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Toxicity evaluation of three pesticides on non-target aquatic and soil organisms: commercial formulation versus active ingredient

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Abstract The Ecological Risk Assessment of pesticides requires data regarding their toxicity to aquatic and terrestrial non-target species. Such requirements concern active ingredient(s), generally not considering the noxious potential of commercial formulations. This work intends to contribute with novel information on the effects of short-term exposures to two herbicides, with different modes of action (Spasor®, Stam Novel Flo 480®), and an insecticide (Lannate®), as well as to corresponding active ingredients (Glyphosate, Propanil and Methomyl, respectively). The microalga *Pseudokirchneriella subcapitata* (growth inhibition), the cladoceran *Daphnia magna* (immobilisation), and the earthworm *Eisenia andrei* (avoidance behaviour) were used as test species. Both herbicides were innocuous to all test organisms at environmentally realistic concentrations, except for Stam and Propanil (highly toxic for *Pseudokirchneriella*; moderately toxic to *Daphnia*). Lannate and Methomyl were highly toxic to *Daphnia* and caused *Eisenia* to significantly avoid the spiked soil at realistic application rates. The toxicity of formulations either overestimated (e.g. Stam/Propanil for *P. subcapitata*) or underestimated (e.g. Stam/Propanil for *D. magna*) that of the active ingredient.

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Introduction

Pesticides and other agrochemicals have been increasingly used given their benefits in controlling pests, pathogens and weeds. These substances constitute a substantial source of contamination of non-target systems due to their overuse and application techniques. Contamination of different environmental matrices (e.g. water, soil), via spray drift, volatilization, run-off and/or leaching, has been widely reported in the literature (e.g., Cerejeira et al. 2003; Wilson and Foos 2006; Tariq et al. 2007). While agricultural soil is the primary recipient for agrochemicals, since most application techniques drive these xenobiotics directly or indirectly into soils, water bodies adjacent to agricultural areas are usually the ultimate recipient for pesticide residues. Both environmental compartments sustain complex living communities interacting within large food webs. Therefore, non-target organisms belonging to both compartments are of primary interest when addressing the potential adverse effects of pesticides.

The authorisation for pesticide commercialisation in Europe (EEC 1991) currently requires previous testing of potential negative effects of the active ingredient(s) on non-target terrestrial (EC 2002a) and aquatic (EC 2002b) organisms. Additionally, under either a predictive or retrospective Environmental Risk Assessment (ERA) framework, ecotoxicological data represent vital information (EC 2002a, b, 2006; Frampton et al. 2006; Jensen and Mesman 2006; Weeks and Comber 2005). Several studies have been conducted on the effects of pesticides in aquatic organisms and comprehensive databases are available (e.g.

Kegley et al. 2007). However, the availability of soil quality criteria for ecological receptors is somewhat deficient when compared with criteria regarding aquatic receptors (O'Halloran 2007). On the other hand, studies performed under EEC and ERA guidelines usually focus on the potential deleterious effects of the active ingredients (a.i.) rather than on the whole marketed products (i.e. commercial formulations). Surfactants and other so-called inert ingredients, which are added to the active ingredient to enhance its chemical and physical efficacy (Cox and Surgan 2006) as a pesticide, may also contribute significantly for its overall toxicity (Cedergreen and Streibig 2005; Krogh et al. 2003; Oakes and Pollak 2000; Pereira et al. 2000; Solomon and Thompson 2003). Hence, toxicity testing of both a.i. and commercial formulation is likely to provide more realistic reports on the overall ecotoxicological impact of pesticides on sensitive non-target organisms.

The present study investigated the toxicity of the pesticides Lannate® (insecticide), Spasor® (herbicide), Stam Novel Flo® (herbicide), and corresponding active ingredients Methomyl, Glyphosate and Propanil, to non-target species (aquatic and terrestrial). We hypothesised that evaluation of the toxicity of active ingredients, which are mandatory for the authorisation of pesticides in Europe (EEC 1991), might underestimate the potential of the commercial formulations to yield deleterious effects on non-target species. Thus, short-term exposures to the above-stated formulations and active ingredients were conducted including relevant ecological receptors and standard ecotoxicological endpoints: (1) growth inhibition of the freshwater green alga *Pseudokirchneriella subcapitata* (OECD 2006); (2) immobilisation of the freshwater cladoceran *Daphnia magna* (OECD 2004); and (3) avoidance behaviour of the earthworm *Eisenia andrei* (ISO 2005). The selected species are recommended as part of the test battery for xenobiotic risk assessment purposes (e.g. Fochtman et al. 2000) and are representative of key links in the energy transfer within aquatic and terrestrial food webs. The simultaneous testing of active ingredients and commercial formulations, using both terrestrial and aquatic test organisms, is an approach that may guarantee more comprehensive and robust support for regulators, decision makers and other competent authorities on the overall management of agricultural practises and related areas.

Materials and methods

The freshwater microalga *Pseudokirchneriella subcapitata* (Chlorophyceae), the zooplankter *Daphnia magna* (Branchiopoda), and the earthworm *Eisenia andrei* (Oligochaeta) were subjected to short-term exposures of one insecticide (Lannate®—Methomyl) and two herbicides (Spasor®—Glyphosate and Stam Novel Flo® 480—Propanil) that have distinct modes of action. Separate experiments were conducted with the commercial formulations and active ingredients. Exposure ranges of commercial formulations were calculated and handled based on the respective concentration of active ingredient (as provided by the manufacturer); this allowed direct comparisons between the toxicity of formulations and isolated a.i.. For text clarity and convenience all concentrations are expressed in mg l^{-1} of Methomyl, Glyphosate and Propanil for aqueous exposures; for soil exposures, concentrations are expressed either in kg ha^{-1} or in mg kg^{-1} in order to facilitate comparison with literature data—conversions from mg kg^{-1} into kg ha^{-1} accounted to the test tray area (2×110 cm^2). The definite concentration ranges used in each test are depicted in Table 1. Regardless of the species used, the treatments in each test were settled by geometric dilutions of a single highly concentrated stock solution, which was freshly prepared either by direct dilution of the commercial formulation in distilled water or by dissolving the required mass of active ingredient (technical grade) in distilled water. Difficulties were found when dissolving Propanil in water; thus, a stock solution was prepared by dissolving Propanil directly in *Daphnia* synthetic test medium (see below), which was later successively diluted with clean medium, eliminating the need of a highly concentrated stock solution. In the *E. andrei* assay, high toxicant concentrations were needed, so acetone was used as an organic carrier when preparing the stock solution (see Appendix for details).

Toxicants

Lannate® (Sapec Agro®, Portugal) is a widely marketed commercial formulation of the monomethyl carbamate insecticide Methomyl [IUPAC name: S-methyl N-(methylcarbamoyloxy)thioacetimidate]. The concentration of this commercial solution is 200 g Methomyl l^{-1} and the active ingredient used in this study (Makhteshim Agan®, Portugal) is reported as 99.5% pure. Methomyl is a contact AChE inhibitor used to control a wide range of insects and spider mites in certain fruit crops, vegetables, ornamentals and field crops (Tomlin 2001). It is a highly water soluble chemical, and has a low octanol:water partition coefficient; this insecticide was found to adsorb poorly to soil organic matter (WHO 1996).

Spasor® (Lactema, Portugal) is a relatively recent commercial compound that uses the broad-spectrum herbicide Glyphosate [IUPAC name: N-(phosphonomethyl)-glycin] as active ingredient. The concentration of this commercial solution is 360 g Glyphosate l^{-1} and the active ingredient used in this study (Sapec Agro®, Portugal) is reported as 95% pure. Glyphosate consists of a

Table 1 Final toxicant concentrations (nominal) used in the definite assays (for calculation of EC_{50} and LOEC values) with the three test species (*P. subcapitata*, *D. magna* and *E. andrei*)

Pseudokirchneriella subcapitata				
mg l^{-1}	Methomyl	62.5; 78.1; 97.7; 122; 153; 191; 238	Lannate	72.5; 94.3; 123; 159; 207; 269; 350
mg l^{-1}	Glyphosate	61.5; 65.5; 81.9; 102; 160; 200	Spasor	49.0; 63.7; 82.8; 108; 140; 182; 237
µg l^{-1}	Propanil	20.7; 24.9; 29.9; 35.8; 43.0; 51.6; 61.9	Stam	0.82; 1.96; 4.71; 11.3; 27.1; 65.1; 156
Daphnia magna				
µg l^{-1}	Methomyl	15.1; 18.1; 22.7; 26.0; 31.3; 38.5; 45.0	Lannate	13.0; 16.9; 22.0; 28.6; 37.1; 48.3
mg l^{-1}	Glyphosate	up to 2,000	Spasor	265; 278; 292; 307; 322; 338; 355
mg l^{-1}	Propanil	1.19; 1.49; 1.87; 2.34; 2.92; 3.65; 4.45	Stam	1.25; 1.87; 2.81; 4.22; 6.33; 9.49
Eisenia andrei				
mg kg^{-1}	Methomyl	2.19; 3.51; 5.62; 8.98; 14.4; 23.0	Lannate	1.36; 1.90; 2.66; 3.72; 5.21; 7.29
kg ha^{-1}		0.39; 0.64; 1.02; 1.63; 2.61; 4.18		0.25; 0.35; 0.48; 0.68; 0.95; 1.32
mg kg^{-1}	Glyphosate	6.15; 9.22; 13.8; 20.7; 31.1; 46.7	Spasor	4.28; 15.4; 27.7; 49.9; 89.8; 162
kg ha^{-1}		1.12; 1.68; 2.51; 3.77; 5.66; 8.49		0.78; 1.40; 2.52; 4.54; 8.17; 14.7
mg kg^{-1}	Propanil	2.2; 3.6; 5.7; 9.2; 14.6; 23.4; 37.5; 60.0	Stam	16.4; 24.6; 36.8; 55.3; 82.9; 124
kg ha^{-1}		0.4; 0.7; 1.0; 1.7; 2.7; 4.3; 6.8; 10.9		2.98; 4.47; 6.70; 10.0; 15.1; 22.6

Concentrations of commercial formulations (Lannate, Spasor and Stam) regard the concentration of active ingredient. For comparative purposes, concentrations in the soil avoidance assays are shown both in mg kg^{-1} and in kg ha^{-1}

glycine moiety and a phosphonomethyl moiety, and it is a systemic herbicide acting in target species through enzyme inhibition in the amino acid metabolism (Herrmann and Weaver 1999; Baylis 2000). It is a water soluble chemical ($\sim$10.5 g l^{-1} at pH = 2 and 20°C); its relatively high Koc values indicate a relevant ability to bind to soil particles, but it is also fairly mobile in certain soil matrices (EC 2002c). Both commercial formulation and a.i. stock solutions exhibited low pH; we adjusted pH with NaOH to comply with guideline requirements (OECD 2004, 2006) and reduce uncertainty in the toxicological assessments.

Stam Novel Flo® (Dow, Portugal) is a commercial formulation of the highly selective contact herbicide Propanil (IUPAC name: 3,4-dichloropropioanilide). The concentration of this commercial solution is 480 g Propanil l^{-1} and the active ingredient used in this study (Sapec Agro®, Portugal) is reported as 97% pure. This anilide is commonly used during the post-emergency of rice (*Oryza sativa*) to control grass and broadleaf weeds through an enzyme-mediated selective inhibition of photosystem II (Tomlin 2001). Additionally, Propanil is highly water soluble (152 mg l^{-1}) and it is not likely to strongly adsorb to soil particles given its low Koc (Kegley et al. 2007).

The three commercial solutions will hereinafter be referred to as Lannate, Spasor and Stam and their toxicity will be compared to that of their respective a.i., Methomyl, Glyphosate and Propanil.

Algal growth assays

The microalga *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) was maintained in nonaxenic batch cultures with Woods Hole MBL medium, at 20 ± 2°C and under permanent illumination (24 h^L). When starting new cultures, algae were harvested while still in the exponential growth phase (5–7 days-old) and inoculated in fresh medium. All the assays followed the OECD (2006) guidelines for algal growth inhibition tests. Algae were exposed for 96 h using a replicated design (three replicate vessels per pesticide concentration). Experiments were carried out under a static design in 100 ml glass vials filled with 40 ml of corresponding test solution (Gonçalves et al. 2005). Clean MBL medium was used as the negative control (optimal growth). Assay conditions were similar to those described above, except that a 16 h^L:8 h^D photoperiod was used, for logistic reasons; to compensate this, we adjusted assay duration to 96 h, instead of the recommended (OECD 2006) 72 h. Test vials were randomly incubated in an orbital shaker (100 rpm), bearing an initial cell density of 10^4 cells ml^{-1}. At the end of the assay, algal density was estimated using a Neubauer haemocytometer; growth inhibition (in %) was estimated by expressing the final cell density of each concentration to that of the control.

Daphnia immobilisation assays

Monoclonal *Daphnia magna* (clone A, *sensu* Baird et al. 1989a) bulk cultures have been continuously reared in our laboratory, under a 16 h^L:8 h^D photoperiod, at a temperature of 20 ± 2°C, in synthetic ASTM hard water medium (ASTM 1980) supplemented with a standard organic additive (Baird et al. 1989b) and vitamins (Elendt and Bias 1990). Cultures were renewed every other day and fed with

P. subcapitata (see rearing procedures above), at a ration of 3.0×10^5 cells ml^{-1}. The acute toxicity of each pesticide to *D. magna* was assessed following standard protocols (e.g. OECD 2004). The 48 h exposures were carried out under a static design using twenty neonates (<24 h old; born between the 3rd and the 5th brood in the bulk cultures) per treatment (five animals randomly assigned to four replicate vessels per treatment). Assay conditions were similar to those described above for culturing procedures. Tests were carried in glass beakers filled with 100 ml of test solution, and clean ASTM hard water medium was used as negative control. Vessels were screened for immobilised individuals after 48 h.

Earthworm avoidance assays

Adult clitellate earthworms (*Eisenia andrei*) were raised as synchronised cultures in large containers, under controlled conditions (temperature 20 ± 2°C; photoperiod 16 h^L:8h^D). The assays were carried out in 110 cm^2 test containers and followed ISO (2005) recommendations. Standard LUFA 2.2 soil (Agricultural Research Centre, Speyer, Germany) was used in all experiments. The soil moisture was adjusted to 40% of the water holding capacity by adding 40 ml of distilled water (control soil) or pesticide solutions (spiked soils). Avoidance was evaluated using a dual-choice test design where half of each test container was filled with 200 g (~325 cm^3) of spiked soil whereas the other half was filled with 200 g of clean (control) soil. After filing the test containers with control and spiked soils, ten adult *E. andrei* (weight: 0.3–0.6 g) were placed on the border line separating the two soils in each test container (see details of the protocol in Antunes et al. 2008). Assays were kept for 48 h under the same incubation conditions as described for *E. andrei* culturing; after this period, the number of earthworms present in each soil (spiked and control) was recorded for each test container. Organisms stretching across the border line between the two soils were assigned as standing in both soils (0.5 individuals in each soil), regardless the relative position of the body in the line.

Statistical analysis

Probit analysis (SPSS 15 for Windows®, SPSS Inc.) was used to estimate EC_{50} values and respective 95% confidence intervals for each tested chemical in acute exposures with algae (growth inhibition) and *Daphnia* (immobilisation).

Avoidance or preference of *E. andrei* for the pesticide-spiked soils was expressed as net response (ranging from +1 to −1), following Antunes et al. (2008):

$$\text{net response} = \frac{C - T}{N},$$

where C is the number of organisms in the control soil, T is the number of organisms in the test (spiked) soil, and N stands for the total number of surviving organisms. Positive values thus account for avoidance of test soil, while neutral or negative responses represent indifference or preference towards it. For each dual-choice experiment, mean net response was computed ($n = 3$) and independent t-tests ($\alpha = 0.05$) were used to determine if net response was significantly higher than zero (H_0: $\mu \leq 0$; H_1: $\mu > 0$). LOEC values were estimated as the lowest concentrations of active ingredient causing significant avoidance of test organisms. For comparative purposes, we also used the habitat function threshold proposed by Hund-Rinke and Wiechering (2001) and recommended by ISO (2005), which states that habitat function of soil is compromised if that soil is avoided by more than 80% of earthworms (corresponding to a net avoidance larger than 0.6—see Antunes et al. 2008).

Results

Table 1 shows the concentrations used for each combination of toxicant and species used in the definite tests. The insecticide Lannate/Methomyl was shown to be very toxic to *D. magna* (formulation: 0.022–0.026 mg l^{-1}; a.i.: 0.019–0.022 mg l^{-1}; these values represent the 95% confidence intervals estimated for the EC_{50}—see Fig. 1), whereas Stam/Propanil (contact herbicide) was moderately toxic to the cladoceran (formulation: 3.2–4.1 mg l^{-1}; a.i.: 1.8–2.5 mg l^{-1}). This contrasted with the pattern observed in *P. subcapitata* growth assays, where the herbicide was highly toxic (formulation: 0.009–0.018 mg l^{-1}; a.i.: 0.023–0.037 mg l^{-1}) and the insecticide was toxic only at high levels (formulation: 164–206 mg l^{-1}; a.i.: 87–126 mg l^{-1}). Both organisms were tolerant to the systemic herbicide Spasor/Glyphosate (after pH adjustment), given the high EC_{50} values estimated for the microalga (formulation: 63–79 mg l^{-1}; a.i.: 108–158 mg l^{-1}) and the crustacean (formulation: 299–315 mg l^{-1}; a.i.: no EC_{50} could be determined up to 2,000 mg l^{-1}) (see Fig. 1).

Although the overall toxicity of commercial formulations versus a.i. was comparable (within the same order of magnitude—see Fig. 1), there were cases where the toxicity of the formulation underestimated that of the a.i. (Lannate/Methomyl for *P. subcapitata* and Stam/Propanil for *D. magna*) and where the toxicity of the formulation overestimated that of the a.i. (Stam/Propanil for *P. subcapitata* and Spasor/Glyphosate for both organisms).

The avoidance behaviour of earthworms (*E. andrei*) was not sensitive to any of the herbicides (Stam/Propanil and Spasor/Glyphosate), even at very high (i.e. unrealistic) concentrations (Fig. 2); the highest concentrations tested

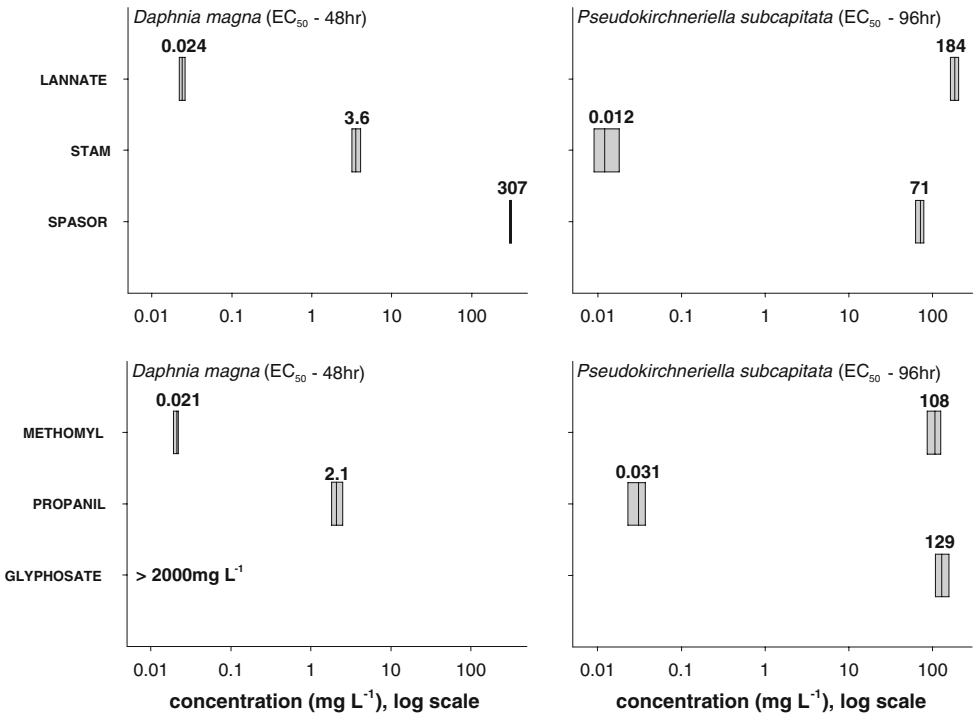

Fig. 1 EC_{50} values (mg l^{-1}) for *Daphnia magna* (*left panel*) and *Pseudokirchneriella subcapitata* (*right panel*) exposed to three pesticides (*top panel*—commercial formulations; *bottom panel*—active ingredients). The actual EC_{50} is depicted above the median of its respective boxplot and the breadth of the boxes represents the corresponding 95% confidence intervals

for all toxicants were roughly three-times higher than the recommended application rates (see "Discussion"). When exposed to Lannate (insecticide), however, *E. andrei* significantly avoided the spiked soil from 0.35 kg ha^{-1} onwards (except at 0.68 kg ha^{-1}, which reflects large heterogeneity among replicates—see Fig. 2). At 0.95 and 1.32 kg ha^{-1}, soil habitat function became compromised (Fig. 2). Thus, for risk assessment purposes, a NOEC of 0.25 kg ha^{-1} and a LOEC of 0.35 kg ha^{-1} should be considered. Similarly, Lannate's a.i. (Methomyl) induced a significant avoidance response in earthworms from 1 kg ha^{-1} onwards (NOEC = 0.64 kg ha^{-1}; LOEC = 1.00 kg ha^{-1}), with soil habitat function also becoming compromised from this concentration onwards (Fig. 2). Although both formulation and a.i. compromised soil habitat function at similar concentrations of Methomyl, the commercial version of the toxicant induced avoidance at lower concentrations.

Discussion

The European Union has demonstrated concerns on the adverse environmental effects of pesticides by regulating its registration and placement on the market (EEC 1991). The REACH directive (EC 2006) is the most recent official EU document where these concerns are expressed, and where the need for generating a comprehensive ecotoxicological database on these chemicals is recognised. In this way, the present study provides relevant information on the deleterious potential of three widely used pesticides in aquatic and terrestrial non-target organisms. In addition, we addressed the inconsistencies that can be found when the toxicity of commercial formulations and corresponding active ingredients is directly compared.

As expected, given its mode of action, Methomyl exhibited very low toxicity to *P. subcapitata* and very high toxicity to *D. magna*. Fernandez-Alba et al. (2002) found a 72-h EC_{50} of 60 mg l^{-1}, using technical grade Methomyl and an Algaltoxkit. Inter-laboratorial variation in methods and the eventual use of a different algal strain may explain the discrepancy between this record and that obtained in our study. Exposure of algae to both active ingredient and tested commercial formulation of the pesticide produced unrealistic (i.e. very high) EC_{50}s. Still, they constitute valuable information for the general ecotoxicological profile of the chemical. Carbamates (such as Methomyl/Lannate) have gained favour over other insecticidal classes worldwide (Ehler 2004); however, these chemicals are not ecologically selective and pose concerns regarding their hazardous potential for non-target arthropods, such as *Daphnia*. The EC_{50} values estimated for *D. magna* after exposure to Methomyl and Lannate were very similar, and denote high sensitivity of the species to the insecticide. These toxicity records were slightly more conservative (presenting lower EC_{50}s) than those reported by other authors [EC_{50}s between 29 and 32 µg l^{-1}, in exposures to the active ingredient (Fernández-Alba et al. 2002; Tomlin 2001; WHO 1996)].

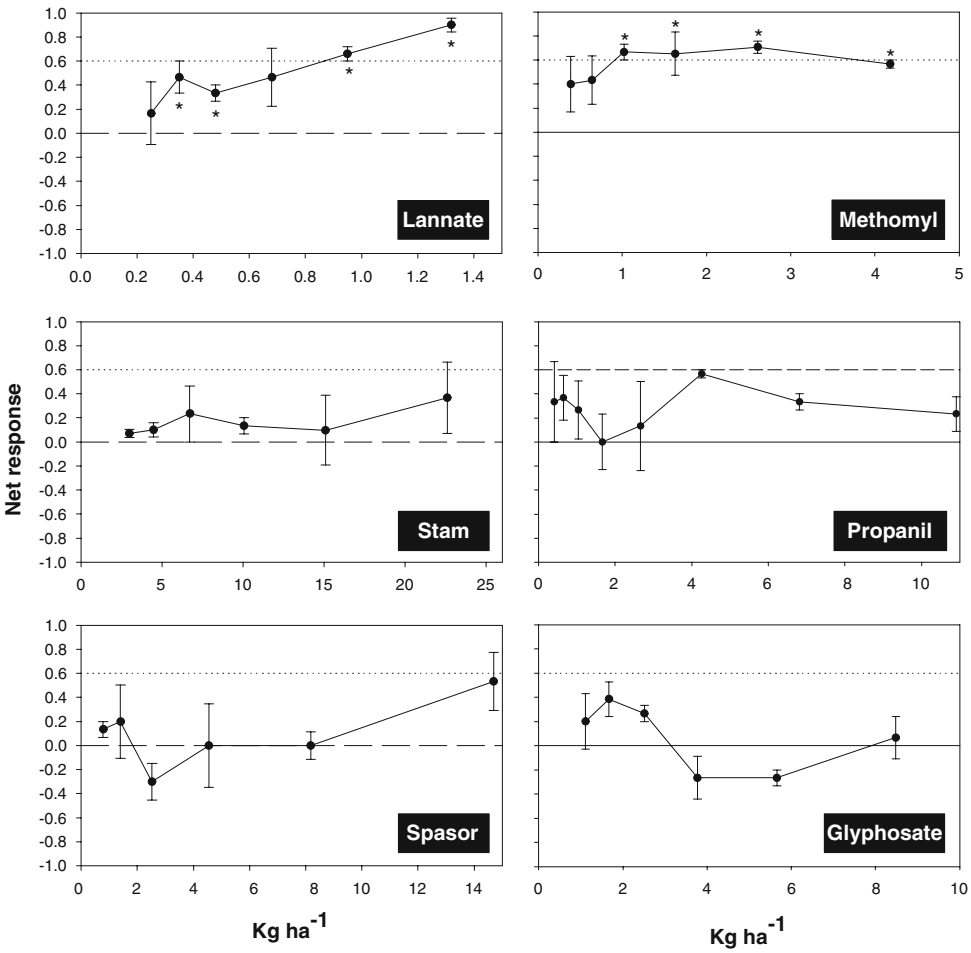

Fig. 2 Net response of *Eisenia andrei* exposed to dual combinations of test soil (LUFA 2.2 spiked with commercial formulations or active ingredients) versus control soil (clean LUFA 2.2). Results are expressed as average values ± standard error. A positive response represents avoidance from test soil and values higher than 0.6 are above Hund-Rinke and Wiechering (2001) habitat function threshold (see text for further explanation). Statistically significant deviations of net response from zero are shown (*) for each toxicant concentration (*t*-tests, $P \leq 0.05$)

Two herbicides with distinct modes of action and different uptake pathways were tested (see "Materials and methods"—*Toxicants* for details). The considerable tolerance to Glyphosate exhibited by *P. subcapitata* should be related to a poor permeability of algal cells to the chemical (in higher plants, Glyphosate enters cells via systemic uptake). In fact, Glyphosate has a zwitterionic structure (Schönherr 2002; Tomlin 2001), which constrains its lipid solubility and, therefore, its diffusion across cell membranes (Schönherr 2002). Glyphosate acts within the shikimate pathway, by specifically inhibiting the enzyme 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase, thus blocking the production of aromatic aminoacids (Baylis 2000; Herrmann and Weaver 1999). The shikimate pathway is a metabolic feature never found in animals (Herrmann and Weaver 1999). Consequently, *D. magna* was found fairly insensitive to Glyphosate and Spasor, as expected. In line with literature data (e.g. Cedergreen and Streibig 2005; EC 2002c; Solomon and Thompson 2003; Tomlin 2001), our records confirm reduced hazard of this herbicide for algae and cladoceran species. However, our results were obtained after pH adjustment, since both forms of the herbicide (a.i. and commercial formulation) are highly acidic. Under high environmental concentrations of Glyphosate (e.g. following application), transient effects due to acidification cannot be excluded.

Propanil and Stam demonstrated high toxicity to *P. subcapitata* and moderate toxicity to *D. magna*. Ferraz et al. (2004) found EC_{50} values for Propanil in different green algae (*Chlorella* spp. and *Scenedesmus* spp.) that are 1–2 orders of magnitude higher than those we found for *P. subcapitata*. The US Environmental Protection Agency (USEPA 2001), however, reported a more concordant 48 h-EC_{50} of 16 μg l^{-1} for a freshwater diatom. These discrepancies suggest large variability in the tolerance of freshwater microalgae to Propanil; for protective purposes, the most sensitive estimates should be taken as reference values within future risk assessment procedures. The EC_{50} values estimated here for *D. magna* to Propanil and Stam were generally within the toxicity range reported in the literature [Propanil EC_{50} for *D. magna* within 1.2–5.0 mg l^{-1} (Pereira et al. 2000; USEPA 2001; Villarroel et al. 2003)]. The specific mechanism of toxicity of Propanil in target species involves an enzyme-mediated disruption of the electron flow in the photosystem II, thereby inhibiting the light reaction of photosynthesis

(Tomlin 2001). In animals, other redox metabolic pathways may be disrupted by Propanil, but available literature only refers the ability of Propanil and other aromatic anilides to promote the increase of methaemoglobin levels (McMillan et al. 1990); such an increase could indirectly affect energy metabolism pathways. USEPA (2001) has expressed its concern on the acute and chronic risks that Propanil, and its major metabolite 3,4-dichloroaniline (3,4-DCA), represents to freshwater invertebrates. In fact, reproductive assays conducted in our lab (Pereira et al. 2007) have shown high chronic toxicity of this herbicide to *Daphnia*. Further studies are clearly needed in order to fully characterise the ecotoxicological profile of this herbicide and accurately estimate its environmental risks.

Earthworms play a major role in the soil ecosystem and are highly sensitive to contaminated soils (e.g., Schaefer 2003; Yeardley et al. 1996), which makes them important ecological receptors and relevant test organisms in Ecotoxicology (Lavelle et al. 2006; Römbke et al. 2005); additionally, the burrowing activities of some species influence the properties and function of soils at different biological, chemical and physical scales (Capowiez et al. 2005). In this way, earthworm avoidance behaviour has recently been considered as a promising endpoint in cost-effective bioassays for screening soil contamination (Antunes et al. 2008; da Luz et al. 2004; Hund-Rinke and Wiechering 2001; Schaefer 2003; Slimak 1997; Yeardley et al. 1996). In this paper, we provide the first data on earthworm avoidance behaviour for the pesticides Methomyl, Glyphosate and Propanil, as well as their corresponding commercial formulations.

Typical application rates of the insecticide Methomyl are of 0.15–1.0 kg ha^{-1} (WHO 1996). Considering Methomyl LC_{50} values available in the literature (Tomlin 2001; WHO 1996) and data available on effects over its population and biomass (detected after unrealistic application rates of 3–3.4 kg ha^{-1}; Tomlin and Gore 1974), it does not seem likely that any hazard exists for earthworm populations, under realistic field scenarios. However, the NOEC and LOEC values we obtained (particularly for the commercial formulation, Lannate) demonstrated higher sensitivity of avoidance behaviour. In fact, our results indicate that soil ecological function may actually become compromised, even if common application rates and techniques are used (i.e., at environmentally realistic concentrations). In addition, ongoing research (unpublished data from our team) has shown that Methomyl neurotoxicity is noticeable earlier than the avoidance response. These evidences raise important concerns about the actual hazardous potential of this xenobiotic to terrestrial communities.

Typical application rates of Glyphosate and Propanil are within the ranges of 1.5–4.3 and 2.5–5.0 kg ha^{-1}, respectively (Tomlin 2001). Considering the avoidance assay data (where higher concentrations were used), it seems safe to say that these herbicides appear to be innocuous for terrestrial earthworm communities. No references were found in the literature addressing the toxicity of Propanil or other anilides to earthworms. Furthermore, Propanil's major metabolite (3,4-DCA) exerted toxicity on *Eisenia* sp. reproduction only at very high concentrations (Hund-Rinke and Simon 2005). Concerning Glyphosate, its low toxicity to earthworms was already reported in studies focusing on mortality or reproduction (EC 2002c). Although the uptake of several contaminants occurs via the thin vascularised cuticle of earthworms (Jager et al. 2003), the passive entrance of Glyphosate through the hypodermis seems unlikely (Bon et al. 2006). Uptake via food particles can, however, constitute an important route of exposure to organic chemicals (Jager et al. 2003).

Marketed pesticides are composed of the active ingredient and a number of other chemicals (generally called inert ingredients) that support its mixing, dilution, application, and stability (Cox and Surgan 2006). Inert ingredients are not supposed to be toxic and their identification and percentages within the formulation are rarely disclosed. However, several authors (e.g. Krogh et al. 2003; Oakes and Pollak 2000; Solomon and Thompson 2003) have already questioned the use of ecotoxicological data obtained using solely active ingredients of pesticides. These authors have shown that the so-called inert ingredients can contribute to the overall toxicity of the formulation, either by exerting toxic activity on their own, or by interacting with the active ingredient. Some studies additionally demonstrated that commercial formulations often exhibit higher toxicity to non-target organisms than the corresponding active ingredients (e.g. Cedergreen and Streibig 2005; Pereira et al. 2000). Our results on the toxicity of Glyphosate/Spasor to aquatic organisms followed this trend, clearly demonstrating that formulation ingredients enhanced herbicide toxicity; in fact, it is recognised that the herbicidal activity of Glyphosate is activated by the remaining ingredients within each commercial formulation (e.g. Baylis 2000).

Apart from the above case, no other combination of active ingredient versus commercial formulation consistently supported our initial hypothesis that the latter would be more noxious to non-target biota, due to the direct and indirect effects of inert ingredients (see above). Nevertheless, differences in EC_{50} (algae and *Daphnia*) or NOEC (earthworms) values between formulations and corresponding active ingredients were frequently noticed in this study (with commercial formulations either overestimating or underestimating the toxicity of their active ingredients). This reinforces concerns on the use of reference toxicity values based in assessments that consider solely the active ingredient(s) of a pesticide. Such an approach may result in

biassed benchmark values for regulatory purposes or for risk assessment procedures on new pesticides.

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Appendix: Methodological details for use of an organic solvent in *E. andrei* exposure to Propanil

The use of organic solvents such as acetone overcomes difficulties in toxicant solubility and often facilitates the full accomplishment of test requirements (OECD 2004). Standard guidelines for toxicity testing with aquatic organisms recommend such a procedure for poorly soluble substances (e.g., OECD 2004; ISO 2005). In the avoidance assay with Propanil, water holding capacity was first adjusted (see "Materials and methods") in both soils (dual-choice: control and test soils). The contaminated half of each replicate was then spiked with a stock solution prepared by dissolving the appropriate amount of Propanil in 1 ml pure acetone (final acetone concentration: 5 ml kg^{-1} dry soil). Test soil was thoroughly mixed immediately after spiking, and it was left to rest (to allow evaporation of acetone) for ca. 1 h prior to the placement of earthworms. In order to discard the hypothesis that acetone could be toxic to earthworms, a dual-choice design (see "Materials and methods") was employed using clean soil vs. soil spiked with 1 ml acetone. Experiments were carried out in decaplicate, following the conditions and procedures described for avoidance tests (see "Materials and methods"—*Earthworm avoidance assays*'). A mean net response of -0.12 ± 0.15 (SE) was obtained and it was found not to deviate significantly from zero (t test, $P > 0.05$). Hence, acetone did not induce avoidance behaviour of earthworms, indicating that the use of acetone as organic carrier, to test the toxicity of poorly soluble chemicals, produces valid test results.

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