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Structural effects of the bioavailable fraction of pesticides in soil: Suitability of elutriate testing

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ABSTRACT

This study focused the ecotoxicological evaluation of four different pesticides (chlorpyrifos, glyphosate, vinclozolin, endosulfan), sprayed into an agricultural soil, using a standard battery of aquatic bioassays for testing of soil elutriates: *Vibrio fischeri* -Microtox®; *Pseudokirchneriella subcapitata* growth inhibition; *Daphnia magna* acute and chronic toxicity. Despite relevant pesticide residues were recovered from the soil matrix (concentrations higher than 1000 $\mu g\,kg^{-1}$), much lower concentrations could be retrieved from elutriates (highest records for endosulfan of ca. 250 $ng\,L^{-1}$ and 1400 $ng\,L^{-1}$; dissolved and particulate concentration, respectively) and little effects were generally found in the bioassays. Lethal effects (*D. magna* 48 h-EC50 of 36.8%) could be noticed following exposure to the endosulfan elutriate. Elutriates induced no toxicity on *V. fischeri*; algal growth was generally inhibited at high elutriate dilutions and stimulated at the lower elutriate dilutions; and no overall impairment of *D. magna* life-history was noticed. Results revealed that cross-contamination during field application, input of organic matter and nutrients by elutriates in test solutions, and choice of test species and endpoints may constrain the ecotoxicological assessment. Suitability of established aquatic bioassay test batteries for these purposes, and questioning on whether direct assays with soil organisms could be more protective tools is discussed.

1. Introduction

During the last quarter of the 20th century concerns were raised about the environmental problems arising from modern agricultural management, e.g. regarding major changes in plant and animal communities as well as the deterioration of soil, water and air quality [1]. These problems relate particularly to the use of pesticides to control weeds and animal pests in crops. Pesticide residues have been found in soil matrices as a consequence of e.g. contamination following ordinary application or sludge-derived soil fertilisation (e.g. [2–10]). The soil is generally the primary sink for agrochemical residues, but, depending on their mobility, solubility and breakdown rates, surface water and groundwater bodies may apply as ultimate recipients. For example, several studies have recorded pesticide levels in surface and groundwater bodies that surpass applicable reference European standards – 0.1 $\mu g\,L^{-1}$ and 0.5 $\mu g\,L^{-1}$ for individual and total products, respectively [11] (e.g. [1,8,12,13]).

Soil contamination by pesticides is addressed within the European Union in the context of the recently adopted regulation No 1107/2009 [14] and the former directive 91/414/EEC [15], which regulate the authorisation of pesticide placement on the market. Within this framework, guidance documents are available that apply e.g. to (i) the assessment of risks of pesticide leaching into groundwater following a detailed physical and chemical scrutiny [16,17], thereby allowing predictions on their potential to contaminate the soil matrix (soil retention function); (ii) the characterisation of active substances and plant protection products in terms of their soil ecotoxicity [18]. Although comprehensive, the former approach provides little evidence on the effective bioavailability and bioaccumulation potential of these contaminants; hence the advantage of performing detailed assessment of actual effects in sensitive biological systems over chemical monitoring [19]. Biologically-based elutriate tests for soil toxicity assessment [20] may provide a measure of the total effect of a given toxicant scenario that considers both its environmental physical and chemical features. Aquatic bioassays with soil elutriates have been included in test batteries for the evaluation of soil retention potential and environmental hazard posed by contaminants available in the soil aqueous phase [19]; these complement information retrieved in

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Table 1
Brief characterisation of four pesticides selected in this study. Unless referred otherwise, the information provided in the table was gathered from Tomlin [61] or from databases developed under the scope of the EU directive 91/414/EEC (EU pesticides database – http://ec.europa.eu/sanco_pesticides/public/index.cfm; FOOTPRINT database – [71]).

Chlorpyrifos (organophosphorus)	O,O-diethyl O-(3,4,6-trichloro-2-pyridinyl) phosphorothioate
Mode of action	Non-systemic with contact, stomach and respiratory action; Cholinesterase inhibitor.
Uses	Control of Coleoptera, Diptera, Homoptera and Lepidoptera pests in soil or on foliage in fruit and vegetable crops.
Water solubility	1.05 mg L^{-1} (20 °C)
Kow; Koc	Log *P* = 4.7 (pH 7; 20 °C); 2785–31000 (non-mobile)
BCF	1374 (threshold for concern)
Soil degradation	$DT_{50field}$ = 2–65 d; $DT_{90field}$ = 55–310 d(1); DT_{90lab} = 141–360 d
Endosulfan (organochlorine)	[(1,4,5,6,7,7-hexachloro-8,9,10-trinorborn-5-en-2,3-ylenebismethylene) sulfite
Mode of action	Non-systemic with contact and stomach action; non-competitive antagonist of the γ-aminobutyric acid (GABA) receptor-chloride channel.
Uses	Control of sucking, chewing, and boring insects and mites in many different crops. Controls also the tsetse flies.
Water solubility	0.32 (20 °C)
Kow; Koc	Log *P* = 3.13 (pH 7; 20 °C); 3000–20000 (non-mobile)
BCF	2755 (threshold for concern)
Soil degradation	$DT_{50field}$ = 62–126 d; DT_{90lab} = 124–426 d(2)
Glyphosate (phosphonoglycine)	N-(phosphonomethyl) glycine
Mode of action	Non-selective systemic, absorbed by the foliage; Inhibition of 5-enolpyruvylshiki- mate-3-phosphate synthase, preventing the synthesis of essential aminoacids.
Uses	Control of several annual and perennial grasses and broad-leaved weeds in a wide range of cropping, industrial and utility situations.
Water solubility	10500 mg L^{-1} (20 °C)
Kow; Koc	Log *P* = −3.2 (pH 7; 20 °C); 884–60000 (moderately mobile to non-mobile)
BCF	0.5
Soil degradation	$DT_{50field}$ = 5–21 d; $DT_{90field}$ = 101(3) – >365 d(4); DT_{90lab} = 40–280 d
Vinclozolin (dicarboximide)	3-(3,5-dichlorophenyl)-5-methyl- 5-vinyloxazolidine-2,4-dione
Mode of action	Non-systemic fungicide; Prevents spore germination and mycelia growth
Uses	Control of *Botrytis*, *Molinia*, *Sclerotinia*, *Helminthosporium*, and *Corticium* spp. in vines, strawberries, oilseed rape, vegetable, fruit, ornamentals, turf, etc.
Water solubility	3.4 mg L^{-1} (20 °C)
Kow; Koc	Log *P* = 3.07 (pH 7; 20 °C); 260–535 (moderately mobile)
BCF	6.5
Soil degradation	$DT_{50field}$ = 34–94 d; DT_{90} field = 100–365 d

(1) elm forest soil [72]; (2) [73]; (3) [74]; (4) railway embankments [75].

direct assays with soil organisms or physical and chemical scrutiny [20–29].

This study is part of a broader research project [REFUSE (III/AMB/2/2005), I.I.I., Portugal] that investigated the link and interdependency between structural and functional effects of pesticides in agricultural soils. Effects of selected pesticides on the organic matter breakdown, on microbial communities as well as on non-target terrestrial invertebrates have hence been addressed under the scope of this project and will be published elsewhere. Our particular focus here is the toxicity assessment of four different pesticides, sprayed into a common agricultural soil, using standard aquatic bioassays for testing of soil elutriates. Chlorpyrifos, glyphosate, vinclozolin and endosulfan were selected as model pesticides considering their significant environmental persistence. Moreover, different chemical classes and modes of action, availability of ecotoxicological information and regarding their high usage rates in the country at the time of the field study argued in favour of this selection. These agrochemicals were generally included on the 'grey area' regarding risk assessment purposes according to the directive 91/414/EEC. Please refer to Table 1 for a brief characterisation of the selected pesticides.

The main aim of this study was to assess the ecotoxicological potential and subsequent structural effects of the bioavailable fraction of the selected pesticides using standard aquatic bioassays. Short-term exposures are usually adopted for these purposes. However, considering the slow degradation rates of the selected pesticides in soil and their generally low mobility (high Koc values) (see Table 1), assessment under long-term exposure should be of higher meaning. Indeed, although their cost- and time-effectiveness, and ease of interpretation, short-term tests have been criticised in terms of their meaning for predictions regarding long-term ecological effects of pesticides including potential changes operating at higher ecosystem levels, e.g. populations (see the review by Newman et al. [30]). Following this discussion, both short- and long-term elutriate exposures of *Daphnia magna* were carried out in this study so that the sensitivity of the assays could be compared. Since actual pesticide residues were assessed in parallel to the test trial both in soil matrices and corresponding elutriates, we additionally discuss the potential and shortcomings of the employed bioassays as part of test batteries for Ecological Risk Assessment of pesticides in soil matrices.

2. Material and methods

2.1. In situ procedure (soil contamination and sampling)

The field trial was performed in the Low Mondego Region on a "set-aside" field located in Coimbra and belonging to the Regional Agricultural Administration in the Littoral-Centre of Portugal (DRABL). The field had been left fallowing for more than 6 years, thus the site soil was assumed as free of pesticide residues. The soil characterisation considering several physical and chemical parameters [16,31] was carried out over a composite sample from the study site prior to the onset of the experiments: grain size distribution – 62.8% coarse sand, 25.9% fine sand, 7% silt, 4.2% clay; pH (H_2O) = 7.48; pH (KCl) = 7.31; bulk density = 2.4 g cm^{-3}; water holding capacity = 70%; cation exchange capacity = 88.6 meq/100 g; organic matter content = 2.4%. Commercial formulations of four different pesticides were used in this study (see Table 2 for details): the herbicide Montana® (a.i. glyphosate), the fungicide Ronilan® (a.i. vinclozolin), and the insecticides Dursban® (a.i. chlorpyrifos) and Thionex® (a.i. endosulfan); these formulated products will be hereinafter referred as M, R, D and T, respectively.

Table 2
Details and application doses regarding the plant protection products (PPP) used in the field contamination trial. Plateau concentration for each pesticide was calculated using the FOCUS model (assuming an annual application of the recommended dose over several years; information on the recommended dose was obtained from the product manufacturer) and according to the guidance document on the litterbag test [31]. Fate parameters used for calculations: ground deposit of 90%, soil depth of 20 cm* and soil density of 2.4 cm m^{-3}. a.i. stands for active ingredient.

Formulated PPP	Recommended dose (g a.i. ha^{-1})	Applied dose (g a.i. ha^{-1}) to attain the plateau concentration
Dursban® (480 g L^{-1} chlorpyrifos) Dow AgroSciences	1920	40; plateau (16.6 μg kg^{-1})
Thionex® (380 g L^{-1} endosulfan) SAPEC	2500	290; plateau (121.4 μg kg^{-1})
Montana® (360 g L^{-1} glyphosate) SAPEC	2520	17; plateau (7.1 μg kg^{-1})
Ronilan® EG (50% vinclozolin) BASF	750	7; plateau (2.9 μg kg^{-1})

(*) According to the guidance document on the litterbag test, the plateau concentration is calculated accounting to 20 cm depth for mechanical tillage operations, but considering full incorporation in the top 10 cm of soil.

The field contamination followed the recommendations made by FOCUS [16] and Römbke et al. [31]. A replicated plot design (5 replicates per treatment) was used: pesticides were sprayed into 25 plots (control, where water was applied instead of pesticide, plus four pesticide treatments) of 25 m^2 (5 × 5 m) randomly distributed within the testing area and separated by 3 m-wide corridors in order to avoid cross-contamination. Two weeks prior to pesticide application, plant cover was cut and the soil was harrowed. The plateau concentration was applied using a PL1 sprayer equipped with ten flat ray spray nozzles commonly used in agriculture (AGROTOP GmbH), and the chemicals were left to incorporate into the soil (10 cm) by surface harrowing. The FOCUS guidance documents [16] were used for calculation of the plateau concentration and annual cumulative application dose (Table 2), taking into account crop interception levels, soil density and pesticide degradation rates. Two days later, and using the same technique, the recommended annual cumulative dose, i.e. the sum of all applications of the plant protection product within a year (Table 2) of each pesticide, was sprayed into the corresponding replicated plots, soughing though a 'worst case scenario'. Pesticide incorporation was assured by simulating a rain event. Two days after the pesticide application, soil samples were collected from each replicated plot (first 10 cm of soil). Samples were transported to the laboratory and stored at −20 °C for a short period until further analysis.

2.2. Elutriates preparation

Collected soils (25 samples; see above) were sieved (2 mm mesh) and stored at 4 °C in dark for preservation until further use. Elutriates were prepared and used for chemical analysis and toxicity testing within the following 8 weeks, as recommended by USEPA [32]. Erlenmeyer glass vessels were used for elutriate preparation, using a 1:4 (w/v) ratio of natural soils to corresponding media (distilled water for Microtox® assay, Woods Hole MBL medium for algae assay, and ASTM hardwater for *Daphnia* assays). Vessels were mechanically shaken for 12 h at room temperature, and then left for deposition for 12 h. The overlying water (elutriate) and settled material were separated by decanting. Elutriates were filtered through a Whatman GF/C filter (1.2 μm porosity, 47 mm diameter) in order to remove suspended matter, and stored at 4 °C in dark until further use. The standard soil LUFA 2.2 (Speyer, Germany) was used as blank in chemical analyses.

2.3. Pesticide extraction – soils

Vinclozolin, chlorpyrifos and endosulfan were extracted from all samples, including the control (total of 20 samples), following the USEPA method [33]. Five grams of soil were extracted 3 times with 20 mL of an hexane:acetone mixture (1:1) in an ultrasonic bath. Extracts were collected after each extraction period and filtered using a Whatman GF/F filter. The volume of extracts was reduced in a rotavapor, and cleaned up using a florisil cartridge (1 g of Supelclean® Florisil) with sodium sulphate on the top. Compounds were eluted using 100 mL of a hexane:ethyl acetate mixture (7:3), the volume was once again reduced and finally the extracts were dried under a gentle stream of nitrogen, followed by re-dissolution in 500 μL of ethyl acetate.

Glyphosate concentration was determined on the corresponding soil samples and in control samples (total of 10 samples) following Aubin and Smith [34]. Five grams of soil were extracted 2 times with 20 mL of sodium hydroxide (NaOH 0.1 M) in a centrifuge tube. The mixture was shaken for 1 h, then centrifuged at 3400 rpm for 10 min and decanted. The collected extracts were filtered through a Whantman GF/F filter and neutralised with HCl to a pH range of 5–7. Extract volume was reduced in a rotavapor and cleaned up using a 500 mg C-18 cartridge (Penomenex).

2.4. Pesticide extraction – elutriates

A second filtration of elutriates was run before extraction (0.45 μm nylon filters, Whatman). Filters were frozen and further ultrasonic extracted for analysis of pesticides in Suspended Particulate Matter (SPM), using the same procedure as for soil samples.

Vinclozolin, chlorpyrifos and endosulfan were extracted from filtered elutriates by solid-phase extraction, using 500 mg C-18 cartridges (Phenomenex). The cartridges were conditioned with 5 mL of ethyl acetate, followed by 5 mL of methanol and 6 mL of Milli-Q water (Millipore system). Sample was then extracted and sorbent was vacuum dried for 20 min. Elution was performed with 5 + 5 mL of ethyl acetate followed by 10 mL of ethyl acetate for rinsing the bottles. A backflush of the cartridge was made using 5 mL of a acetone:tetrahydrofuran (1:1) solution. Volume of extracts was reduced using a rotavapor and cleaned up using a florisil cartridge (1 g of Supelclean® Forisil) with sodium sulphate on the top. Pesticides were eluted with 20 mL hexane:ethyl acetate (10:1) and 10 mL hexane:ethyl acetate (7:3), extracts were then dried by a nitrogen stream and re-dissolved in 500 μL of ethyl acetate. For glyphosate, the volume of elutriates was reduced to 50 mL using a rotavapor, and then freeze-dried.

2.5. Pesticide analysis

Vinclozolin, chlorpyrifos and endosulfan were analysed using a Shimadzu Corporation GC/MS-QP5050A equipped with a SPB-5 fused silica capillary column (30 m × 0.25 mm i.d.; 0.25 μm film thickness, Supelco) and helium as carrier gas. Samples (1 μL) were injected into the GC using the splitless mode (injector temperature = 280 °C; interface temperature = 300 °C). The temperature program of the column consisted in 2 min at 50 °C, increased at a rate of 15 °C min^{-1} until 150 °C, increased again at 10 °C min^{-1} until 280 °C. Acquisition was programmed to monitor selected ion (SIM mode) using one quantification ion and two confirmation ions. A PAHs-deutered mixture (Supelco) was added to each sample before analysis to be used as internal standard for quantification purposes. For glyphosate analysis, extracts were derivatised, as described by Khrolenko and Wieczorek [35]. The analytical instrumentation

Table 3
Actual pesticide (active ingredient) concentrations in soil from each sampling plot (GC–MS or HPLC analysis). Highlighted values (underlined) correspond to the soil samples selected to produce elutriates for further use in *Daphnia* acute toxicity assays. Vin – Vinclozolin; Clp – Chlorpyrifos; Gly – Glyphosate; Ens – Endosulfan.

	Samples	Active ingredient ($\mu g\,Kg^{-1}$, dry weight)					
		Vin (DL = 0.5)	Clp (DL = 0.2)	Gly (DL = 260)	Ens		
					α (DL = 1.4)	β (DL = 0.7)	(α + β)
C Control	1	6.18	9.59	bdl	24.5	6.75	31.2
	2	bdl	0.77	bdl	3.93	2.31	6.23
	3	1.89	2.48	bdl	bdl	6.44	6.44
	4	0.81	1.10	bdl	2.43	4.65	7.08
	5	4.52	6.54	bdl	14.5	6.46	21.0
Average		3.35	4.10	–	11.3	5.32	14.4
Standard deviation		2.45	3.83	–	10.3	1.88	11.3
D Dursban® (a.i. Clp)	6	bdl	785	–	13.4	21.3	34.7
	7	bdl	71.6	–	bdl	bdl	–
	8	bdl	1045	–	5.56	3.15	8.71
	9	1.46	926	–	3.32	4.40	7.73
	10	1.19	640	–	2.51	2.48	4.99
Average		1.33	694	–	6.19	7.84	14.0
Standard deviation		0.19	379	–	4.96	9.02	13.9
T Thionex® (a.i. Ens)	21	1.84	1.85	–	102	139	241
	22	bdl	7.50	–	113	145	257
	23	9.02	3.57	–	1716	1181	2897
	24	bdl	3.61	–	132	126	258
	25	0.54	2.11	–	540	530	1,069
Average		3.80	3.73	–	521	424	944
Standard deviation		4.57	2.26	–	693.	456	1147
M Montana® (a.i. Gly)	11	2.50	3.95	700	16.3	4.41	20.7
	12	0.81	1.58	340	1.34	1.38	2.72
	13	bdl	1.44	1100	bdl	8.20	8.20
	14	0.70	1.48	1170	1.47	1.89	3.36
	15	0.96	2.28	1230	1.42	4.99	6.41
Average		1.24	2.15	908	5.12	4.17	8.27
Standard deviation		0.85	1.06	379	7.43	2.74	7.29
R Ronilan® (a.i. Vin)	16	327	3.98	–	7.39	11.0	18.35
	17	133	3.54	–	9.86	12.0	21.81
	18	241	10.5	–	bdl	2.78	2.78
	19	1060	1.60	–	bdl	2.45	2.45
	20	481	15.3	–	28.6	11.0	39.6
Average		448	6.98	–	15.3	7.83	17.0
Standard deviation		365	5.73	–	11.6	4.78	15.4

bdl = below detection limit; DL = detection limit ($\mu g\,kg^{-1}$).

included an HPLC Jasco model with a Rheodyne 7125 injector and a loop size of 20 μL coupled to an UV detector (Chrom-A-Scope, BarSpec) operating at 240 nm. The analytical column was a Luna-C18 (100 Å, 250 × 4.6, 5 μm, Phenomenex), with a guard column of the same material. The mobile phase consisted in 85% Phosphate buffer (0.06 M) and 15% of acetonitrile, at a flow rate of 1 mL min^{-1}.

All pesticides stock standards were purchased from Supelco (Pestanal®) and prepared as the samples: vinclozolin, chlorpyrifos and endosulfan standards were diluted in ethyl acetate; gliphosate standards were prepared in water and then derivatised. Only LC grade solvents from Labscan were used. Recovery of all analytes was measured by extracting fortified samples and a reference material (CRM-804), ranging between 70 ± 3% (chlorpyrifos) and 90 ± 6% (endosulfan). Replicate samples showed a relative standard deviation bellow 10%, and the signal of blanks (elutriates prepared using LUFA 2.2.) was subtracted to the samples.

2.6. Ecotoxicological analysis

2.6.1. Test organisms and culture conditions

Monoclonal cultures of *D. magna* (clone A, *sensu* Baird et al. [36]) were reared under a 16 h^L:8 h^D photoperiod cycle and a temperature of 20 ± 2 °C for several generations in our laboratory. Daphnids were cultured in ASTM hardwater medium [37] enriched with an organic additive [38], and were fed with *Pseudokirchneriella subcapitata*. All experiments were initiated with neonates (<24 h old), born between the 3rd and 5th brood in cultures. The algae *P. subcapitata* were maintained in nonaxenic batch cultures with Woods Hole MBL medium, at 20 ± 2 °C and under permanent illumination. The bioluminescent bacteria *Vibrio fischeri* was afforded from Azur Environmental, USA, as lyophilised material kept at −20 °C until testing.

2.6.2. Lethal toxicity bioassays

A preliminary ecotoxicological survey on the soil samples was performed using *D. magna* lethality as a standard endpoint. *D. magna* acute toxicity tests were performed in accordance with standard protocols [32,39], under the same temperature and photoperiod regimes as described for rearing procedures. In brief, a static design was employed using 20 animals (4 replicated vessels, each comprising 5 animals ageing less than 24 h) per treatment [39]. Treatments consisted in an ASTM control and successive elutriate dilutions in this synthetic medium (100 mL final volume). Bearing a worst case scenario in mind, 1 elutriate derived only from 1 soil replicate per pesticide was tested. The replicate chosen was the one showing the highest pesticide concentration recorded (see highlighted values in Table 3; chosen samples were C1, D8, M15, R19, T23). For each elutriate, 6–8 test concentrations were established. Range-finding bioassays were carried out to set the definite test dilutions (C1, M15, R19, T23 – 0.0%, 25.0%, 50.0%, 75.0%, 100.0%

elutriate; D8 – 20.00%, 23.20%, 26.91%, 31.22%, 36.21%, 42.01%, 48.73%, 56.52% elutriate). After a 48 h exposure-period, vessels were checked for immobilised individuals, which were counted for further determination of EC_{50} values and corresponding confidence intervals through Probit analysis [40].

2.6.3. Sub-lethal toxicity bioassays

As a second stage, a series of sub-lethal toxicity bioassays were carried out using aquatic organisms previously recommended as part of standard batteries for soil toxicity assessment (e.g., [22,23]): (I) *V. fischeri* liquid-phase luminescent inhibition test–Microtox® [41]; (II) microalgae (*P. subcapitata*) growth inhibition test [42,43]; (III) *D. magna* chronic toxicity test [44]. These bioassays were carried out using elutriates prepared from composite samples of soils (i.e., the 5 soil replicates within each soil sample were homogenised). This allowed maximisation of the number of assays performed with the limited soil samples available for testing.

Standard Microtox® liquid-phase assays [41] were performed using elutriates C, D, R, M and T. Procedures followed instructions by the manufacturer and luminescence inhibition was measured after 5 and 15 min exposure-period. The solid-phase assay was not applied since the related protocol consists in the exposure of the bacteria to a soil extract rather than directly to the soil; since there was no chemical analysis over this soil extract, little parallel could be traced with results from the remaining bioassays that comprised exposure to prepared soil elutriates.

Growth inhibition of the microalga *P. subcapitata* (formerly known as *Selenastrum capricornutum*) was assessed following USEPA [32] and OECD [43] guidelines, with adaptation for microplate use [42]. Algae were exposed for 72 h to dilutions of each elutriate solution (0%, 12.5%, 25%, 50%, 75%, 100% elutriate diluted in MBL). A replicated design (5 replicates per treatment) was applied, using 300 μL final test volume per well and 50 μL inoculum per well with an initial cell density of 10^4 cells mL^{-1}. Clean MBL medium was used as negative control (optimal growth). The microplates were incubated in a orbital shaker at 25 °C and a 24 h^L:0 h^D photoperiod. At the beginning and the end of the assay absorbance was read at 440 nm as a measure of algal growth (microplate spectrophotometer; Labsystems, Multiscan EX), and growth rates were calculated according to the following expression:

$$GR = \frac{\ln(Abs_f) - \ln(Abs_i)}{\Delta t}$$

where Abs_f stands for absorbance (440 nm) at the end of the test, Abs_i is the absorbance (440 nm) corresponding to the cell density used to initiate the test (10^4 cells mL^{-1}), and Δt is the time interval (days). Data were analyzed using a one-way analysis of variance (ANOVA), followed by a Dunnett test (if applicable), in order to determine significant effects induced by the elutriate over the endpoint.

Life-history effects (reproduction, somatic growth and population growth) of elutriates were assessed using the *D. magna* chronic toxicity test [32,44]. A semi-static design was employed, using 10 individualised animals randomly assigned to each treatment [44]. An ASTM control and successive elutriate dilutions in this synthetic medium were used as treatments: 12.5%, 25.0%, 50.0%, 75.0% and 100.0% was the dilution range used for all elutriates, except D, where the dilution range was adjusted to 2% ,4%, 8%, 16% and 32% in agreement with the acute test results. Test solutions (50 mL) were supplemented with the organic additive (concentrated as for cultures). Daphnids were transferred to freshly-prepared test solutions every other day, and were fed daily with a fixed *P. subcapitata* ration (3×10^5 cells mL^{-1}). Animals were checked daily for mortality and reproductive state and, if released, offspring were counted and immediately discarded. The following life-history parameters were recorded: total number of offspring and number of broods. The somatic growth rate (SGR) was calculated from measurements made on *Daphnia* females at the beginning and at the end of the test, according to the following expression:

$$SGR = \frac{\ln(l_f) - \ln(l_i)}{\Delta t}(day^{-1})$$

where l_f stands for body size (mm) of the test organism at the end of the test, l_i is the average body size (mm) of a subsample ($n = 20$) of neonates coming from the same batch of neonates that initiated the test, and Δt is the time interval (days). Survival and fecundity estimates were used to compute the rate of population increase (r), which was iterated from the Euler–Lotka equation:

$$1 = \sum_{x=0}^{n} e^{-rx} l_x m_x,$$

where r is the rate of population increase (day^{-1}), x is the age class in days, lx is the probability of surviving to age x, and mx is the fecundity at age x. Standard errors for r were estimated using the jack-knifing technique described by Meyer et al. [45]. Data from each endpoint were analyzed using a one-way analysis of variance (ANOVA), followed by a Dunnett test (if applicable), in order to determine significant effects induced by each elutriate over the endpoint.

3. Results

3.1. Chemical analysis

This study involved actual pesticide application in the field. Even accounting to safe distances between application plots, some cross-contamination was expected. Hence, soil samples from all replicates within each pesticide treatment were screened for the four applied pesticides (see Table 3). Glyphosate was an exception to this screening procedure, being quantified only in the samples where Montana® was applied and in the control samples. As expected, the soil samples from control plots showed low or undetectable pesticide concentrations. On the other hand, consistency was registered between the chemical analysis and the pesticide application i.e. the highest active ingredient records were obtained in the soil samples where the corresponding pesticide formulation was applied. All pesticides were found in concentrations higher than 1000 μg kg^{-1} in the corresponding soil samples; particularly high endosulfan concentrations were retrieved in soil T (2897 μg kg^{-1} α + β endosulfan). One should recognise that some variation in pesticide concentrations exists between replicates within the same soil treatment (Table 3). This variation was confirmed in repeated analytical quantifications performed over selected samples, which teases apart eventual flaws occurring in single readings. Pesticides other than the pesticide applied in a given sampling plot were found, which is very likely to result from cross-contamination during field application. However, these pesticides were always found in residual concentrations (<35 μg kg^{-1}) similar to those retrieved in control plots, which validates further experiments of this study.

The actual concentration of the pesticides (including chlorpyrifos) in elutriates used for these experiments was generally very low (see Table 4). Results of pesticide analysis on elutriates obtained from composite samples prepared for chronic assays are shown in Table 4. Glyphosate was not detected in any of the forms measured (dissolved or aggregated in suspended matter). Active ingredients of the remaining pesticides were detected in very low concentrations in the dissolved form (highest records were found for endosulfan α and β – 116 ng L^{-1} and 156 ng L^{-1}, respectively). However, records of pesticides in suspended matter were con-

Table 4
Pesticide concentrations in elutriates used for the lethal (dissolved concentration) and sub-lethal (dissolved and particulate concentration i.e. adsorbed to suspended organic matter) bioassays with bacteria, algae and daphnids.

	Pesticide (a.i.)	Dissolved concentration ($ng\,L^{-1}$)		Particulate concentration ($ng\,L^{-1}$)
		Lethal assays	Sub-lethal assays	Sub-lethal assays
D	Chlorpyrifos	88	53	134
T	Endosulfan α	48	116	261
	Endosulfan β	104	156	1448
	Endosulfan α + β	151	272	1708
M	Glyphosate	bdl	bdl	bdl
R	Vinclozolin	22	57	25

bdl = below detection limit; Detection Limit (glyphosate) = 1.5 $\mu g\,L^{-1}$.

siderable higher for all samples (highest values for endosulfan β ≈ 1400 $ng\,L^{-1}$).

3.2. Lethal bioassays

Elutriates obtained from soils C, R, M and T showed no acute toxicity to *D. magna* after 48 h exposure. However, the short-term exposure to elutriate obtained from sample D8 (Dursban®, a.i. chlorpyrifos) allowed an estimation of a 48 h immobilisation-EC_{50} of 36.8% with a 95% confidence interval ranging between 32.8% and 41.7%. Further studies included the assessment of sub-lethal toxicity of all soil elutriates.

3.3. Sub-lethal bioassays

No toxicity was induced by the composite elutriate samples on the luminescence of *V. fischeri* i.e. no luminescence-IC_{50} could be determined neither after 5 min nor after 15 min exposure-period. The bioassays with *P. subcapitata* followed the same trend and no growth inhibition IC_{50} could be estimated (Fig. 1 and Table 5). However, microalgae growth was significantly affected by different soil elutriates (Table 5). Generally, growth was significantly inhibited by the lowest soil elutriate concentration (e.g. 12.5% for T, R, C) and significantly stimulated by the highest concentration (100%) for all tested soil elutriates.

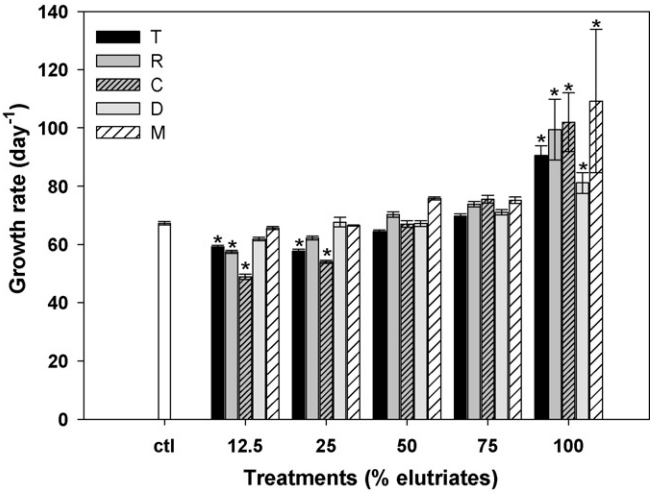

Fig. 1. Growth rate (day^{-1}) ($n = 3$) of *Pseudokirchneriella subcapitata* exposed to soil elutriates (C – Control; M – Montana; R – Ronilan; T – Thionex; D – Dursban). Error bars represent standard error, and '*' assigns differences between elutriate dilutions and negative control (one-way ANOVA followed by Dunnet test; $P \leq 0.05$).

No overall impairment of *D. magna* life-history was found. Both the reproductive endpoints and the growth rates generally denote stimulatory effects driven by increasing elutriate concentrations (Fig. 2; Table 5). The soil elutriates C and R provide the most clear picture of this phenomenon: e.g. as elutriate concentrations increase, significantly higher offspring production and population growth rates (r) could be recorded almost in all test treatments. The individuals also grew at significantly higher rates in all tests as elutriate concentration increased. It should be noticed that elutriate R was the single showing higher ability to consistently stimulate

Table 5
One-way ANOVA summary regarding the sub-lethal effects of soil elutriates (C – Control; M – Montana; R – Ronilan; T – Thionex; D – Dursban) in daphnids and microalgae. Statistically significant changes in life-history responses of *Daphnia magna* and algal growth are reported (df – degrees of freedom).

Endpoint	Elutriate	df	$MS_{residual}$	F ratio	P value
D. magna Fecundity	C	5, 54	215.3	4.775	0.001
	D	5, 54	304.5	2.307	0.057
	T	5, 53	360.2	0.306	0.907
	M	5, 54	142.2	3.882	0.004
	R	5, 52	406.8	15.87	<0.001
D. magna Number of broods	C	5, 54	0.181	7.494	<0.001
	D	5, 54	0.235	1.261	0.294
	T	5, 53	0.175	9.375	<0.001
	M	5, 54	0.104	18.90	<0.001
	R	5, 52	0.152	1.312	0.273
D. magna Somatic growth rate	C	5, 54	$1.25\,e^{-6}$	4.366	0.002
	D	5, 54	$3.36\,e^{-7}$	4.905	<0.001
	T	5, 52	$7.08\,e^{-7}$	11.39	<0.001
	M	5, 54	$1.01\,e^{-6}$	9.620	<0.001
	R	5, 52	$5.64\,e^{-7}$	44.31	<0.001
D. magna Population growth rate (r)	C	5, 54	$3.79\,e^{-4}$	8.918	<0.001
	D	5, 54	$7.60\,e^{-4}$	1.592	0.178
	T	5, 54	$4.42\,e^{-4}$	1.537	0.194
	M	5, 54	$5.38\,e^{-4}$	2.368	0.052
	R	5, 54	$6.34\,e^{-4}$	8.025	<0.001
Algal growth inhibition	C	5, 22	30.84	40.90	<0.001
	D	5, 22	23.79	6.04	0.001
	T	5, 22	12.87	37.71	<0.001
	M	5, 22	52.30	13.61	<0.001
	R	5, 22	27.25	25.64	<0.001

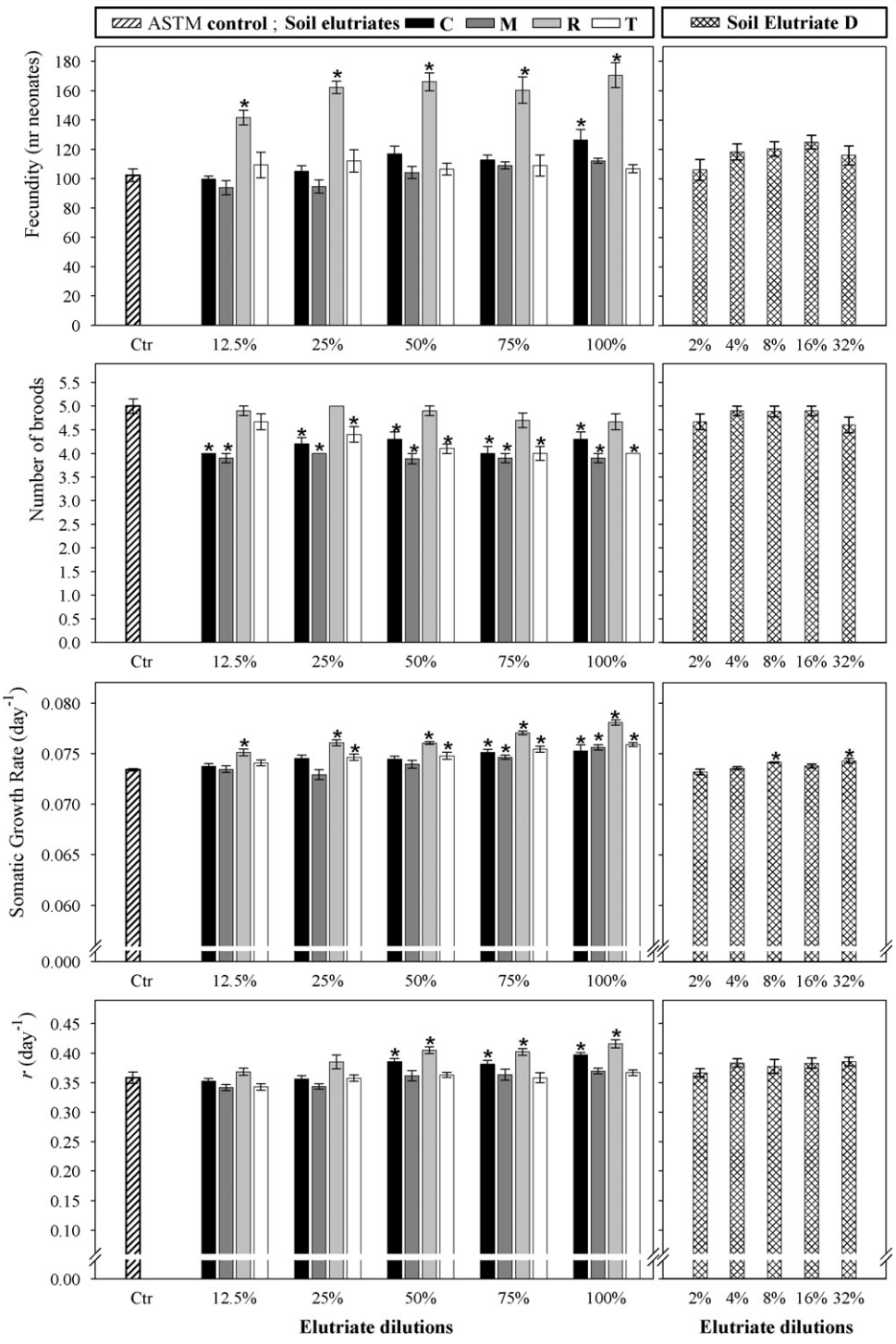

Fig. 2. Long-term effects of soil elutriates (C – Control; M – Montana; R – Ronilan; T – Thionex; D – Dursban) in *Daphnia magna* ($n = 10$), namely on its reproductive output, somatic growth rate and population growth rate (*per capita* rate of population increase; r). Error bars represent standard error, and '*' assigns differences between elutriate dilutions and negative control for each trait (one-way ANOVA followed by Dunnet test; $P \leq 0.05$).

D. magna reproduction and growth; indeed, statistically significant stimulatory effects were noticed even in the exposure to the highest elutriate dilution (12.5%), concerning fecundity and somatic growth rate. Despite the stimulation in offspring production and r, significant decrease in the number of broods yielded by the females during the test period was observed in all elutriate tests except in elutriate R (where the number of broods also decreased relatively to control, but not significantly). This indicates that the addition of soil elutriates to the test solution promoted the increase of the net reproductive output through the production of fewer but larger egg clutches.

Assays with elutriate D (a.i. chlorpyrifos) were performed using higher dilution rates relatively to those used for testing of the remaining pesticides. This was a methodological option taken because of the acute toxicity records previously obtained (see above). However, no deleterious effects were observed in the chronic assay up to a concentration of 32%, even though this is very close to the acute EC_{50} (36.8%) found in prior exposures. The sig-

nificant stimulation of the somatic growth rate at high elutriate concentrations (8% and 32%) should additionally be noticed (Fig. 2, Table 5).

4. Discussion

Pesticides are certainly key agents in agriculture worldwide to keep satisfactory production levels. However, large discussion has been conducted by both the scientific community and regulatory agencies on whether the benefits of these compounds pay-off for the environmental impacts of their residues; and in parallel, effort has been put on the development of safer alternatives. Detailed physical and chemical characterisation of every pesticide, as well as its general ecotoxicological profiling is required by the EU before placement on the market [15]. This study focuses on this latter requirement regarding the soil matrix of an arable soil and the potential of chlorpyrifos, glyphosate, vinclozolin and endosulfan as disrupters of the soil quality. Indeed, risk assessment of contaminated soils should not be only based on the chemical determination of the total content or concentration of contaminants, since biological effects only relate to their bioavailable fraction [46]. Together with studies on mobility and uptake kinetics, exposure of sensitive organisms to soil elutriates to monitor ecotoxicological effects is seen as a major complement to chemical analyses to address bioavailability of contaminants [22,23,46].

The luminescent inhibition test (e.g. Microtox®), the algae growth inhibition test and the *Daphnia* immobilisation assay are part of test batteries for assessment of soil retention function regarding different contaminants [22,23]. Generally, bacteria and algae have shown higher sensitivity than daphnids in such bioassays [22,23]. However, considering the chemical classes and mode of action of the insecticides tested here, deleterious effects promoted by elutriates of these toxicants in survival, reproduction, and growth of the crustaceans were expected. Judging from our first-stage survey on lethal effects of elutriates to *D. magna* it could be assumed that soil D8 (spiked with the insecticide Dursban®; a.i. chlorpyrifos) would represent important harm to the biota, thus supporting the view that elutriate bioassays with daphnids could be sensitive for studies involving insecticides. On the contrary, when testing the other insecticide Thionex® (a.i. endosulfan), no acute toxicity could be observed.

As contact and ingestion pesticides, both the insecticides are expected to be highly toxic to non-target arthropods such as *Daphnia*. The cholinesterase inhibitor chlorpyrifos has already shown high acute toxicity to *D. magna* with recorded 48 h-immobilisation EC_{50} values or LC_{50} values ranging 0.74–1 $\mu g\,L^{-1}$ following standardised bioassays using waterborne chemical solutions [47,48]. Moreover, the organic matter content, which should be considered in tests with elutriates, has already been shown to reduce the acute toxicity of chlorpyrifos to freshwater crustaceans [49]. Considering these records, the acute toxicity found for soil D8 can hardly be explained. The elutriate (corresponding to 100% treatment) shows much lower concentration of dissolved chlorpyrifos (Table 4), thus the acute effects noticed in our test are very likely to result from additional factors other than or interacting with chlorpyrifos toxicity.

Jones and Huang [50] tested chlorpyrifos toxicity using the Microtox® assay and found EC_{50} values corresponding to 25% and 31% of an initial solution at 0.84 $mg\,L^{-1}$. Moreover, they found that, as humic substances were added to the solutions, significant increases in the EC_{50} values were recorded. If one regards the chlorpyrifos concentration quantified in our soil elutriates (dissolved concentration; 53 $ng\,L^{-1}$) as a parallel to the contaminant fraction available for bacteria during the assay, it should be concluded that the results (no toxicity) agree with previous data from the literature; and that organic matter present in the soil samples should be of crucial importance in keeping the pesticide sequestered in non bioavailable fraction for *V. fischeri*). Growth of *P. subcapitata* was not significantly reduced by chlorpyrifos in elutriate test solutions, which is consistent with the results reported by Van Donk et al. [51] noticing impairment following exposures of *Selenastrum capricornutum* to 1 $mg\,L^{-1}$ waterborne chlorpyrifos and higher. These authors also argued that chlorpyrifos carriers used in Dursban® should have an important role in decreasing its toxicity by enhancing phosphorous concentration in the medium. It is likely that in our study, where Dursban® was also used, this could have add to the general increase on dissolved nutrients (mobilisation from soil into the water phase) in stimulating algae growth at the highest elutriate concentrations. The increase of organic matter and nutrients dissolved in media, as more soil elutriate is added, should also apply to explain the stimulation observed in *D. magna* reproductive output and growth rates.

Although endosulfan is a disrupter of the neuronal function similarly to chlorpyrifos, the γ-aminobutyric acid (GABA) receptor/chloride ionophore complex is its primary target; GABA acts as an antagonist by stabilising non-conductive conformations of the chloride channel, though reducing neuronal inhibition [52]. In fact, α and β endosulfan have already shown lower toxicity than chlorpyrifos in similar assays, by recording *D. magna* 48 h-EC_{50} values within the range 810–1920 $\mu g\,L^{-1}$ (being the α-isomer slightly more toxic than the β-isomer) [53]. Adsorption to organic matter and mobilisation of endosulfan and chlorpyrifos are fairly similar (see Kow and Koc in Table 1). Thus, the higher concentrations found in elutriate solutions, with particular emphasis on the β isomer (Table 4), should be related to the higher recommended dose applied (Table 2), rather than reflect more efficient mobilisation into the water column during the preparation of elutriates. The lower reported toxicity of endosulfan relatively to chlorpyrifos clearly compensated for the higher concentrations retrieved in elutriate solutions, hence no immobilised daphnids could be found after acute exposure to elutriate T.

Previous assessment of endosulfan toxicity through the Microtox® bioassay reports 5 min- and 15 min-EC_{50} values of 330 $mg\,L^{-1}$ and 370 $mg\,L^{-1}$, respectively [54]. Consistently, since endosulfan was found dissolved in elutriates at concentrations below 1 $\mu g\,L^{-1}$ (Table 4), effects in *V. fischeri* luminescence where not noticed here. On the other hand, despite endosulfan has previously shown little toxicity for *P. subcapitata* (growth-EC_{50} and NOEC of 428 $mg\,L^{-1}$ and 130 $mg\,L^{-1}$, respectively; [55]), the elutriate T impaired significantly alga growth until a threshold concentration (50% elutriate) where the nutrient input into the water column should have compensated for the toxicant effects. This apparent inconsistency may result from cross-contamination in the field during pesticide application; i.e., residues of the remaining pesticides may exert additional toxicity *per se* or as interacting chemicals and promote the observed growth inhibition.

Chuah et al. [56] found fecundity of a cladoceran other than *D. magna* (*Moina macrocopa*) reduced by 70% after 15 days exposure to 0.4 $\mu g\,L^{-1}$ waterborne endosulfan. Based on these records, and if one considers that in our study daphnids were exposed to dissolve plus particulate concentrations of endosulfan (Table 4), deleterious effects in fecundity would be expected. Number of broods was the single reproductive parameter impaired after long-term exposure of *D. magna* to elutriate T, whereas somatic growth was stimulated as elutriate concentration increased. In daphnids, this pattern is generally consistent with a response to deteriorated environmental conditions through investment on growth and maintenance rather than on offspring production [57]. Elutriate T neither decreased offspring production nor affected population growth, thus daphnids apparently were able to cope with changed, although not necessarily worst, environmental conditions.

D. magna has been found fairly insensitive to acute exposures of glyphosate (e.g., EC_{50} of 307 mg L^{-1}; [58]). Indeed, glyphosate acts through preventing the synthesis of essential aminoacids in target plant weeds [59] within a metabolic pathway that does not exists in animals [60]. Moreover, and despite this pesticide was applied in the field at higher dosage, it was not found in elutriate samples. Hence no lethal effects were expected nor were recorded in this study following acute exposure of *D. magna* to elutriate M. Glyphosate is a systemic herbicide [61] with a zwitterionic structure that constrains its diffusion across cell membranes [62]. Hence its low toxicity to *P. subcapitata* as previously shown in studies testing waterborne solutions (growth inhibition EC_{50} of 129 mg L^{-1}; [58]) and here by the unchanged growth rates of the microalgae following exposure to elutriate M. When systemic pesticides are concerned, replacement of algae with a macrophyte representing the producers' level in test batteries for ecotoxicological assessment should be considered if a conservative approach is intended. The systemic specificity and poor liposolubility (as measured by the Kow; Table 1) of glyphosate is also likely to constrain the sensitivity of bacteria toxicity tests within test batteries. Consistently, the pesticide has shown little toxicity to *V. fischeri* in the study by Amorós et al. [63] (EC_{50} of 36.4 mg L^{-1}) and promoted no changes in luminescence here. Despite the very low concentrations of glyphosate found in elutriate M (below 1.5 μg L^{-1}), noxious effects of the pesticide in *D. magna* reproduction were noticed. These results contrast with those by Abrantes et al. [64], who obtained a LOEC value for *D. magna* offspring production and daily growth rate of 37.5 mg L^{-1} glyphosate, using the formulation Roundup®. Considering these records, the impairment observed in our study regarding fecundity and number of broods can hardly be directly linked to glyphosate itself but may rather reflect exposure to a different chemical environment (e.g., different formulating products contributing for the overall toxicity record).

Vinclozolin is a dicarboximide that inhibits spore germination and mycelia growth in phytopathogenic fungi, but its primary mode of action and namely its interaction with lipid peroxidation and oxygen free radicals production is still unclear [65]. Similarly to glyphosate, vinclozolin shows little acute toxicity to *D. magna* (EC_{50} higher than 3 mg L^{-1}; [66]) thus no effects in short-term survival were expected in this study given the low concentration of the pesticide retrieved in elutriate R. As far to our knowledge, no literature records exist on the toxicity of vinclozolin to *V. fischeri* or regarding luminescence metrics. Results from a study on changes in the soil microbial community after vinclozolin application at normal rates show adverse effects but only at the functional structure of the bacteria community [67]. Toxicity records concerning microalgae were also not found in literature. In this way, and taking into account that no consistent growth inhibition was measured further considerations on microalgae sensitivity to vinclozolin would be speculative. The soil elutriate R produced no significant adverse reproductive effects on *D. magna*. Vinclozolin is able to disrupt the species sex ratio in chronic exposures at 1 mg L^{-1} [66], which would be a meaningful parameter replacing fecundity or even the population growth rate (despite being integrative, it does not discriminates the relevance of an increased male production, thus increased probability of a diapause event) in conservative ecotoxicological assessments.

The general trend in ecotoxicological assessment of contaminated environmental matrices is the application of a standard battery of bioassays with known sensitivities and order of sensitivity to different toxicants, such as argued by e.g. Achazi [22] and Hund-Rinke et al. [23]. From a retrospective point of view, i.e. when contaminated land is being assessed and no full knowledge is available on the existent toxic levels, a standard battery would probably be the best approach as a first biological screening of occurring adverse impacts. However, if a prospective analysis is envisaged and a conservative approach is aimed (e.g. for gathering data to support regulation), details on the chemical mode of action and known mechanism of toxicity should contribute to establish the most appropriate battery of bioassay. For example, glyphosate toxicity may be underestimated based on results of toxicity testing made with algae rather than macrophytes such as *Lemna* spp., given its systemic nature; as well, because of its peculiar mechanism of toxicity, vinclozolin toxicity may be underestimated if reproductive parameters other than changes in population sex ratio are analysed. It should be noticed in addition that despite bacteria luminescence has been argued as one of the most responsive ecotoxicological endpoints being sensitive enough to become part of standard test batteries [22,23], here Microtox® was proven to be the least sensitive bioassay. This raises concerns on the suitability of a standardised test battery for screening of soil contamination and on the feasibility of results based on such a non-customised tool as basis for further decision-making processes.

The use of aquatic tests with soil elutriates to address retention of contaminants is also controversial. Several authors have used aquatic bioassays with elutriates in parallel with direct exposure of terrestrial model organisms to the contaminated soil (e.g., [21,25,68,69]); such an approach should be a valuable tool that combines assessment at both the solid- and the liquid-phase of the soil matrix sequestering pollutants. If, in some cases, testing with elutriates was more responsive than direct testing (e.g., [25]), in other cases, the opposite was verified (e.g., [69]). Despite comparisons of our data with results from direct assessment of soil toxicity cannot be done (ongoing studies), one should recognise that aquatic bioassays done here with elutriates were generally of little responsiveness. An apparent stimulatory effect by increasing elutriate concentrations was observed in our study both in algal growth and in daphnid reproduction/growth, which has constrained conclusions on their toxicity. In line with the arguments provided in previous studies [69,70], one should conclude that nutrient enrichment and additional suspended organic matter in media provided by soil had a determinant role in inducing the observed stimulation in algae and in cladoceran assays, respectively. Indeed, the use of aquatic bioassays to complement soil ecotoxicological assessment should be held with care and conclusions should take into account the related uncertainty.

This study provides discussion on two fundamental items regarding assessment of pesticide toxicity to the non-target biota, namely on the relevance of field-scaled studies to address pesticide structural effects in soil ecosystems; and on the adjustment of test batteries and test methods for this purpose. We found that some elutriate treatments from the control soil (i.e. field plot where no pesticide was applied) were able to induce noxious effects–see in particular algae growth and *D. magna* number of broods. Cross-contamination during pesticide application in the field should be the main factor contributing for such results. This is a limitation which is not easily surpassed given that there is no absolute control over all environmental variables constraining the experimental trial at the moment of and on the few hours after application (e.g., climate conditions such as wind speed). Based on our results, and assuming that it is not pleasing to lose the realism provided by field-scaled studies, the increase of buffer areas between different experimental plots should be seriously considered in further similar studies, in order to eliminate cross-contamination effects in test results. Alternatively, the use of actual barriers (e.g. plastic dividers) that can better isolate experimental plots could apply depending on the equipment available for the pesticide application and on the plot area. Another factor that may confound results is the use of commercial formulations rather than active ingredients in ecotoxicological studies, provided that its composition is generally not fully disclosed. Indeed, adjuvants that add to the active ingredient(s) in commercial pesticide formulations to enhance the product

efficiency have been proven to change their toxicity to non-target species (e.g. [58]).

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