_{10} << Pprosaro EC_{10}), converging to similar toxicity at higher concentrations ($EC_{50/90}$), although in terms of reproduction, Aamistar was around 2 times more toxic than Pprosaro.

It is difficult to compare the magnitude of the results obtained for these formulations FPs with literature data because the very few studies on pesticides formulations FPs on soil oligochaetes (e.g. (Rico et al., 2016; Velki et al., 2019)) report either acute toxicity based on filter paper contact test and/or were performed on formulations FPs containing a single a.i, (and the toxicity results are expressed as mg a.i/kg). In our study, the ECx calculated for the formulations FPs Aamistar and Pprosaro correspond to the entire formulation FPs (based on

its density, thus including all, a.i.(s) and the chemicals such as the adjuvants, solvents, etc.). Similar to ours, a study on the collembolan *Folsomia candida* reported LC/ECx for a wood preservative [containing two a.i.(s) (tebuconazole and 3-iodo-2-propynyl N-butylcarbamate – IPBC)] based on the total ~~formulation~~ FP mass (Guimarães et al., 2018). Despite slightly higher (LC_{50} = 821 mg/kg; EC_{10} = 545 mg/kg), the effect concentrations are in the same order of magnitude as ours.

Azoxystrobin was the most toxic a.i. when tested alone (EC_{50} of 39 and 37mg/kg for survival and reproduction, respectively), showing higher toxicity than reported by Leitão et al. (2014) also for *E. crypticus* (reproduction EC_{50} of 99 mg/kg). The differences are likely due to the different soil types used, LUFA 2.2 versus a natural Mediterranean soil, where e.g. organic matter content differs between 2 and 5%, respectively. Although no effects were observed for survival and reproduction at 5 mg azoxystrobin/kg soil, this concentration was shown to already significantly disturb the gut microbiota of *E. crypticus*, with enriched antibiotic resistance genes (Zhang et al., 2019); such an impact can spread through the food chain and amplify the ecological risks of azoxystrobin. In comparison to other soil invertebrates results from Leitão et al. (2014) place *E. crypticus* and *F. candida* at similar levels, while *E. andrei* was more sensitive to azoxystrobin. Our results are similar to those reported for *E. andrei* (EC_{50} = 42 mg /kg) in a different soil (Leitão et al., 2014). Azoxystrobin appears to cause acute toxicity to *E. crypticus* as observed from severe effects on survival (similar ECx for survival and reproduction) and hence reproduction effects are probably a consequence of adults' mortality. The LC_{50} (EC_{50} for survival) observed here is around 10 times lower than the one reported for *E. fetida*, in OECD soil (14 d LC_{50} of 372 mg /kg (Wang et al., 2012)), ~~indicating that *E. crypticus* is more sensitive to azoxystrobin compared to *E. fetida*,~~ although direct comparison cannot be done since~~, again~~ different soils (LUFA 2.2 versus OECD) and test durations (21 d for enchytraeids and 14 d for earthworms) were used~~, hence no direct~~

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~~comparison should be done~~. However, the chronic 28 d LC_{50} > 500 mg/kg for *E. andrei* (Leitão et al., 2014), although in different soil types, support the indication of higher sensitivity of *E. crypticus* survival. Cyproconazole alone was not toxic to *E. crypticus* within the tested concentrations (up to 70 mg/kg). On the other hand, for *E. fetida*, cyproconazole was more toxic than azoxystrobin (14 LC_{50} of 212 mg cyproconazole/kg and 372 mg azoxystrobin/kg (Wang et al., 2012)). For the collembolan *F. candida* azoxystrobin seems to be more toxic than cyproconazole (28d no observed effect concentration – NOEC of 25 and 55.8 mg/kg for azoxystrobin and cyproconazole, respectively) (Registration report ADD-F2-003, 2018) ~~although this is a rather weak evidence being NOEC based~~ with the flaws present when comparing NOEC values.

Regarding the comparison between the a.i.(s) and ~~formulations~~FPs, the effect curves for azoxystrobin and A~~a~~mistar (Fig 1) are very close, this for the endpoint reproduction, indicating that azoxystrobin is the main responsible for the toxicity caused by A~~a~~mistar to *E. crypticus*. In terms of survival, azoxystrobin alone was more toxic than A~~a~~mistar (azoxystrobin LC_{50} = 39 mg/kg, Amistar LC_{50} corresponds to 58 mg azoxystrobin/kg). ~~and,~~Even though natural variation cannot be ruled out (as depicted in Fig. 1, the effect curves are close)~~.~~ for this endpoint, the possible reduction of toxicity can be either due to a "dilution" effect with the adjuvants, or due to a low antagonism effect (i.e., the combined effect of two or more chemicals is less than the sum of the effects of each individually) in the mixture (cyproconazole, the other a.i., did not induce toxicity to *E. crypticus*). The higher toxicity of the pure a.i. compared to the ~~formulation~~ FP has been previously reported, e.g. in *E. crypticus* for atrazine in comparison to the atrazine based ~~formulation~~ FP "G~~g~~esaprim" (Gomes et al., 2019), in this case the ~~formulation~~ FP contains a single a.i..

Tebuconazole induced reproductive toxicity to *E. crypticus* while prothioconazole, the other a.i. of P~~p~~rosaro did not (up to 250 mg/kg). The toxicity of tebuconazole was at the same level

as azoxystrobin for reproduction, but 4 times less lethal (LC_{50} of 164 and 39 mg/kg for tebuconazole and azoxystrobin, respectively). This can have implications for the risk assessment, which can be expected higher for more lethal compounds applied similarly. Further, higher toxicity of low-toxic compounds cannot be excluded if exposure is extended during more generations and longer term. In agreement with our results, a comparative study on pesticides toxicity to *E. fetida* showed that azoxystrobin (14 d LC_{50} of 327 mg/kg) was more toxic than tebuconazole (14 d LC_{50} of 895 mg/kg) (Wang et al., 2012), but other studies reported lower 14 d LC_{50} values for tebuconazole (287 mg/kg in Chen et al. (2018), and 180 mg/kg in Rico et al. (2016) the last one tested as ~~formulation~~ FP - Folicur 25 EW). The reproductive toxicity to *E. crypticus* is at the same level as the reported for *F. candida* in OECD soil (EC_{10} = 44.5 mg/kg (6.2 < CI < 82.8 mg/kg)), however tebuconazole is more lethal to *E. crypticus* than to *F. candida* (LC_{50} of 164 and > 1250 mg/kg, respectively) (Guimarães et al., 2018). The absence of toxicity of prothioconazole in *E. crypticus* is in agreement with the acute lethality assessed in *E. fetida* (14d LC_{50} > 1000mg/kg (PPDB: DataBase). ~~However, a chronic (reproductive) NOEC of 1.33 mg/kg (PPDB: DataBase) *E. fetida* indicates its potentially higher sensitivity compared to *E. crypticus*.~~ As observed for A~~a~~mistar, the effects on reproduction caused by P~~p~~rosaro seem to be explained by one of the a.i., in this case tebuconazole, as the effect curves for P~~p~~rosaro and tebuconazole are very close (Fig 1). However, in terms of adults´ survival, P~~p~~rosaro is more toxic than any of its a.i.(s) alone. This can be due to toxic effects caused by the adjuvants (often called inert ingredients) present in the ~~formulation~~ FP and/or by synergistic effects (i.e., the combined effect of two chemicals is greater than the sum of the effects of each given chemical alone) between the two a.i.(s) or the a.i.(s) and the adjuvants. In any case, it seems to preferentially affect the adults (the effects on reproduction were similar between P~~p~~rosaro and the a.i. tebuconazole). Studying a wood preservative ~~formulation~~ FP containing tebuconazole and

IPBC as a.i.(s.), Guimarães et al. (2018) found that IPBC was the principal contributor to lethal toxicity to *F. candida*, while the effects on reproduction were fairly predictable based on the mixture concentration addition (CA) model, with slight underestimation. This underestimation is suggested to result from the contribution of the formulation's FP's additives to the reproductive toxicity (Guimarães et al., 2018). The formulation's FP's additives seem to trigger an avoidance behaviour that was not predictable based on the effect of the a.i.(s) on organisms' survival or reproduction (Guimarães et al., 2018). Similarly, the higher avoidance response of *E. andrei* to the herbicide formulation FP Viper in comparison to its a.i. penoxsulam, has been attributed to the adjuvants present in the formulation FP (Marques et al., 2009). Previously, Cox and Surgan (2006) highlighted that the inert ingredients can increase toxicity and ecotoxicity of pesticides formulationsFPs, and that it is unclear if inert ingredients increase toxicity directly or by interacting with the a.i.(s). Since not all the inert ingredients are often clearly identified in the product labels, and so not easy to assess its toxicity, it is of utmost importance to test also the formulationsFPs, and not only the a.i.(s) (Cox and Surgan, 2006). Further, there is the case of the formulations FPs containing more than one a.i. (as the ones tested here) for which, in addition to the problematic of the adjuvants, there is also the possible interaction between the a.i.s (and of course between the a.i.s and the adjuvants). Even though within the EU regulation, evaluation is required for the a.i.(s) (at the EU level) and for the FPs (at Member State level), the information might not be easily available for the (scientific) community. It was not possible to find ecotoxicological information for soil invertebrates on Amistar ® XTRA and Prosaro ® 250 EC safety datasheets, and our study shows that the toxicity of the formulation FP Pprosaro was not predicted by the toxicity of its a.i.(s). As recommended, testing various species is of high relevancy (Frampton et al., 2006) to protect soil biodiversity and implement SSD based ERA. It may be of interest to realise that if these compounds were to be evaluate

as chemicals, the fact that only 1 (or two species) were present would indicate a safety factor of 100 or 10 (depending in further evidence), making it close to the reported PECs and possibly also considering the uncertainty of the ECx estimates.

5. Conclusions

To our best knowledge, this is the first study reporting toxicity data for the fungicide's ~~formulations~~ FPs Amistar ® XTRA and Prosaro ® 250 EC to a soil invertebrate (non-target organism). Overall A~~a~~mistar was more toxic than P~~p~~rosaro, particularly considering the endpoint reproduction.

By studying, simultaneously, the toxicity of the a.i.(s) of each ~~formulation~~FP, indications are that the reproductive toxicity of A~~a~~mistar is explained by the a.i. azoxystrobin while ~~p~~Prosaro's is explained by the a.i. tebuconazole. However, in terms of survival, the toxicity of the ~~formulations~~ FPs was not predicted by the toxicity of the a.i.(s), particularly in the case of P~~p~~rosaro, which was more toxic than any of its a.i.(s). This highlights the importance of testing the ~~formulations~~FPs, rather than the a.i.(s) alone, to assess or predict the risk of commercial pesticide products to the environment. Further, testing various species is recommended and key to implement SSD based ERA, meaning that the present results could indicate safety factors of 100 or 10 (depending in further evidence), making it close to the reported PECs. Last, chronic testing is recommended and more relevant than acute, although acute effects are usually also covered during a chronic endpoint test. Also some sublethal endpoints might be implemented in future studies, such as biomarkers of genotoxicity, oxidative stress, immune system etc. to further understand toxic effects.

Declaration of competing interest

356 The authors declare no conflict of interest.

357

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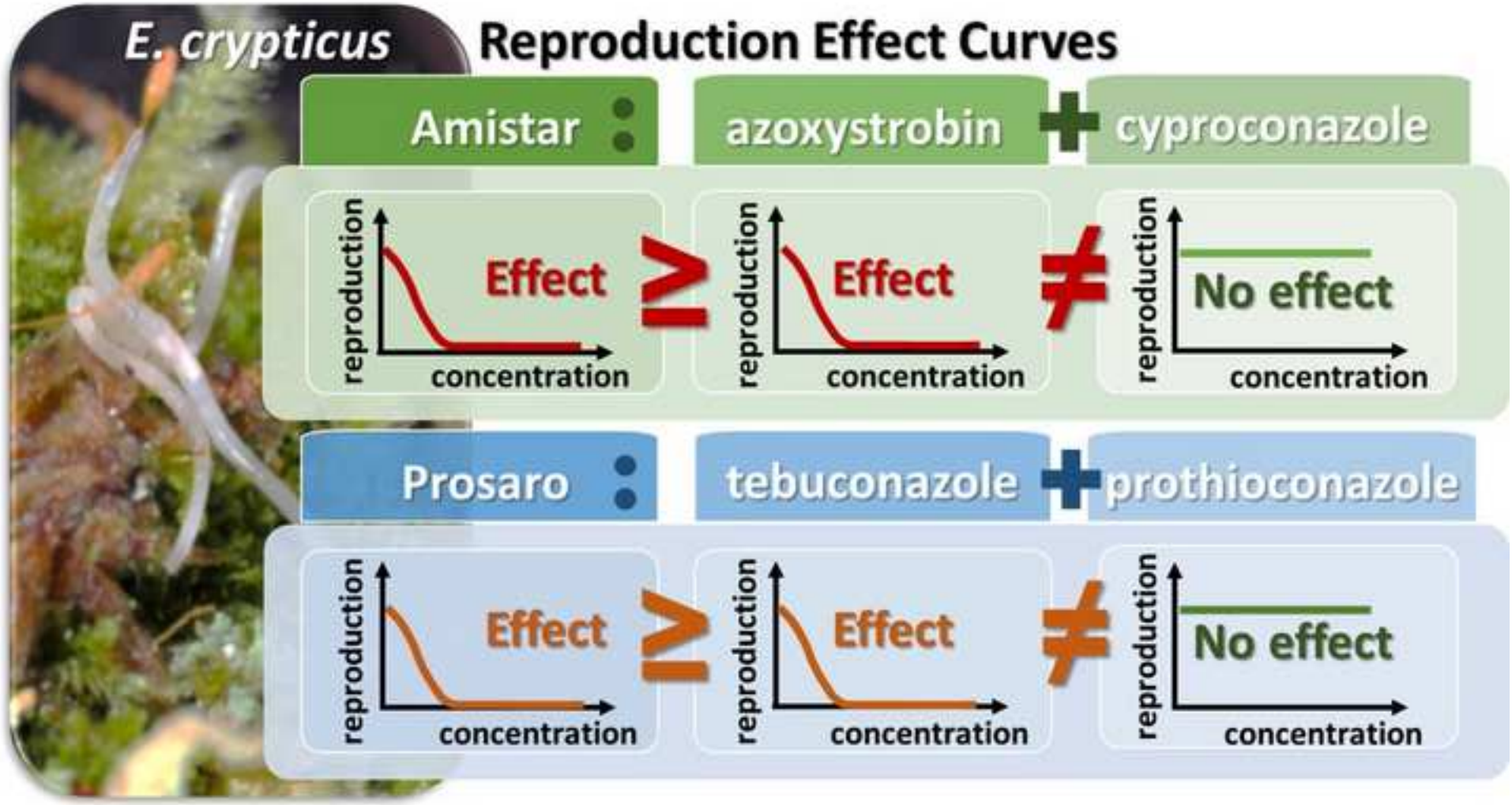
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457

*Graphical Abstract



*Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)

Highlights

. Toxicity of the formulations Amistar and Prosaro were studied vs its a.i.s

. Amistar was more toxic than prosaro to the non-target oligochaete *E. crypticus*

. formulations effects were mainly explained by azoxystrobin and tebuconazole

. Lethal effects of the formulations were not fully predicted by its a.i.s

- formulations toxicity must be explored if to predict realistic environmental hazard

*Manuscript (double-spaced and continuously LINE and PAGE numbered)-for final publication
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Toxicity of fungicides to terrestrial non-target fauna – formulated products versus active ingredients (azoxystrobin, cyproconazole, prothioconazole, tebuconazole) – a case study with *Enchytraeus crypticus* (Oligochaeta)

Susana I.L. Gomes [a1], Anna Ammendola [a1], Silvia Casini [b] and Mónica J.B. Amorim [a]*

[a] Department of Biology & CESAM, University of Aveiro, 3810-193 Aveiro, Portugal

[b] Department of Physical, Earth and Environmental Sciences, University of Siena, via Mattioli, 4, 53100 Siena, Italy

[1] Both first authors

* corresponding author at: Department of Biology & CESAM, University of Aveiro, 3810-193 Aveiro, Portugal. E-mail address: mjamorim@ua.pt (MJB Amorim)

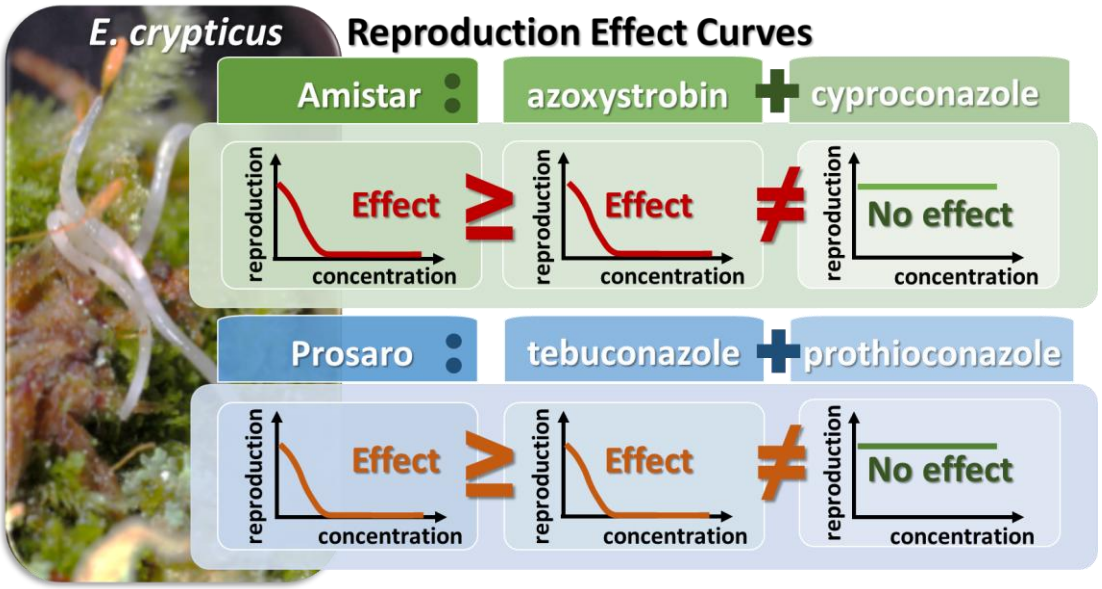
Abstract

Despite the high usage of pesticides in current agricultural practices, its effects to humans and to the environment (non-target species) are a continuous concern. Soil dwelling organisms are among the first in line of exposure to pesticides, however their risks are often based on the pure active ingredient (a.i.) and not on the commercial formulated products (FPs) actually applied in the fields. In the present study, we investigated the effects of two fungicide FPs versus its a.i.(s): Amistar® XTRA and the respective a.i.(s) azoxystrobin and cyproconazole, and Prosaro® 250 EC and the respective a.i.(s) prothioconazole and tebuconazole, to the non-target soil oligochaete *Enchytraeus crypticus*. The standard Enchytraeid Reproduction Test was used to assess effects on survival and reproduction. Results showed that Amistar was more toxic than Prosaro, particularly for reproduction (EC_{50} = 161 mg Amistar/kg soil, EC_{50}

= 350 mg Prosaro/kg soil). For both FPs, reproductive effects were mainly related to one of its a.i.(s) (azoxystrobin [EC_{50} = 37 mg azosxystrobin/kg soil] for Amistar, and tebuconazole [EC_{50} = 41 mg tebuconazole/kg soil] for Prosaro), while lethal effects were not predicted by the toxicity of its a.i.(s) (particularly in the case of Prosaro, which was more toxic than its a.i.(s)). These findings highlight the need to further explore the toxicity data of the FPs compared to the a.i.(s), aiming to predict a more realistic environmental hazard of pesticides.

Keywords: pesticides; Amistar®; Prosaro®; survival; reproduction; formulated products

TOC



1. Introduction

Plant protection products (PPPs) have a very important role in modern agriculture to reduce plant pests and diseases to improve crop yields. However, most of the applied PPPs affect non-target species. For example, it is estimated that less than 0.1% of the applied insecticides reach their target (Pimentel, 1995), and for fungicides this percentage can be even lower (Duke, 2017). Fungicides/bactericides represent 44% of the pesticides sold in the European Union (EU), in 2018(followed by herbicides, haulm destructors and moss killers, with around 34 % of sales (Eurostat dataset)). The toxic effects of pesticides, including fungicides, are far more studied in earthworms (e.g. (Rico et al., 2016; Velki et al., 2019; Wang et al., 2012)) [also because the assessment of ecotoxicological effects to the earthworm *Eisenia fetida* is required within e.g. EU regulation for PPPs] than in enchytraeids (e.g. (Römbke et al., 2017)). Even though earthworms (Lumbricidae) are probably the most used bioindicators of soil quality, enchytraeids, which fulfil the similar criteria for good indicators of the biological quality of soils, are particularly relevant in soils with lower abundance of earthworms, being widely distributed and abundant (for review see Pelosi and Römbke (2016)). A clear advantage in the use of enchytraeids is the shorter generation time, which allows the performance of standard chronic (reproductive) tests in 4-6 weeks or less (OECD 220, 2016), ca. half the time used for earthworms (8 weeks) (OECD 222, 2016). Hence all good arguments for performing also tests with enchytraeids. Further, the testing of more species will contribute to support a distribution-based approach for pesticide testing (Frampton et al., 2006) which offers a better estimation of effect to other non-target organisms.

Even though the approval of PPP in the EU requires i) the approval of the a.i. at the EU level, and ii) the authorization of the FP at the Member State level, and despite the overall knowledge on pesticides toxicity to soil oligochaetes, the inspection between formulated products (FPs) versus active ingredients (a.i.(s)) is often lacking in the literature. However, a

study by Mesnage et al. (2014) on three human cell lines showed that 8 out of 9 FPs tested (3 from each category: fungicides, herbicides, and insecticides) were more toxic than the reported a.i. alone, highlighting the importance of the direct study of the commercial FP (which includes also the so called adjuvants, additives or inert substances) in regulatory tests. The need for a careful assessment of the impacts of formulated products to soil invertebrates has been highlighted before, e.g. Marques et al. (2009) who tested the effects of two commercial herbicides and the respective a.i.(s) in the earthworm *Eisenia andrei* using avoidance response as endpoint. Still, toxicity studies including both FPs and a.i.(s) are rare.

In this study we assessed the effects of the two commercial fungicides Amistar® XTRA and Prosaro® 250 EC (hereinafter referred to as Amistar and Prosaro), in comparison to its respective active ingredients azoxystrobin and cyproconazole (for Amistar), and prothioconazole and tebuconazole (for Prosaro) on the soil model invertebrate *Enchytraeus crypticus*, based on the standard Enchytraeid Reproduction Test - ERT (OECD 220, 2016). Both fungicide FPs are widely used across the world to protect (mainly) cereal cultures. Although the current reported PECs (predicted environmental concentrations) in soils are ca. 0.3 mg azoxystrobin/kg and 0.1 mg cyproconazole/kg (Germany National Assessment, 2018), and ca. 0.08-0.16 mg tebuconazole/kg and 0.08 mg prothioconazole/kg (Netherlands National Assessment, 2017), there is little information to non-target species. In terms of toxicity, to the best of our knowledge, only the a.i.(s) were tested on soil oligochaetes. In a study by Wang et al. (2012), azoxystrobin and cyproconazole were classified as extremely toxic to *Eisenia fetida* (48h LC_{50} (50% lethal concentration) = 2.72 and 8.48 μg/cm^2, respectively), based on a filter paper contact test; using the same method, tebuconazole was classified as very toxic (48h LC_{50} = 31.57 μg/cm^2); in OECD soil, the same compounds were acutely toxic to *E. fetida* (14d LC_{50}) at 327.4, 211.8, and 895.2 mg/kg of azoxystrobin, cyproconazole, and tebuconazole, respectively (Wang et al., 2012). Other studies reported higher toxicity for

tebuconazole to *E. fetida*, with 48h LC_{50} [filter paper contact test] of 5.7 μg/cm^2 (Chen et al., 2018), and 14d LC_{50} [OECD soil] of 287 mg/kg (Chen et al., 2018) and 180 mg/kg (Rico et al., 2016). In addition to the complete absence of data on the FP's toxicity, data on chronic exposure (reproductive effects) is also very scarce. Among the test substances, only azoxystrobin was tested in *E. crypticus*, causing 50% effects on reproduction (EC_{50}) at 99.2 mg/kg (in a natural Mediterranean soil) (Leitão et al., 2014). In comparison to acute data in soil, results from Leitão et al. (2014) indicate that reproduction is more sensitive, with an EC_{50} of 42 mg/kg for *E. andrei*. Therefore, the relevance to produce toxicity data for pesticides FPs in comparison to its a.i.(s), based on chronic (reproductive) effects to non-target soil species, is still imperative.

2. Material and methods

2.1. Test organism

The test species *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae) was used. The cultures were kept in agar, consisting of Bacti-Agar medium (Oxoid, Agar No. 1) and a sterilized mixture of four different salt solutions at the final concentrations of 2 mM CaCl2·2H2O, 1 mM MgSO4, 0.08 mM KCl, and 0.75 mM NaHCO3, under controlled conditions of temperature (19 ± 1 °C) and photoperiod (16:8 hours light:dark). The cultures were fed with ground autoclaved oats twice per week.

2.2. Test soil

The standard LUFA 2.2 natural soil (Speyer, Germany) was used. The main characteristics are pH (0.01 M CaCl2) of 5.5, 1.77% organic matter, 10.1 meq/100 g CEC (cation exchange

capacity), 44.8% WHC (water holding capacity), 7.3% clay, 13.8% silt, and 78.9% sand regarding grain size distribution.

2.3. Test materials and spiking

The commercial fungicides Amistar® XTRA (Syngenta, 18.2% azoxystrobin and 7.3% cyproconazole) was purchased from Syngenta Italia (S.p.A, Milan, Italy) and Prosaro® 250 EC (Bayer, 12.5% prothioconazole and 12.5% tebuconazole) was purchased from Bayer CropScience (S.r.l., Milan, Italy). The pure active ingredients azoxystrobin (Pestanal ®, analytical standard, ≥ 98.0%), cyproconazole (Pestanal ®, analytical standard, ≥ 98.0%), prothioconazole (Pestanal ®, analytical standard, ≥ 98.0%), and tebuconazole (Pestanal ®, analytical standard, ≥ 98.0%), were purchased from Merk. The recommended application doses in the field are 0.6-1 L Amistar /ha and 1 L Prosaro /ha, i.e. ca. 1.5 to 7.3 mg/kg if assuming a 1 to 5 cm mixing depth in the soil, or 73.3 mg/kg in the soil surface (1 mm).

For the commercial fungicides the tested concentrations were 0, 9.6, 29.9, 96, 299, and 960 mg Amistar/kg soil and 0, 19.7, 61.5, 197, 615, and 1970 mg Prosaro/kg soil. The pure a.i.(s) were tested at the same concentrations, i.e. as present each of the FPs, resulting in the tested concentration of 0, 0.55, 1.75, 5.5, 17.5, 55, and 175 mg azoxystrobin/kg soil, 0, 0.22, 0.7, 2.2, 7, 22, and 70 mg cyproconazole/kg soil, and 0, 0.78, 2.5, 7.8, 25, 78, and 250 mg/kg soil for prothioconazole and for tebuconazole. The fungicides Amistar and Prosaro are water soluble, so they were serially diluted and added to the pre-moistened soil (batches of soil per concentration). The soil was homogeneously mixed and deionised water was added until 50% of soil's maximum water holding capacity (maxWHC). The soil was mixed again, divided into each test vessel, and was allowed to equilibrate for 1 day prior to the start of the tests. The a.i.(s) azoxystrobin, cyproconazole, prothioconazole and tebuconazole were dissolved in

acetone, due to their low solubility in water, and serially diluted to the desired test concentrations (as stated above), homogeneously mixed into the batches of soil (per concentration), and left to evaporate in a fume hood for 24 h. Solvent (acetone) controls were prepared in parallel, adding acetone alone to the soil, in the equivalent volume as that used for the concentration ranges. After 24 h, the soil was moistened (with deionised water) until 50% of soil's maxWHC, and introduced in each test vessel. The exposure started immediately thereafter.

2.4. Test procedures

The standard guideline for the Enchytraeid Reproduction Test (ERT) (OECD 220, 2016) was followed. In short, 10 adults with well-developed clitellum were placed in each test vessel containing 20 g of moist soil and food supply (24 ± 1 mg, autoclaved rolled oats). Test ran at 20 ± 1 ºC and 16:8 h photoperiod during a period of 21 days. Food (12 ± 1 mg) and water were replenished every week, based on weight loss. Four replicates per treatment were done. At the test end, the organisms were fixed with ethanol and stained with Bengal rose (1% in ethanol). After 24 h, soil samples were sieved through meshes with decreasing pore size (1.6, 0.5, and 0.3 mm) to separate the enchytraeids from most of the soil and facilitate counting. Adult and juvenile organisms were counted using a stereo microscope and survival and reproduction assessed.

2.5. Data analysis

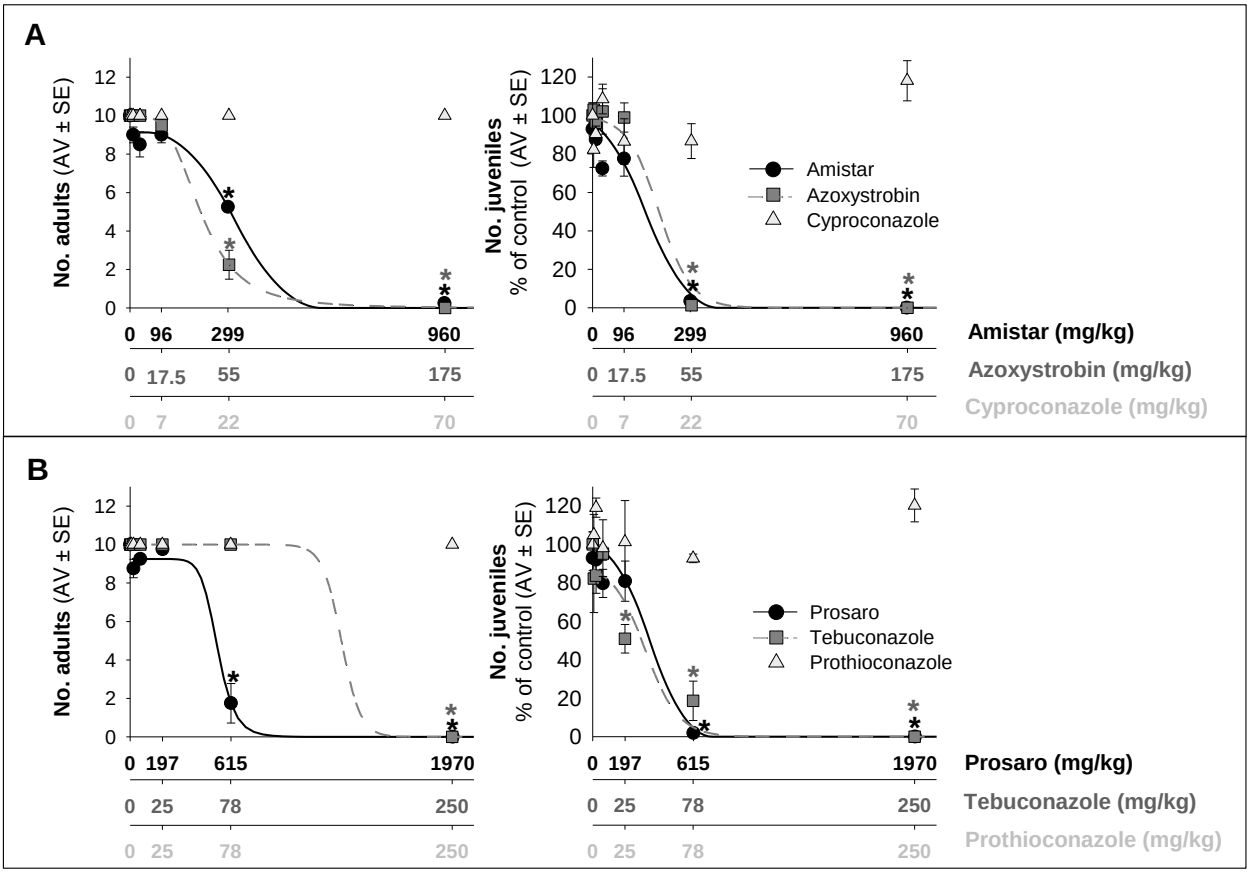
To assess differences between treatments and controls, one-way analysis of variance (ANOVA) was performed, followed by the post hoc Dunnett's method for multiple comparisons at a significance level of 0.05 (SigmaPlot 11.0). For the a.i.(s) tests, water and

158 solvent controls were compared using the t-test, at a significance level of 0.05. As there were
159 no significant differences between controls, they were pooled prior to the performance of
160 ANOVA, as described above. Effect concentrations (ECx) were calculated modelling data to
161 logistic or threshold sigmoid 2 parameters regression models, as indicated in Table 1, using
162 the Toxicity Relationship Analysis Program (TRAP 1.30) software. To improve model fitting
163 for azoxystrobin and Prosaro survival data, the variable concentration was log transformed.

164

165 ## 3. Results

166 All the tests fulfilled the validity criteria as described in OECD 220 (2016) with adult
167 mortality below 20% and the number of juveniles higher than 50, with a coefficient of
168 variation lower than 50%, in controls. There were no significant differences between the
169 controls and solvent controls (acetone), hence they were pooled in the graphs (and statistical
170 analysis). The results on adults' survival and reproduction are shown in Fig. 1 and the ECx
171 calculated are shown in Table 1.



172

173 **Fig.1.** Results of the standard Enchytraeid Reproduction Test (ERT) in terms of survival and
174 reproduction of *Enchytraeus crypticus* exposed in LUFA 2.2 soil to A) the pesticide
175 formulated product (FP) Amistar, and its active ingredients (a.i.(s)) Azoxystrobin and
176 Cyproconazole; B) the pesticide FP Prosaro, and its a.i.(s) Tebuconalzole and
177 Prothioconazole. The results are presented as average ± standard error (n = 4). *p < 0.05
178 (Dunnett's method). The additional x axis shows the correspondence between a.i.(s) and FP
179 concentrations. Lines represent the model fit to data.

180

181 Amistar induced a decrease in the number of adults and juveniles at 299, and 960 mg/kg, with
182 higher effects on reproduction than on survival (EC_{50} = 161 and LC_{50} = 320 mg/kg).
183 Concerning Amistar' a.i.(s), azoxystrobin decreased the number of adults and juveniles at 55
184 and 175 mg/kg (with similar effects on survival and reproduction LC_{50} = 39 and EC_{50} = 37

mg/kg); while cyproconazole caused no effects. Prosaro induced a decrease in the number of adults and juveniles at 615, and 1970 mg/kg, with higher effects on reproduction than on survival (EC_{50} = 350 and LC_{50} = 533 mg/kg). Concerning Prosaro' a.i.(s), tebuconazole decreased the number of adults at 250 mg/mg and the number of juveniles at 25, 78 and 250 mg/kg, showing higher toxicity in terms of reproduction (EC_{50} = 41 and LC_{50} = 164 mg/kg); while prothioconazole caused no effects.

Table 1 Summary of the effect concentrations (ECx with 95% confidence intervals – CI), expressed as mg test substance per kg soil (dry weight), for *Enchytraeus crypticus* exposed to the pesticides FPs Amistar and Prosaro (square brackets show the equivalent ECx based on the active ingredients concentrations present in the FPs – Az: Azoxystrobin, Cy: Cyproconazole, Te: Tebuconazole, and Pr: Prothioconazole), and the respective active ingredients (a.i.(s)) Azoxystrobin and Cyproconazole (Amistar' a.i.(s)) in LUFA 2.2 soil. The models used are Threshold sigmoid 2 or 3 parameters (Thres2P or 3P) or Logistic 2 parameters (Log2P). S: slope; y0: top point; n.d.: not determined.

Endpoint	**Test material**	**EC10 (95% CI)**	**EC50 (95% CI)**	**EC90 (95% CI)**	**Model & parameters**
Survival	Amistar [Az + Cy]	172 (74 - 269) [31 Az + 13 Cy]	320 (286 - 355) [58 Az + 23 Cy]	412 (316 - 508) [75 Az + 30 Cy]	ThreSig2P S:3.7E-3; y0:9.1; r^2:1
	Azoxystrobin	21 (15 - 30)	39 (34 - 45)	71 (62 - 83)	Log2P S:2.1; y0:10; r^2: 1
	Cyproconazole	> 70	> 70	> 70	
Reproduction	Amistar [Az + Cy]	38 (6 - 70) [6.9 Az + 2.8 Cy]	161 (130 - 193) [29 Az + 12 Cy]	238 (154 - 322) [43 Az + 17 Cy]	ThreSig2P S:4.5E-3; y0:100; r^2:0.9
	Azoxystrobin	17 (2 - 33)	37 (25 - 49)	57 (40 - 74)	Log2P
	Cyproconazole	> 70	> 70	> 70	S:2.8E-2; y0:100; r^2:0.8
Survival	Prosaro [Te + Pr]	429 (n.d.) [55 Te + 55 Pr]	533 (n.d.) [68 Te + 68 Pr]	662 (n.d.) [84 Te + 84 Pr]	Log2P S:5.8; y0:9.3; r^2:1
	Tebuconazole	148 (145 - 150)	164 (162 - 166)	180 (178 - 183)	Log2P

	Prothioconazole	> 250	> 250	> 250	S:3.4E-2; y0:10; r^2:1
Reproduction	Prosaro [Te + Pr]	139 (48 - 229) [18 Te + 18 Pr]	350 (262 - 438) [45 Te + 45 Pr]	480 (178 - 783) [61 Te + 61 Pr]	ThreSig2P S:2.6E-3; y0:99.5; r^2:0.9
	Tebuconazole	12 (-6 - 31)	41 (16 - 66)	69 (21 - 118)	Log2P
	Prothioconazole	> 250	> 250	> 250	S:1.9E-2; y0:90.3; r^2:0.7

200

201 **4. Discussion**

202 The toxicity of Amistar and pPosaro FPs seemed mostly associated to one of their a.i.(s),
203 azoxystrobin (Az) and tebuconazole (Te) respectively. Reproduction confirmed to be a more
204 sensitive endpoint than survival. This is often the case shown in literature for exposure to
205 various classes of chemicals, pesticides included, not only for enchytraeids (Amorim et al.,
206 2005; Castro-Ferreira et al., 2012; Novais et al., 2011, 2010), but also for earthworms (e.g.
207 (Leitão et al., 2014; Römbke et al., 2007)). Regarding the endpoint survival, Amistar was
208 more toxic than Prosaro at lower concentrations (Amistar EC_{10} << Prosaro EC_{10}), converging
209 to similar toxicity at higher concentrations ($EC_{50/90}$), although in terms of reproduction,
210 Amistar was around 2 times more toxic than Prosaro.

211 It is difficult to compare the magnitude of the results obtained for these FPs with literature
212 data because the very few studies on pesticides FPs on soil oligochaetes (e.g. (Rico et al.,
213 2016; Velki et al., 2019)) report either acute toxicity based on filter paper contact test and/or
214 were performed on FPs containing a single a.i, (and the toxicity results are expressed as mg
215 a.i/kg). In our study, the ECx calculated for the FPs Amistar and Prosaro correspond to the
216 entire FPs (based on its density, thus including all, a.i.(s) and the chemicals such as the
217 adjuvants, solvents, etc.). Similar to ours, a study on the collembolan *Folsomia candida*
218 reported LC/ECx for a wood preservative [containing two a.i.(s) (tebuconazole and 3-iodo-2-
219 propynyl N-butylcarbamate – IPBC)] based on the total FP mass (Guimarães et al., 2018).

220 Despite slightly higher (LC_{50} = 821 mg/kg; EC_{10} = 545 mg/kg), the effect concentrations are
221 in the same order of magnitude as ours.

222 Azoxystrobin was the most toxic a.i. when tested alone (EC_{50} of 39 and 37mg/kg for survival
223 and reproduction, respectively), showing higher toxicity than reported by Leitão et al. (2014)
224 also for *E. crypticus* (reproduction EC_{50} of 99 mg/kg). The differences are likely due to the
225 different soil types used, LUFA 2.2 versus a natural Mediterranean soil, where e.g. organic
226 matter content differs between 2 and 5%, respectively. Although no effects were observed for
227 survival and reproduction at 5 mg azoxystrobin/kg soil, this concentration was shown to
228 already significantly disturb the gut microbiota of *E. crypticus*, with enriched antibiotic
229 resistance genes (Zhang et al., 2019); such an impact can spread through the food chain and
230 amplify the ecological risks of azoxystrobin. In comparison to other soil invertebrates results
231 from Leitão et al. (2014) place *E. crypticus* and *F. candida* at similar levels, while *E. andrei*
232 was more sensitive to azoxystrobin. Our results are similar to those reported for *E. andrei*
233 (EC_{50} = 42 mg /kg) in a different soil (Leitão et al., 2014). Azoxystrobin appears to cause
234 acute toxicity to *E. crypticus* as observed from severe effects on survival (similar ECx for
235 survival and reproduction) and hence reproduction effects are probably a consequence of
236 adults' mortality. The LC_{50} (EC_{50} for survival) observed here is around 10 times lower than
237 the one reported for *E. fetida*, in OECD soil (14 d LC_{50} of 372 mg /kg (Wang et al., 2012)),
238 although direct comparison cannot be done since different soils (LUFA 2.2 versus OECD)
239 and test durations (21 d for enchytraeids and 14 d for earthworms) were used. However, the
240 chronic 28 d LC_{50} > 500 mg/kg for *E. andrei* (Leitão et al., 2014), although in different soil
241 types, support the indication of higher sensitivity of *E. crypticus* survival. Cyproconazole
242 alone was not toxic to *E. crypticus* within the tested concentrations (up to 70 mg/kg). On the
243 other hand, for *E. fetida*, cyproconazole was more toxic than azoxystrobin (14 LC_{50} of 212
244 mg cyproconazole/kg and 372 mg azoxystrobin/kg (Wang et al., 2012)). For the collembolan

F. candida azoxystrobin seems to be more toxic than cyproconazole (28d no observed effect concentration – NOEC of 25 and 55.8 mg/kg for azoxystrobin and cyproconazole, respectively) (Registration report ADD-F2-003, 2018) with the flaws present when comparing NOEC values.

Regarding the comparison between the a.i.(s) and FPs, the effect curves for azoxystrobin and Amistar (Fig 1) are very close, this for the endpoint reproduction, indicating that azoxystrobin is the main responsible for the toxicity caused by Amistar to *E. crypticus*. In terms of survival, azoxystrobin alone was more toxic than Amistar (azoxystrobin LC_{50} = 39 mg/kg, Amistar LC_{50} corresponds to 58 mg azoxystrobin/kg). Even though natural variation cannot be ruled out (as depicted in Fig. 1, the effect curves are close) for this endpoint, the possible reduction of toxicity can be either due to a "dilution" effect with the adjuvants, or due to a low antagonism effect (i.e., the combined effect of two or more chemicals is less than the sum of the effects of each individually) in the mixture (cyproconazole, the other a.i., did not induce toxicity to *E. crypticus*). The higher toxicity of the pure a.i. compared to the FP has been previously reported, e.g. in *E. crypticus* for atrazine in comparison to the atrazine based FP "Gesaprim" (Gomes et al., 2019), in this case the FP contains a single a.i..

Tebuconazole induced reproductive toxicity to *E. crypticus* while prothioconazole, the other a.i. of Prosaro did not (up to 250 mg/kg). The toxicity of tebuconazole was at the same level as azoxystrobin for reproduction, but 4 times less lethal (LC_{50} of 164 and 39 mg/kg for tebuconazole and azoxystrobin, respectively). This can have implications for the risk assessment, which can be expected higher for more lethal compounds applied similarly. Further, higher toxicity of low-toxic compounds cannot be excluded if exposure is extended during more generations and longer term. In agreement with our results, a comparative study on pesticides toxicity to *E. fetida* showed that azoxystrobin (14 d LC_{50} of 327 mg/kg) was more toxic than tebuconazole (14 d LC_{50} of 895 mg/kg) (Wang et al., 2012), but other studies

reported lower 14 d LC_{50} values for tebuconazole (287 mg/kg in Chen et al. (2018), and 180 mg/kg in Rico et al. (2016) the last one tested as FP - Folicur 25 EW). The reproductive toxicity to *E. crypticus* is at the same level as the reported for *F. candida* in OECD soil (EC_{10} = 44.5 mg/kg (6.2 < CI < 82.8 mg/kg)), however tebuconazole is more lethal to *E. crypticus* than to *F. candida* (LC_{50} of 164 and > 1250 mg/kg, respectively) (Guimarães et al., 2018). The absence of toxicity of prothioconazole in *E. crypticus* is in agreement with the acute lethality assessed in *E. fetida* (14d LC_{50} > 1000mg/kg (PPDB: DataBase). As observed for Amistar, the effects on reproduction caused by Prosaro seem to be explained by one of the a.i., in this case tebuconazole, as the effect curves for Prosaro and tebuconazole are very close (Fig 1). However, in terms of adults´ survival, Prosaro is more toxic than any of its a.i.(s) alone. This can be due to toxic effects caused by the adjuvants (often called inert ingredients) present in the FP and/or by synergistic effects (i.e., the combined effect of two chemicals is greater than the sum of the effects of each given chemical alone) between the two a.i.(s) or the a.i.(s) and the adjuvants. In any case, it seems to preferentially affect the adults (the effects on reproduction were similar between Prosaro and the a.i. tebuconazole). Studying a wood preservative FP containing tebuconazole and IPBC as a.i.(s), Guimarães et al. (2018) found that IPBC was the principal contributor to lethal toxicity to *F. candida*, while the effects on reproduction were fairly predictable based on the mixture concentration addition (CA) model, with slight underestimation. This underestimation is suggested to result from the contribution of the FP's additives to the reproductive toxicity (Guimarães et al., 2018). The FP's additives seem to trigger an avoidance behaviour that was not predictable based on the effect of the a.i.(s) on organisms' survival or reproduction (Guimarães et al., 2018). Similarly, the higher avoidance response of *E. andrei* to the herbicide FP Viper in comparison to its a.i. penoxsulam, has been attributed to the adjuvants present in the FP (Marques et al., 2009). Previously, Cox and Surgan (2006) highlighted that the inert

ingredients can increase toxicity and ecotoxicity of pesticides FPs, and that it is unclear if inert ingredients increase toxicity directly or by interacting with the a.i.(s). Since not all the inert ingredients are often clearly identified in the product labels, and so not easy to assess its toxicity, it is of utmost importance to test also the FPs, and not only the a.i.(s) (Cox and Surgan, 2006). Further, there is the case of the FPs containing more than one a.i. (as the ones tested here) for which, in addition to the problematic of the adjuvants, there is also the possible interaction between the a.i.s (and of course between the a.i.s and the adjuvants). Even though within the EU regulation, evaluation is required for the a.i.(s) (at the EU level) and for the FPs (at Member State level), the information might not be easily available for the (scientific) community. It was not possible to find ecotoxicological information for soil invertebrates on Amistar ® XTRA and Prosaro ® 250 EC safety datasheets, and our study shows that the toxicity of the FP Prosaro was not predicted by the toxicity of its a.i.(s). As recommended, testing various species is of high relevancy (Frampton et al., 2006) to protect soil biodiversity and implement SSD based ERA. It may be of interest to realise that if these compounds were to be evaluate as chemicals, the fact that only 1 (or two species) were present would indicate a safety factor of 100 or 10 (depending in further evidence), making it close to the reported PECs and possibly also considering the uncertainty of the ECx estimates.

5. Conclusions

To our best knowledge, this is the first study reporting toxicity data for the fungicide's FPs Amistar ® XTRA and Prosaro ® 250 EC to a soil invertebrate (non-target organism). Overall Amistar was more toxic than Prosaro, particularly considering the endpoint reproduction.

By studying, simultaneously, the toxicity of the a.i.(s) of each FP, indications are that the reproductive toxicity of Amistar is explained by the a.i. azoxystrobin while Prosaro's is explained by the a.i. tebuconazole. However, in terms of survival, the toxicity of the FPs was not predicted by the toxicity of the a.i.(s), particularly in the case of Prosaro, which was more toxic than any of its a.i.(s). This highlights the importance of testing the FPs, rather than the a.i.(s) alone, to assess or predict the risk of commercial pesticide products to the environment. Further, testing various species is recommended and key to implement SSD based ERA, meaning that the present results could indicate safety factors of 100 or 10 (depending in further evidence), making it close to the reported PECs. Last, chronic testing is recommended and more relevant than acute, although acute effects are usually also covered during a chronic endpoint test. Also some sublethal endpoints might be implemented in future studies, such as biomarkers of genotoxicity, oxidative stress, immune system etc. to further understand toxic effects.

Declaration of competing interest

The authors declare no conflict of interest.

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CRediT authorship contribution statement

SIL Gomes: Investigation, Formal analysis, Writing - Original Draft, Review & Editing **Anna Ammendola:** Investigation, Formal analysis; **S Casini:** Editing; **MJB Amorim:** Conceptualization, Supervision, Project administration, Funding acquisition, Draft, Review & Editing.

***Declaration of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

