Ecotoxicology and Environmental Safety 195 (2020) 110504

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Sediment toxicity of the fungicide fludioxonil to benthic macroinvertebrates -evaluation of the tiered effect assessment procedure

Theo C.M. Brock[a], João Romão[a,b], Xiao Yin[a,c], Rima Osman[a], Ivo Roessink[a,*]

[a] Wageningen Environmental Research, Wageningen University and Research, P.O. Box 47, 6700, AA Wageningen, the Netherlands
[b] Current Address: Department of Biology, University of Aveiro, 3810-193, Aveiro, Portugal
[c] Current Address: Zhe Jiang Agriculture and Forestry University, College of Agricultural and Food Science, 88 North Road of Huan Cheng, Lin'an, Hangzhou, Zhe Jiang, 311300, China

ARTICLE INFO

Keywords:
Sediment ecotoxicology
Fungicide exposure
Standard test species
Species sensitivity distributions
Weight of evidence approach

ABSTRACT

28-Day sediment-spiked laboratory toxicity tests with eight benthic macroinvertebrates and the lipophilic fungicide fludioxonil were conducted to verify the proposed tiered sediment effect assessment procedure as recommended by the European Food Safety Authority (EFSA). The test species were the oligochaetes *Lumbriculus variegatus* and *Tubifex tubifex*, the insects *Chironomus riparius* and *Caenis horaria*, the crustaceans *Hyalella azteca* and *Asellus aquaticus* and the bivalves *Corbicula fluminalis* and *Pisidium amnicum*. Toxicity estimates were expressed in terms of total concentration of dry sediment as well as in pore water concentration. Field-collected sediment, also used in a previously performed sediment-spiked microcosm experiment, was used in tests with all species. *L. variegatus* and *C. riparius* had similar lowest 28d-L(E)C_{10} values when expressed in terms of total sediment concentration, but in terms of pore water concentration *L. variegatus* was more sensitive. Three of the six additional benthic test species (*A. aquaticus*, *C. horaria*, *C. fluminalis*) had 28d-EC_{10} values a factor of 2–6 lower than that of *L. variegatus*. Comparing different effect assessment tiers for sediment organisms, i.e. Tier-0 (Modified Equilibrium Partitioning approach), Tier-1 (Standard Test Species approach), Tier-2 (Species Sensitivity Distribution (SSD) approach) and Tier-3 (Model Ecosystem approach), it is concluded that the tiers based on sediment-spiked laboratory toxicity tests provide sufficient protection when compared with the Tier-3 Regulatory Acceptable Concentration (RAC). Differences between Tier-1 and Tier-2 RACs, however, appear to be relatively small and not always consistent, irrespective of expressing the RAC in terms of total sediment or pore water concentration. Derivation of RACs by means of the SSD approach may be a challenge, because it is difficult obtaining a sufficient number of valid chronic EC_{10} values with appropriate 95% confidence bands for sediment-dwelling macroinvertebrates. Therefore, this paper proposes a Tier-2 Weight-of-Evidence approach to be used in case an insufficient number of valid additional toxicity data is made available. Similar studies with pesticides that differ in fate properties and toxic mode-of-action are necessary for further validation of the tiered effect assessment approach for sediment organisms.

1. Introduction

Sediment-dwelling invertebrates play important roles in the functioning of freshwater ecosystems, such as food-web support, nutrient cycling, decomposition of organic matter and bioremediation of pollutants (e.g., Wall, 2004; Diepens et al., 2017). However, sediments of edge-of-field surface waters are reported to be both a sink and source of lipophilic chemicals, including pesticides, potentially leading to long-term effects on benthic organisms and their communities (e.g. Warren et al., 2003; Schäfer et al., 2011; Diepens et al., 2014; Hunt et al., 2016; Moran et al., 2017; Huff Hartz et al., 2019). To protect benthic organisms from potential exposure, a prospective environmental risk assessment (ERA) for sediment-dwelling organisms has to be conducted in the authorisation procedure of pesticides if, after intended agricultural use, the substance is indicated or predicted to accumulate in the sediment compartment (EFSA, 2013). In the European Union, the development of prospective ERA procedures for pesticides, including decision schemes for sediment organisms, falls under the mandate of the European Food Safety Authority (EFSA). Effect assessment tiers for sediment organisms described in EFSA (2015) comprise Tier-0 (Modified Equilibrium Partitioning approach), Tier-1 (Standard Test Species approach), Tier-2 (based on toxicity data for standard and additional

* Corresponding author.
E-mail address: ivo.roessink@wur.nl (I. Roessink).

https://doi.org/10.1016/j.ecoenv.2020.110504
Received 23 October 2019; Received in revised form 9 March 2020; Accepted 16 March 2020
Available online 25 March 2020

test species, e.g. the Species Sensitivity Distribution (SSD) approach) and Tier-3 (Model Ecosystem approach).

To date, sediment effect assessments for pesticides in freshwater ecosystems are mainly based on results of laboratory tests with a few standard test species, such as larvae of the non-biting midge *Chironomus* (predominantly *C. dilutus* and *C. riparius*) and the amphipod *Hyalella azteca* (Deneer et al., 2013). The EFSA Aquatic Guidance Document (EFSA, 2013) mentions that either a chronic toxicity value for the insect *C. riparius* (most frequently tested) or for the oligochaete worm *Lumbriculus variegatus* (up till now hardly tested) should be supplied if a sediment assessment is triggered. A more recent scientific opinion (EFSA, 2015) proposes to use different combinations of two benthic standard test species in the Tier-1 effect assessment for sediment-exposure to different types of pesticides, viz., *C. riparius* (or another OECD recommended *Chironomus* species) and *H. azteca* for compounds with insecticidal activity, the oligochaete *Lumbriculus variegatus* (or *Tubifex tubifex*) and *Chironomus* sp. (or *H. azteca*) for compounds with fungicidal/biocidal properties and the rooted macrophyte *Myriophyllum* sp. and one of the benthic invertebrates mentioned above for compounds with herbicidal properties. It is, however, uncertain whether the derivation of a Regulatory Acceptable Concentration (RAC) based on chronic sediment toxicity data for these standard benthic test species is sufficiently protective for a wider array of freshwater benthic organisms and their communities in edge-of-field surface waters. In addition, in higher-tier sediment toxicity testing for modern pesticides, predominantly insecticides have received some attention in the scientific literature (e.g., Boyle et al., 2016; Brock et al., 2016; Brock et al., 2018; Rogers et al., 2016), while information on sediment-exposure to fungicides and responses of benthic invertebrates other than the standard test species *Chironomus* spp. is scarce (Deneer et al., 2013; Yin et al., 2018).

We selected the lipophilic fungicide fludioxonil (log P_{OW} 4.17; EFSA, 2007) as benchmark substance to evaluate the prospective ERA procedure for sediment organisms as proposed by EFSA (2013; 2015). Fludioxonil [4-(2,2-difluoro-1,3-benzodioxol-4-yl)-1H-pyrrole-3-carbonitrile] is a non-systemic phenylpyrrole fungicide widely used as a foliar, seed and post-harvest treatment application to control diseases caused by fungi in the class of Ascomycetes, Basidiomycetes and Deuteromycota (Fungi imperfecti) (EFSA, 2007). Many fungicides, however, are reported to have biocidal properties in the sense that they affect a wider array of aquatic organisms (Maltby et al., 2009; Zubrod et al., 2019; Rico et al., 2019). Fludioxonil has a low solubility in water (1.8 mg a. s./L) and a high mean partition coefficient between soil organic carbon and water (K_{foc} = 145,600 mL/g OC) (EFSA, 2007). This implies that, when present in edge-of-field surface waters, fludioxonil will readily adsorb to sediment from the water phase.

Within the context of the research project on sediment ecotoxicology of pesticides of our laboratory, a previous paper dealt with exposure and effects of sediment-spiked fludioxonil on benthic macroinvertebrates and zooplankton in outdoor microcosms (Yin et al., 2018). That study showed that fludioxonil persisted in the sediment and that measured concentrations were 53–82% of the initial concentrations after 84 days. Of the sediment-dwelling macroinvertebrates, oligochaete worms, particularly *Dero digitata*, showed the most pronounced treatment-related decline in abundance. The population-level NOEC (No Observed Effect Concentration) of this species, as well as the NOEC for the sediment-dwelling macroinvertebrate community, was 14.2 mg fludioxonil/kg dry sediment, corresponding to 88.8 μg a. s./L in pore water (expressed in terms of geometric mean measured concentrations for the exposure period 0–28 days). The NOEC for the most sensitive pelagic species was 1.6 μg a. s./L overlying water, corresponding to 5.0 mg a. s./kg dry sediment (Yin et al., 2018). The responses of nematodes to the fungicide fludioxonil in the sediment-spiked outdoor microcosm study revealed a sediment NOEC for the total nematode community and the most sensitive nematode population approximately a factor of 3–4 higher than that for the benthic macroinvertebrate community (Höss et al., 2019).

The present paper complements those of Yin et al. (2018) and Höss et al. (2019) and aims to evaluate the consistency and protectiveness of the tiered sediment effect assessment procedure currently used (EFSA, 2013) and proposed (EFSA, 2015) for prospective environmental risk assessment for pesticides and sediment organisms in Europe. For this reason, we conducted 28-d sediment-spiked laboratory toxicity tests with the standard Tier-1 benthic test species *C. riparius* and *L. variegatus* and six additional benthic macroinvertebrates (one oligochaete worm, one insect, two molluscs and two crustaceans). In these tests field-collected sediment similar to that used in the previously performed sediment-spiked microcosm experiment was used to facilitate the comparison of toxicity data and regulatory acceptable concentrations (RACs). The results for standard and additional benthic test species thus obtained are used to (i) discuss the reliability of tests with non-standard test species, (ii) to compare the sensitivity of the additional test species relative to standard test species in terms of total sediment concentration and pore water concentration, (iii) to evaluate the consistency and protectiveness of the tiered effect assessment approach as proposed by EFSA (2015) for sediment organisms, and (iv) to discuss alternative approaches that might be used in the Tier-2 assessment based on standard and additional test species.

2. Materials and methods

2.1. Test species

The benthic test species of macroinvertebrates were selected (i) as the standard EU test species for sediment-dwelling organisms and fungicides, viz., *C. riparius* and *L. variegatus* (EFSA, 2013; 2015), (ii) additional test species to give a total of 8 different benthic species belonging to at least six different orders/families to allow the Species Sensitivity Distribution (SSD) approach (EFSA, 2013). An important criteria was the availability of the test species either from laboratory or for them to be collected from the field in sufficient numbers from nearby freshwater ecosystems. The selected test species were two insects, *C. riparius* (Chironomidae) and *Caenis horaria* (Ephemeroptera), two oligochaete worms, *L. variegatus* (Lumbriculidae) and *Tubifex tubifex* (Tubificidae), two bivalves, *Corbicula fluminalis* (Cyrenidae) and *Pisidium amnicum* (Sphaeriidae) and two crustaceans, *Asellus aquaticus* (Isopoda) and *Hyalella azteca* (Amphipoda). Information on their origin, size/life-stage and test conditions can be found in Table 1.

2.2. Sediment preparation and spiking

With all benthic species mentioned in section 2.1, 28-d laboratory toxicity tests were conducted using field-collected sediment. This sediment was also used in a previously performed sediment-spiked outdoor microcosm experiment with the fungicide fludioxonil (Yin et al., 2018). The sediment originated from a few experimental ditches at the Sinderhoeve field station (Renkum, The Netherlands) not used for ecotoxicological experiments after the introduction of sediment from an unpolluted lake in 2008. The wet sediment contained, on average 53.8% w/w water and had an organic carbon (OC) content of 1.85% w/w dry sediment.

For the toxicity tests using the test species *L. variegatus*, *T. tubifex*, *A. aquaticus*, *H. azteca*, *C. riparius* and *C. horaria* (see Table 1), the same fludioxonil-spiked sediment was used as in the outdoor microcosm experiment. For a detailed description of the spiking procedure see Yin et al. (2018). After spiking (June 2016), the sediment to be used in the laboratory toxicity tests was stored for approximately a year at −20 °C in polyethylene containers in portions of approximately 3 and 6 L. We conducted two sediment-spiked toxicity tests at the same time. The spiked sediment needed for each set of two tests was removed from the freezer and placed in the dark at room temperature (approximately 20 °C). The period that the sediment was in un-frozen condition

Table 1
Species name, origin, life-stage and testing conditions for selected benthic species in the 28-d sediment-spiked toxicity tests with fludioxonil using field-collected sediment.

Taxon	Origin test species	A: Mean length in mm B: Mean dry weight in mg	No of replicate test systems to study treatment-related effects	Individuals per test vessel (% mortality in controls at day28)	Treatments: Geometric mean test concentrations A: mg a.s./kg dry weight sediment B: µg a.s./L pore water	DO (mg/L) ± SD pH ± SD Temp (°C) ± SD
Lumbriculus variegatus (Oligochaeta)	Laboratory culture at Wageningen UR, NL	A: not measured B: 0.805 mg/ind.	Controls (n = 5) Treatments (n = 3)	10 (0%)	A: 0–1.09–4.60–13.41–43.92–138.20–482.70 B: 0–7.65–26.74–83.38–419.1 – 1077.0–2198.1	pH = 8.2 ± 0.2 DO = 8.4 ± 0.4 T = 20.6 ± 0.1
Tubifex tubifex (Oligochaeta)	Purchased commercial culture	A: not measured B: 0.421 mg/ind.	Controls (n = 5) Treatments (n = 3)	20 (7%)	A: 0–1.47–4.76–13.65–46.88–143.6–506.4 B: 0–8.70–27.86–86.26–455.7–1215.0–2199.0	pH = 8.2 ± 0.1 DO = 8.5 ± 0.3 T = 20.1 ± 1.0
Corbicula fluminalis (Mollusca)	River Nederrijn near Wageningen, NL	A: 4.77 ± 0.49 mm B: 12.98 mg/ind.	Controls (n = 5) Treatments (n = 3)	10 (16%)	A: 0–0.76–2.41–5.61–14.31–50.48–155.35 B: 10.44–23.75 – 63.53–307.1 – 568.6–1169.1	pH = 8.3 ± 0.2 DO = 9.0 ± 0.5 T = 18.0 ± 1.3
Pisidium amnicum (Mollusca)	Outdoor experimental ditches of Wageningen UR, NL	A: 8.08 ± 0.61 mm B: 24.53 mg/ind.	Controls (n = 5) Treatments (n = 3)	10 (22.5% in controls and 7% in lowest treatment)	A: 0–0.74–1.88–5.00–15.77–55.69–164.4 B: 0–11.15–28.12–60.47–305.1–551.0–1141.4	pH = 8.4 ± 0.1 DO = 9.1 ± 0.2 T = 18.1 ± 0.1
Asellus aquaticus (Crustacea)	Outdoor *Glyceria maxima* culture at experimental ditches of Wageningen UR, NL	A: 5.09 ± 0.57 mm B: 1.53 mg/ind.	Controls (n = 5) Treatments (n = 3)	10 (4%)	A: 0–0.95–1.87–6.84–21.40–71.65–254.2 B: 0–11.68–29.84–59.28–302.3–752.3–1168.0	pH = 8.3 ± 0.1 DO = 8.8 ± 0.2 T = 19.2 ± 0.2
Hyalella azteca (Crustacea)	Laboratory culture at Wageningen UR, NL	A: 4.01 mm B: 0.219 mg/ind.	Controls (n = 5) Treatments (n = 3)	10 (10%)	A: 0–1.20–3.54–10.68–32.75–98.83–379.7 B: 0–11.01–33.51–122.5–387.3–774.5–1297.0	pH = 8.3 ± 0.2 DO = 8.4 ± 0.3 T = 20.6 ± 0.1
Chironomus riparius (Insecta)	Laboratory culture, University of Amsterdam, NL	A: first instar larvae B:	Controls (n = 5) Treatments (n = 4)	20 (27%) 71% emergence in controls	A: 0–0.90–2.48–8.82–30.23–70.84–338.61 B: 0–10.83–22.81–69.08–327.2–775.7–1078.4	pH = 8.3 ± 0.1 DO = 8.7 ± 0.3 T = 19.5 ± 1.3
Caenis horaria (Insecta)	Outdoor experimental ditches of Wageningen UR, NL	A: 3.42 ± 0.63 mm B: 0.12 ± 0.05 mg	Controls (n = 5) Treatments (n = 3)	10 (18%)	A: 0–0.88–1.61–6.04–18.47–68.49–302.8 B: 0–11.90–30.13–68.21–296.9–745.6–1084.0	pH = 8.4 ± 0.2 DO = 8.9 ± 0.1 T = 19.2 ± 0.2

between spiking and use in the laboratory toxicity test (ageing period) was approximately 7–8 days in all tests.

Approximately 40 L of the same well-mixed field-collected sediment that was not spiked (sampled in June 2016) was stored at −20 °C in polyethylene containers in portions of approximately 3 and 6 L, to allow the conduct of future toxicity tests with the same field-collected sediment. This frozen sediment was used for tests with the bivalves *Corbicula fluminalis* and *Pisidium amnicum*. The frozen sediment was thawed at room temperature (approximately 20 °C) before the spiking procedure and for each test concentration in each toxicity test, portions of 5 kg (solvent controls) and 3 kg (remaining six test concentrations) of wet sediment were used. Dosing was done by manually mixing a specific dosing solution in the sediment with a hand-held electric cement mixer for 10 min. After spiking, the sediment was aged for 7 days before use. Before introducing this sediment in the test systems, it was thoroughly mixed again for approximately 10 min.

To prepare the spiking solutions, technical grade fludioxonil was used and acetone as solvent. The spiking treatments included a solvent control (sediment spiked with 1.48 mg acetone/L wet sediment), and six treatment-levels of fludioxonil. The six fludioxonil treatments received the same amount of acetone as the solvent controls. The spiking was conducted from low to high concentrations, to minimize cross-contamination (for sediment exposure concentrations in each laboratory toxicity test see Table 1).

2.3. Design of sediment-spiked laboratory toxicity tests

The 28-d sediment-spiked toxicity tests were all conducted in the same type of test system in the laboratories of Wageningen Environmental Research. The test systems consisted of 1.5 L glass jars (height 20 cm; diameter 10 cm), containing 200 g of control or treated wet sediment and approximately 1 L of Sinderhoeve well water. The test systems were placed in a water bath (temperature 20 ± 2 °C) under a 16:8h light:dark regime. During the test, the overlying water was not renewed, but well water was added to compensate for evaporation in the test vessels. Aeration of the overlying water was provided through an S-shaped stainless-steel tube, avoiding sediment resuspension (see Fig. 1). The water quality parameters pH, dissolved oxygen (DO) and temperature were measured weekly in the overlying water of each test system.

The test design for *C. riparius* was based on OECD test guideline 218

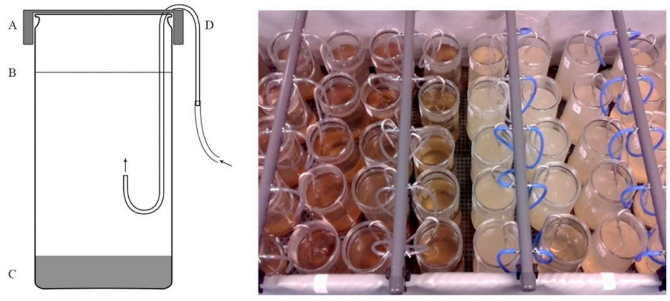

Fig. 1. Left: Schematic overview of an individual test system used in the laboratory single species tests (A, lid of mesh gauze used in tests with emerging insects; B, overlying water level; C, sediment layer; D, stainless-steel aeration tube. Right: Photograph of test systems of the *Lumbriculus variegatus* bioassay placed in the water bath and in which field-collected sediment (darker colour) and artificial OECD sediment (lighter colour) was used. Note that the results of the tests using artificial sediment will be published in another paper. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(OECD, 2004), but deviated by using larger test systems in terms of quantities of sediment and water and slightly adapted feeding conditions. A larger water volume was used to facilitate aeration of the overlying water and to avoid less optimal environmental conditions for the test organisms (e.g. oxygen depletion, high ammonium levels). As food, 280 mg of a mixture of Trouvit and Tetraphyl in a ratio of 20:1 was mixed in the spiked sediment of each test system at the start of the experiment following the recommendations by Marinković et al. (2011). In addition, 35 mg of the same food was added to each test system on days 7 and 14. Twenty first-instar larvae were added to each test system. Measured endpoints were emergence of adults, time of emergence (development time) and survival at day 28 (the sum of surviving larvae, pupae and emerged adults). Following OECD test guideline 233, we considered the *C. riparius* test valid if the mean emergence in controls by the end of the test was at least 70% and 85% of the adults had emerged between day 12 and 23 (OECD, 2010).

The test design for *L. variegatus* and all other non-standard benthic species mentioned in Table 1 was based on OECD test guideline 225 (OECD, 2007), but again deviated by using a larger volume of test system, similar to that used for *C. riparius*. Following the guideline recommendation (OECD, 2007), at the start of the experiment (after spiking) 0.5 g of nettle powder (0.25% of the wet sediment weight) was mixed in the spiked sediment of each test system as a potential food source and a further 0.5 g added via the water column on day 14. Measured endpoints for oligochaete worms, bivalves and crustaceans were number of surviving individuals and relative rate in daily dry weight biomass increase (for the EC_x calculations the biomass on day 0 was set at 100% and the total increase in biomass of the test organisms on day 28 in each test system was expressed as a percentage of the start biomass) and where appropriate, net daily increase in numbers (reproduction). Measured endpoints for the ephemeropteran *C. horaria* were emergence of adults and survival on day 28 (the sum of surviving larvae, pupae and emerged adults). The tests with the oligochaete worms *L. variegatus* and *T. tubifex* were considered valid in case of an increase of living worms in the controls by a factor of 1.8 (OECD, 2007). In case of other non-standard test species, for the test to be considered valid, survival in control test systems should be higher than 80% at the end of the test (see USEPA, 2000).

2.4. Sediment fludioxonil analysis

At the start (day 0) and the end (day 28) of the laboratory toxicity tests, concentrations of fludioxonil were measures in the total sediment and in sediment pore water in all treatments. For the fludioxonil measurements on day 28, one extra test system for each treatment was prepared in each test to sample sediment. On each sampling date and for each treatment-level approximately 200 g well-mixed wet sediment was sampled, placed in 250 mL high density polyethylene (HDPE) bottles and stored at −20 °C until chemical analysis. From each thawed sample of sediment, one well-mixed subsample of approximately 20 g wet sediment was transferred into a centrifuge tube and centrifuged for 8 min at 2500 rpm to separate pore water from solid sediment. About 2.0 mL of pore water from each subsample was collected, of which 0.5 mL was used for chemical analysis. Then the remaining wet sediment was extracted by adding approximately 30 mL acetonitrile containing 1% formic acid (v/v). After shaking for 2 h on a flat-bed shaker these samples were centrifuged again (2500 rpm for 8 min). The supernatant was transferred to a 25 mL tube and the remaining sediment was dried for 24 h in an oven at 105 °C.

All pore water samples (0.5 mL) were immediately diluted 1:1 with acetonitrile in order to be analysed by Agilent 1260 Infinity 6460 Triple Quad LC-MS (Agilent Technologies, Germany). When necessary (sample concentration above the highest standard of the calibration curve) the samples were further diluted with acetonitrile:Milliq-water (50:50 v/v). All the dilutions were done directly in GC vials using a Hamilton 600 dilutor. All sediment extracts were also diluted in the same way. For a description and analysis of quality control samples see Yin et al. (2018). The limit of detection (LOD) and limit of quantification (LOQ) for fludioxonil in pore water samples of our experiments were 0.06 μg a. s./L and 0.19 μg a. s./L, respectively. For sediment samples the LOD and LOQ were 2.0 and 6.5 μg a. s./kg dry sediment, respectively.

We express the toxicity values of the fungicide in terms of (i) μg a. s./kg dry sediment, and (ii) μg a. s./L sediment pore water. For this the geometric mean concentrations of fludioxonil calculated from measurements on days 0 and 28 in each laboratory toxicity test are used (see Table 1 and Supporting Information).

2.5. Data analysis

28-day LC_x and EC_x values (and 95% confidence bands) for the benthic test species were estimated using the MOSAIC web-interface for statistical analyses in ecotoxicology (Charles et al., 2018). MOSAIC is available at http://pbil.univ-lyon1.fr/software/mosaic/. The calculations within MOSAIC are based on the R package 'morse' (Delignette-Muller et al., 2016).

Hazardous concentrations to 5% (HC_5) and 50% (HC_{50}) of the species tested were calculated from species sensitivity distribution (SSD) curves. These HC_p values, and their 95% confidence bands, were calculated using $MOSAIC_{SSD}$ (Charles et al., 2018 and link above) by selecting the log-normally distributed model (also see Aldenberg and Jaworska, 2000).

3. Results

3.1. 28-Day L(E)C_{10} and L(E)C_{50} data for the benthic species tested

All 28d-L $(EC)_{10}$ and 28d-L(E)C_{50} values (and 95% confidence bands) for the eight benthic macroinvertebrates and the endpoints measured, are presented in Table 2. Information on CRED reporting recommendations (Moermond et al., 2016) for our tests is presented in the Supporting Information (Appendix A), including basic data for measured exposure concentrations and biological measurement endpoints in the laboratory toxicity tests (Table SI 1–9).

3.1.1. Lumbriculus variegatus

With 0% mortality and an increase of living worms by more than a factor of 1.8 in control test systems, the *L. variegatus* test met the OECD validity criteria. In controls mean % dry weight biomass increase on day 28 relative to day 0 was 335% (Tables SI–2).

Both in terms of total sediment concentration and pore water concentration, the endpoint 'relative rate in daily biomass increase' showed the lowest toxicity values, followed by the endpoint 'reproduction'. When expressed in terms of concentration in total sediment, the LC_{10} value for the 'survival' endpoint was approximately a factor of 20 higher than the EC_{10} for the 'relative rate in daily biomass increase' endpoint. In terms of pore water concentration, the LC_{10} value was approximately 9 times higher than the corresponding EC_{10}. All toxicity data showed relatively small confidence bands, with a spread (ratio between upper and lower value of the 95% confidence band) of less than 3 (Table 2).

3.1.2. Tubifex tubifex

In control test systems, on average 93% of the individuals incubated at the start of the test were still alive on day 28, while the mean % dry weight increase on day 28 relative to day 0 was 158%. We were not able to assess the reproduction endpoint, since juvenile individuals could not be separated from each other without breaking them, hampering an accurate count of juvenile numbers (Tables SI–3).

28d-EC_{10} values for the endpoint 'relative rate in biomass increase' were 2.9 (pore water) and 4.6 (total sediment) times lower compared to the corresponding 28d-LC_{10} values. The 28d-EC_{10} values showed the

Table 2
28d-L(E)C_x values (and 95% confidence bands) for eight benthic invertebrates in laboratory toxicity tests using field-collected sediment spiked with the fungicide fludioxonil. The 28d-L(E)C_x values are expressed in terms of geometric mean fludioxonil concentration during the test in the total dry sediment (mg a.s./kg DW) and in sediment pore water (μg a.s./L).

Taxon Endpoint	Toxicity value	Total sediment (mg a.s./kg DW)	Pore water (μg a.s./L)
Lumbriculus variegatus (Oligochaeta)			
Relative rate in daily biomass increase	28d-EC_{10}	17.7 (9.87–28.3)	208 (123–313)
	28d-EC_{50}	44.9 (33.3–60.5)	428 (329–561)
Reproduction	28d-EC_{10}	35.6 (24.8–87.5)	369 (277–880)
	28d-EC_{50}	67.8 (55.7–109)	611 (577–989)
Survival	28d-LC_{10}	368 (202–471)	1850 (1260–2150)
	28d-LC_{50}	541 (481–859)	2360 (2190–3290)
Tubifex tubifex (Oligochaeta)			
Relative rate in daily biomass increase	28d-EC_{10}	19.2 (5.54–43.6)	243 (41.9–427)
	28d-EC_{50}	33.5 (16.7–46.0)	362 (135–448)
Survival	28d-LC_{10}	87.6 (43.7–164)	714 (387–1140)
	28d-LC_{50}	733 (474–1430)	3080 (2230–5510)
Corbicula fluminalis (Mollusca)			
Relative rate in daily biomass increase	28d-EC_{10}	7.49 (1.61–22.4)	118 (14.5–287)
	28d-EC_{50}	11.1 (4.93–27.6)	193 (64.4–311)
Survival	28d-LC_{10}	2.14 (0.673–5.76)	65.5 (13.8–167)
	28d-LC_{50}	12.9 (7.72–21.6)	231 (121–351)
Pisidium amnicum (Mollusca)			
Relative rate in daily biomass increase	28d-EC_{10}	21.0 (4.61–47.8)	329 (47–505)
	28d-EC_{50}	26.0 (14.4–52.6)	377 (214–564)
Reproduction	28d-EC_{10}	48.3 (0.196–117)	487 (10.1–881)
	28d-EC_{50}	61.2 (13.8–127)	585 (254–939)
Survival	28d-LC_{10}	24.1 (11.4–40.1)	339 (215–456)
	28d-LC_{50}	59.2 (42.4–81.9)	588 (485–727)
Asellus aquaticus (Crustacea)			
Relative rate in daily biomass increase	28d-EC_{10}	4.44 (0.046–27.0)	35.0 (1.57–254)
	28d-EC_{50}	7.47 (0.923–45.4)	67.8 (12.9–318)
Reproduction	28d-EC_{10}	7.03 (0.048–26.2)	6.48 (3.37–11.1)
	28d-EC_{50}	10.1 (1.39–45.1)	15.8 (11–22.6)
Survival	28d-LC_{10}	20.6 (5.74–55.2)	351 (102–688)
	28d-LC_{50}	153 (94.5–279)	1010 (777–1480)
Hyalella azteca (Crustacea)			
Relative rate in daily biomass increase	28d-EC_{10}	39.1 (18.2–81.1)	405 (246–653)
	28d-EC_{50}	45.1 (30.9–85.1)	451 (361–680)
Survival	28d-LC_{10}	54.7 (28.5–91.9)	542 (364–731)
	28d-LC_{50}	75.1 (53.1–95.7)	656 (526–755)
Chironomus riparius (Insecta)			
Emergence	28d-EC_{10}	23.7 (14.3–49.8)	637 (244–747)
	28d-EC_{50}	52.0 (41.8–66.5)	737 (487–771)
Emergence + survival larvae	28d-LC_{10}	17.6 (9.76–27.1)	300 (173–591)
	28d-LC_{50}	53.0 (41.5–67.3)	568 (447–744)
Caenis horaria (Insecta)			
Emergence	28d-EC_{10}	2.90 (0.354–14.7)	43.7 (4.99–229)
	28d-EC_{50}	13.6 (5.27–33.0)	176 (65.2–399)
Emergence + survival nymphs	28d-LC_{10}	107 (70.2–237)	837 (748–987)
	28d-LC_{50}	122 (75.2–251)	884 (781–1020)

widest confidence bands (with spreads of 7.9 and 10.2 for total sediment and pore water data, respectively) (Table 2).

3.1.3. Corbicula fluminalis

In control test systems, on average 84% of the incubated individuals were still alive on day 28, while the mean % dry weight increase on day 28 relative to day 0 was 81%. This clam species did not reproduce during the test (Tables SI–4).

The number of surviving individuals, as well as dry weight biomass increase, showed a general decline with increasing exposure concentration, but variability between replicates within the 2.41 and 14.31 mg a. s./kg treatments was relatively high (see Tables SI–4). This explains the rather wide confidence bands of the 28d-L(E)$_{10}$ estimates, particularly when expressed in terms of pore water concentration (spreads of 19.7 and 12.1 for EC_{10} and LC_{10} values, respectively). The calculated median 28d-LC_{10} values were lower than the corresponding 28d-EC_{10}'s for the endpoint 'relative rate in daily biomass increase', although their confidence bands overlapped, particularly when expressed in terms of pore water concentrations (Table 2).

3.1.4. Pisidium amnicum

With a control mortality of 22.5%, strictly speaking our validity criterion of less than 20% mortality in control test systems was not met for the bivalve *P. amnicum* (see Table 1). In test systems of the six fludioxonil treatment-levels, however, mean mortality of *P. amnicum* was respectively 7%, 20%, 10% and 16%, 57% and 93% when going from low to high exposures (see Tables SI–5). For this reason, we considered that this test can be used in the effect assessment. In addition, during the test this clam produced offspring and relative to controls this offspring was clearly reduced at the highest two treatment levels (Tables SI–5). Although a clear trend of a treatment-related negative effect on net dry weight biomass increase was observed at the two highest treatment levels, the values for '% dry weight increase on day 28 relative to day 0' were relatively small and variable within controls and lower treatments (Tables SI–5).

The endpoint 'relative rate in daily biomass increase' resulted in the lowest median 28d-EC_{10} values (spread of confidence band somewhat higher than 10 for both total sediment and pore water values). Differences between median 28d-EC_{10} values of the 'relative rate in

daily biomass increase' endpoint and median 28d-LC_{10} values were relatively small. The median 28d-EC_{10} values for the endpoint reproduction were the highest, although these values also had a very wide confidence band. Overall, differences between median L(E)C_{10} and corresponding median L(E)C_{50} estimates were relatively small (Table 2).

3.1.5. Asellus aquaticus

In control test systems, on average 96% of the incubated individuals were still alive on day 28, while the mean % dry weight increase on day 28 relative to day 0 was 80%. This isopod reproduced during the test, particularly in the controls and lowest treatment (Tables SI–6).

When expressed in terms of total sediment concentration, the lowest toxicity values were observed for the endpoint 'relative rate in daily biomass increase'. In contrast, when expressed in terms of pore water concentration, the lowest toxicity values were observed for the endpoint 'reproduction'. The highest toxicity values were calculated for the endpoint 'survival'. Overall, the confidence bands of the 28d-L(E)$_x$ values for pore water showed a smaller spread that those for total sediment. Nevertheless, all 28d-EC_{10} values, except that for the endpoint reproduction and pore water, had very wide confidence intervals (spread > 160). The spreads of the confidence bands of the 28d-LC_{10} estimates all were smaller than 10 (Table 2).

3.1.6. Hyalella azteca

On average, 90% of the incubated individuals were still alive on day 28 in controls, while the mean % dry weight increase on day 28 relative to day 0 was 180%. This amphipod did not reproduce during the 28-day test (Tables SI–7).

The 28d-EC_x values for the endpoint 'relative rate in daily biomass increase' were somewhat lower than the corresponding LC_x values, irrespective whether expressed in terms of total sediment or pore water concentration. In addition, the spreads of the confidence bands in all tests were small (< 5) (Table 2).

3.1.7. Chironomus riparius

The OECD validity criterion of at least 70% emergence in control test systems was met. On average, emergence was 71% by day 28 in control test systems. Emergence took place between days 15 and 24. At the end of the experiment a few surviving larvae were still present in the sediment compartment of the test systems (Tables SI–8).

The 28d-LC_{10} values (defined as the sum of emerged individuals and surviving larvae on day 28) were lower than the 28d-EC_{10} values for the endpoint emergence. This difference was largest when expressing the toxicity in terms of pore water concentration. The confidence bands of all 28d-L(E)C_{50} values were small, with spreads not exceeding 3.5 (Table 2).

3.1.8. Caenis horaria

On average, emergence of this sediment-dwelling ephemeropteran was approximately 50% by day 28 in control test systems and those nymphs that did not emerge largely were alive at the end of the experiment. Survival, here defined as the sum of emerged individuals and surviving nymphs on day 28, was on average 82% (Tables SI–9).

The median 28d-EC_x values for the endpoint emergence were considerably lower than the corresponding LC_x values, irrespective whether they were expressed in terms of total or pore water concentration. The 28d-EC_{10} values showed relatively wide confidence bands (spread > 40) in contrast to the 28d-LC_{10} values (spread < 4).

3.2. Species sensitivity distributions (SSDs)

To illustrate the position of the different benthic species within the sensitivity distribution, SSD curves constructed with 28-d EC_{10} values and 28-d LC_{10} values, as reported in Table 2, are presented in Fig. 2. To achieve optimal comparability in 28d-EC_{10} values, we selected the endpoint 'emergence' for insects and 'relative rate in daily biomass increase' for all other taxa. The 28-d LC_{10} values for all species are highly comparable, since they all relate to the survival endpoint.

The available 28d-EC_{10} estimates expressed in terms of fludioxonil concentration in total dry sediment showed the following order: C. horaria < A. aquaticus < C. fluminalis < *L. variegatus* < T. tubifex < P. amnicum < C. riparius < H. azteca. (Fig. 2A). Expressing the 28d-EC_{10}'s in terms of μg/L pore water this order slightly shifted, viz., A. aquaticus < C. horaria < C. fluminalis < *L. variegatus* < T. tubifex < P. amnicum < H. azteca < C. riparius (Fig. 2C). The position of the standard test species C. riparius in the SSDs constructed with 28d-EC_{10} values was always high in the curve, while the other standard benthic test species *L. variegatus* had a middle position. The species *A. aquaticus* and *C. horaria* could always be found in the tail of the SSDs constructed with 28d-EC_{10} values, but note that the EC_{10} value for these taxa showed a much larger spread in 95% confidence band than observed for the other benthic species (Table 2). The higher the spread between lower and upper value of the 95% confidence band, the higher is the uncertainty of the toxicity estimate.

The available 28d-LC_{10} estimates expressed in terms of mg a. s./kg dry sediment, showed the following order: *C. fluminalis* < *C. riparius* < *A. aquaticus* < *P. amnicum* < *H. azteca* < *T. tubifex* < *C. horaria* < *L. variegatus* (Fig. 2B). Expressing the 28d-LC_{10}'s in terms of μg/L pore water, the sensitivity order of species slightly shifted in that *P. amnicum* was more sensitive than *A. aquaticus* (Fig. 2 D). In the SSD curves constructed with 28d-LC_{10} values it appears that the standard test species *L. variegatus*, as well as the other oligochaeta *T. tubifex*, has a position high in the curve, in contrast to the standard test species *C. riparius*, which can be found on second position in the tail of the SSDs.

3.3. Hazardous concentrations

HC_5 values differ when different toxicity values are used to construct the SSD (see e.g. Fig. 2). To derive chronic Regulatory Acceptable Concentrations (RACs) for pesticides and invertebrates it is common practise to use chronic EC_{10} (or NOEC) values as input for SSDs. Since our chronic toxicity data are based on several endpoints (see Table 2), selections have to be made. In Table 3, three options for data selection are presented to construct SSDs for HC_5 and HC_{50} calculation, viz., A) 28d-EC_{10} values for the endpoint 'emergence' in case of insects and 'relative rate in daily biomass increase' for other taxa, B) lowest 28d-EC_{10} values for each benthic species, irrespective of the sublethal endpoint, and C) lowest 28d-L(E)C_{10} for each benthic species, irrespective of endpoint.

The median HC_5 values in terms of total sediment concentration varied between 1.7 mg a. s./kg (option C) and 3.1 mg a. s./kg (options A and B). The confidence band of the option C HC_5 value, however, was relatively wide (spread of 36.7) compared to that of the HC_5 value of options A and B (spread of 5.3). In terms of pore water concentration, the median HC_5 of option A was relatively high (35 μg a. s./L) compared to options B and C (14 and 13 μg a. s./L, respectively). The HC_5 value of option B had the widest confidence band (spread 53.6), but its lower limit (2.8 μg a. s./L) was similar to that of the confidence band of option C (3.0 μg a. s./L). HC_{50} values were a factor of 4.2–10 higher than corresponding HC_5's. The spread of the 95% confidence band was consistently lower for the HC_{50} estimates compared to those of the HC_5's.

4. Discussion

4.1. Sensitivities of standard test species

Although an OECD test guideline is available to conduct sediment-spiked toxicity tests with *L. variegatus*, and this species is one of the recommended Tier-1 benthic test species, hardly any sediment toxicity data for this species and fungicides could be found in the published

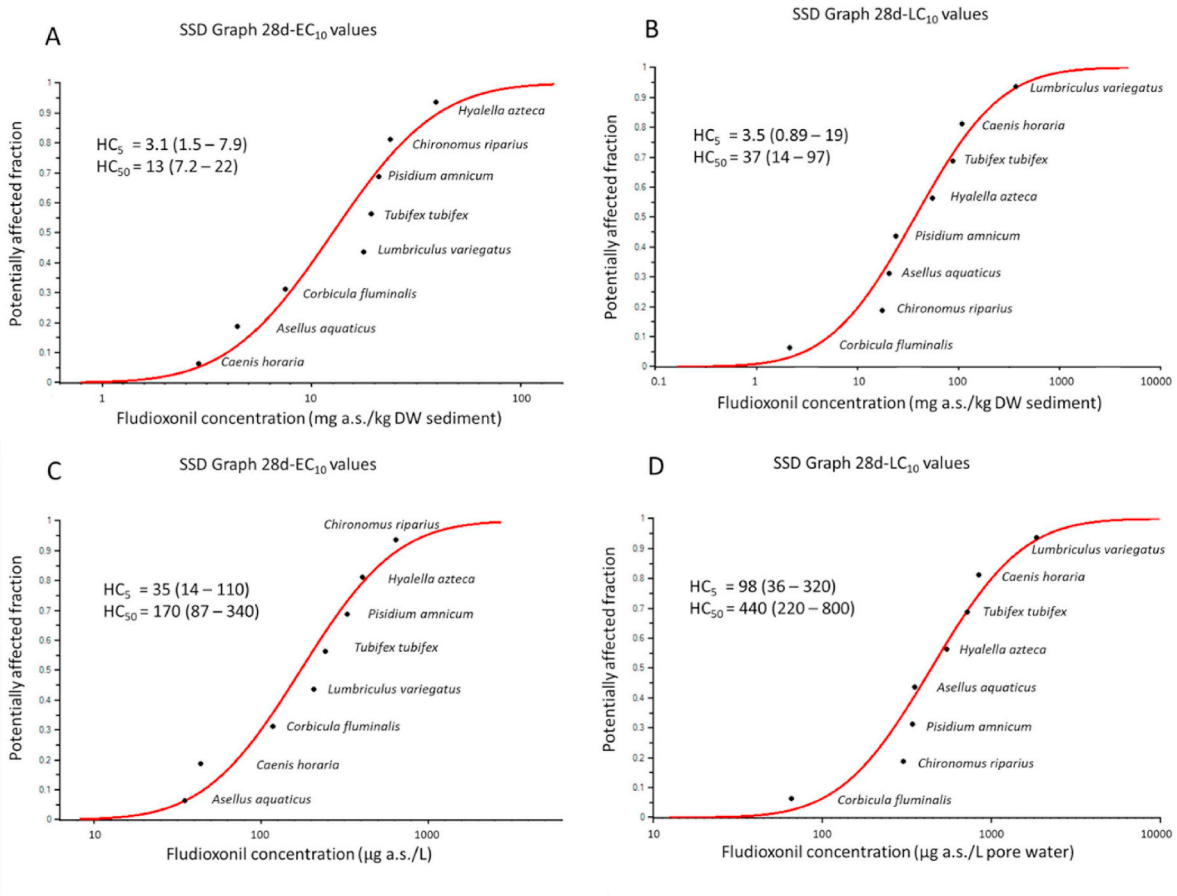

Fig. 2. Species sensitivity distributions (SSDs) constructed with 28d-EC_{10} values (panels A, C) or 28d-LC_{10} values (panels B, D) for 8 different benthic invertebrates and derived from laboratory sediment toxicity tests using field-collected sediment, spiked with the fungicide fludioxonil. The 28d-L(E)C_{10} values are expressed in terms of total sediment concentration (mg a. s./kg DW; panels A and B) and in sediment pore water concentration (µg a. s./L; panels C and D). For input data see Table 2. As 28d-EC_{10} input data for panels A and C, the 'emergence' endpoint is selected for insects and the 'relative rate in daily biomass increase' endpoint for all other taxa. Hazardous concentrations to 5% (HC_5) and 50% (HC_{50}) of the species and their 95% confidence bands calculated with $MOSAIC_{SSD}$ are presented in each panel.

literature (see e.g., Deneer et al., 2013), and also not in the dossier of fludioxonil (EFSA, 2007). This is because currently the Tier-1 sediment effect assessment for fungicides can be based on a single chronic toxicity test with a *Chironomus* species (EFSA, 2013) and that the recommendation to also test *L. variegatus* (EFSA, 2015), is not yet a strict requirement.

In our study and both for total sediment and pore water concentration, the lowest 28d-EC_{10} values for *L. variegatus* (endpoint relative rate in daily biomass increase) were slightly lower than the *C. riparius* 28d-EC_{10} values for emergence (Table 2). In addition, our 28d-EC_{10} value of 23.7 mg a. s./kg dry sediment for *C. riparius* and the endpoint emergence is considerably lower than the 28d-NOEC of 160 mg a. s./kg dry sediment (the highest concentration tested) for the same endpoint reported in the fludioxonil dossier (EFSA, 2007). In addition, our 28d-EC_{10} value is also lower than the 28d-NOEC of 40 mg a. s./kg dry sediment for the endpoint 'effects on emerged midges' that is selected by EFSA (2007) in the sediment effect assessment for fludioxonil. The original report (personal communication Mick Hamer from Syngenta) on which this NOEC of 40 mg a. s./kg dry sediment is based gives a sediment OC content of 1.7%, almost similar to the OC content of the sediment used in our tests (1.85%). Nevertheless, the observed differences in toxicity described above may be explained, at

Table 3
Hazardous concentrations to 5% (HC_5) and 50% (HC_{50}) of the species tested and their 95% confidence bands for fludioxonil, calculated with the computer program $MOSAIC_{SSD}$. Input data were taken from Table 2.

Option input data	Type of concentration	HC_5 derived from SSD constructed with median 28d-L(E)C_{10}'s	HC_{50} derived from SSD constructed with median 28d-L(E)C_{10}'s
A: 28d-EC_{10} values for the endpoint 'emergence' in case of insects and 'relative rate in daily biomass increase' for other taxa (Fig. 2A & C)	mg a.s./kg dry sediment	3.1 (1.5–7.9)	13 (7.2–22)
	µg a.s./L pore water	35 (14–110)	170 (87–340)
B: Lowest 28d-EC_{10} values, irrespective of endpoint	mg a.s./kg dry sediment	3.1 (1.5–7.9)	13 (7.2–22)
	µg a.s./L pore water	14 (2.8–150)	140 (49 - 320
C: Lowest 28d-L(E)C_{10} values, irrespective of endpoint	mg a.s./kg dry sediment	1.7 (0.82–7.2)	9.9 (4.7–20)
	µg a.s./L pore water	13 (3–110)	120 (44–260)

least in part, by the fact that we used field-collected sediment, while the *C. riparius* test reported in the dossier was conducted on artificial sediment. Differences in toxicities for the same benthic test species and the same pesticide between laboratory single-species tests with field-collected and artificial sediment have been reported in several studies (see e.g., Fleming et al., 1998; Goedkoop et al., 2005; Brock et al., 2018).

4.2. Sensitivities of standard and additional test species

For all species tested and reported in this paper, the same homogenised field-collected sediment was used, the same as that used in the sediment-spiked microcosm experiment reported by Yin et al. (2018). This minimized the influence of sediment type on concentration-response relationships in our experiments. Three of the six additional benthic test species (*A. aquaticus*, *C. horaria*, *C. fluminalis*) had 28d-EC_{10} values a factor of 2–6 lower than that of the overall most sensitive standard test species *L. variegatus*. The observed differences in toxicity values between species of our sediment-spiked laboratory toxicity tests with the same field-collected sediment most likely can be explained by the combination of (i) intrinsic differences in sensitivity, (ii) differences in considered measurement endpoints between species, and (iii) differences in autecological characteristics of the selected benthic species that influenced exposure.

In our laboratory toxicity tests with *C. riparius* we observed that at the highest fludioxonil treatments, a larger proportion of larvae avoided burrowing in the sediment. Although this behaviour was not exactly quantified (not selected as measurement endpoint), this observation suggests active avoidance behaviour when larvae experience high fludioxonil concentrations in sediment.

The observed differences in sensitivity between test species observed in our sediment-spiked laboratory toxicity tests (this paper), sometimes differed markedly from the responses of these species observed in the sediment-spiked outdoor microcosm experiment (Yin et al., 2018). In the outdoor microcosm experiment, population densities of *L. variegatus* were hardly affected by long-term sediment exposure concentrations up to 495.5 mg a. s./kg dry sediment (corresponding to 1451.3 µg a. s./L pore-water), a concentration much higher than the *L. variegatus* 28d-EC_{10} value of 17.7–35.6 mg a. s./kg dry sediment (corresponding to 208–369 µg a. s./L pore-water) observed in our laboratory tests with field collected sediment. Possibly, the release in competition by a treatment-related decline in other oligochaetes compensated the direct toxic effects of fludioxonil on the *L. variegatus* population in the sediment-spiked microcosm experiment. For example, the oligochaetes *Dero digitata* with a microcosm NOEC of 14.2 mg a. s./kg dry sediment (corresponding to 88.8 µg a. s./L pore-water) and Tubificidae with a microcosm NOEC of 46.5 mg a. s./kg dry sediment (corresponding to 410.8 µg a. s./L pore-water) showed a pronounced treatment-related decline (Yin et al., 2018). Another observation is that in the sediment-spiked outdoor microcosm experiment, Sphaeridae (largely *P. amnicum*) showed an increase at treatment levels higher than 14.2 mg a. s./kg dry sediment, while in our single species test *P. amnicum* was negatively impacted at concentrations higher than of 21.0 mg a. s./kg dry sediment (corresponding to 377 µg a. s./L pore-water). In the outdoor microcosm experiment, however, *P. amnicum* was predominantly found on macrophytes and filamentous algae above the sediment, while in laboratory single species test this species was more or less forced to remain in contact with the sediment as no other structures were present to support the animals. It cannot be excluded that sediment avoidance behaviour of *P. amnicum* reduced exposure to fludioxonil to a greater extent in the outdoor microcosm experiment than in the more simple laboratory toxicity test.

4.3. Calibration of the EFSA effect assessment approaches for sediment macroinvertebrates

The results of the sediment-spiked laboratory single species tests presented in this paper, and the results of the sediment-spiked outdoor microcosm experiment published by Yin et al. (2018), can be used to evaluate the protectiveness of the tiered approach for sediment effect assessment for fungicides as proposed by EFSA (2015).

As a screening step to assess the risk to benthic organisms, EFSA (2015) proposed to use the chronic Regulatory Acceptable Concentration for pelagic organisms (= $RAC_{sw;ch}$) and the Equilibrium Partitioning (EqP) approach, applying an additional factor of 10 to cover the uncertainty of exposure due to sediment ingestion (Tier-0, modified EqP approach). For a theoretical background of the EqP concept see Di Toro et al. (1991). This Tier-0 $RAC_{sed;EqP}$ (initially expressed in terms of concentration per g OC in dry sediment) can be calculated with the following formula: ($RAC_{sw;ch}$ * K_{oc})/10. Using the information presented in EFSA (2007), the Tier-1 $RAC_{sw;ch}$ for fludioxonil can be derived by selecting the lowest chronic NOEC/EC_{10} value available for pelagic Tier-1 test species (= 21-d NOEC for *Daphnia magna* of 5 µg/L) and by applying an assessment factor (AF) of 10 ($RAC_{sw;ch}$ = 0.5 µg/L). Selecting the mean K_{foc} value of 145,600 L/kg OC reported for fludioxonil in EFSA (2007) as a proxy for the K_{oc}, the Tier-0 $RAC_{sed;EqP}$ = (0.5*145,600)/10 = 7280 µg a. s./kg OC. Since the sediment in our tests had an OC content of 1.85%, the Tier-0 $RAC_{sed;EqP}$ in terms of total sediment concentration is 0.135 mg a. s/kg dry sediment (Fig. 3A).

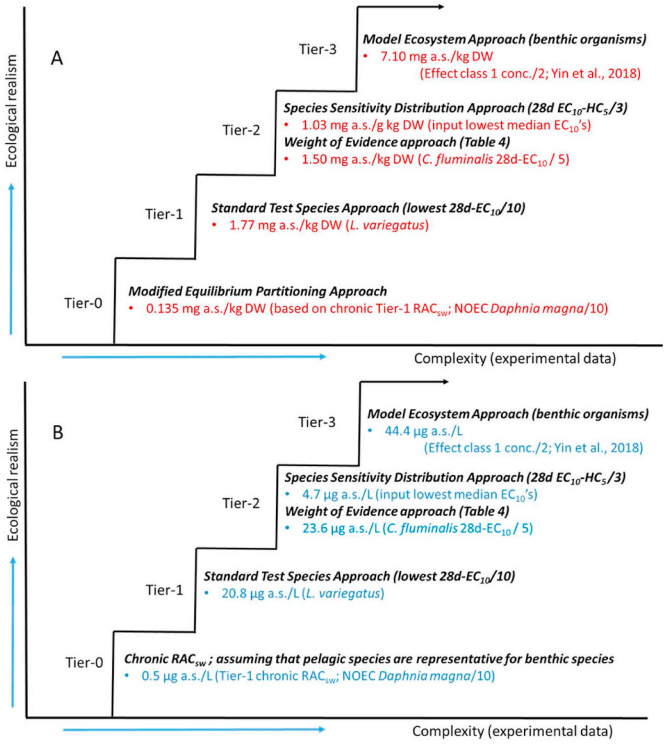

Fig. 3. Overview of possible Regulatory Acceptable Concentrations in sediment (RAC_{sed}) for the fungicide fludioxonil and derived for several effect assessment tiers to methods described in EFSA (2015) and the proposed Weight-of-Evidence approach described in Table 4. Panel A: RAC_{sed} estimates expressed in terms of fludioxonil concentration in mg a. s./kg dry weight sediment. Panel B: RAC_{sed} estimates expressed in terms of fludioxonil concentration in the sediment pore water. The Tier-1 and Tier-2 RAC_{sed} estimates are based on toxicity tests with field-collected sediment to facilitate the comparison with the Tier-3 RAC_{sed} estimate derived from the sediment-spiked microcosm experiment in which the same field-collected sediment was used. The Tier-2 RAC_{sed} derivation is based on the lowest 28d-EC_{10} values reported in Table 2 and the HC_5 calculations for option B in Table 3.

Adopting the EqP concept assumes that the sensitivity distribution of pelagic water organisms in terms of µg/L overlying water is similar to that of sediment-dwelling organisms in terms of µg/L sediment pore water. For that reason the Tier-1 $RAC_{sw;ch}$ value mentioned above is used as a Tier-0 proxy for the RAC_{sed} (in terms of pore water concentration) in Fig. 3B.

In the chronic Tier-1 RAC_{sed} derivation proposed by EFSA (2013), either the 28d EC_{10}/NOEC of *C. riparius* or that of *L. variegatus* can be used. EFSA (2015) proposed to use the lower of the two 28d NOEC/EC_{10} values for *L. variegatus* and *C. riparius* and the application of an assessment factor (AF) of 10. From our data *L. variegatus* overall is the most sensitive standard test species for fludioxonil. Following the proposal of EFSA (2015) and selecting the lowest 28d-EC_{10} value of *L. variegatus*, the Tier-1 RAC_{sed} becomes then 1.77 mg a. s./kg dry sediment (Fig. 3A) or 20.8 µg a. s./L sediment pore water (Fig. 3B).

When applying the SSD approach in the chronic Tier-2 effect assessment procedure for pesticides it is recommended in EFSA (2013) to select the median HC_5 derived from SSD's constructed with chronic EC_{10} or NOEC values and the application of an AF of 3. In addition, the SSD should be constructed with toxicity data for at least 8 different benthic species, that for biocidal fungicides like fludioxonil should represent taxa belonging to at least five (EFSA, 2015) to six (EFSA, 2013) different orders/families. We met both these criteria. Of the options presented in Table 3 to derive HC_5 values, we selected option B (SSD based on lowest 28d-EC_{10} value of each benthic species tested, irrespective of the sublethal endpoint). The resulting Tier-2 RAC_{sed} value is 1.03 mg a. s./kg dry sediment (Fig. 3A) or 4.7 µg a. s./L (Fig. 3B).

It can be argued that not all 28d-EC_{10} values presented in Table 2 have a sufficiently high quality. For example, the 28d-EC_{10} values calculated for *A. aquaticus* and *C. horaria* are surrounded by a large uncertainty as indicated by their wide 95% confidence bands. Excluding these toxicity data implies that for only 6 benthic taxa, representing six different taxonomic orders/families, valid 28d-EC_{10} values are available. According to EFSA (2013, 2015) this number of toxicity data is too low to derive a RAC by means of the SSD approach. As suggested by EFSA (2015), a Tier-2 Weight-of-Evidence (WoE) approach might be applied when, besides toxicity data for the standard test species, toxicity data are made available for additional species, but the data set is incomplete for the SSD approach. In the proposed WoE approach, the toxicity value for the most sensitive species is selected but the Tier-1 AF is reduced, dependent on the number and quality of the toxicity data made available. EFSA (2015) proposed that in sediment ERA based on chronic toxicity data the AF can maximally be reduced from 10 to 4. A decision scheme how to select a correct AF in the WoE approach, however, is not provided. For sediment effect assessment based on sediment-spiked chronic toxicity data we propose the decision scheme presented in Table 4 to derive the Tier-2 WoE AF. Following this proposal, and with six valid 28d-EC_{10} values available for six different taxonomic orders of benthic invertebrates, the appropriate assessment factor is 5. Excluding the less valid toxicity values for *A. aquaticus* and *C. horaria*, the lowest 28d-EC_{10} value in Table 2 is that of the clam *C. fluminalis*, both for total sediment concentration (7.49 mg a. s./kg dry sediment) and pore water concentration (118 µg/L). The resulting alternative Tier-2 RAC_{sed} WoE value becomes then 1.50 mg a. s./kg dry sediment (Fig. 3A) or 23.6 µg a. s./L (Fig. 3B).

According to EFSA (2013, 2015) a Tier-3 RAC_{sed} can be derived from an appropriate micro-/mesocosm experiment by selecting the sediment concentration (of an appropriate time-window, e.g. the initial 28 days) of the highest treatment-level that did not result in significant population-level effects on benthic organisms and the application of an AF of 2. The NOEC (Effect class 1) of the most sensitive benthic population (the oligochaete *Dero digitata*) in the sediment-spiked outdoor microcosm experiment reported by Yin et al. (2018) was 14.20 mg a. s./kg dry sediment or 88.8 µg a. s./L in sediment pore water. Applying the AF of 2 the possible Tier-3 RAC_{sed} values become 7.10 mg a. s./kg dry sediment (Fig. 3A) or 44.4 µg a. s./L in sediment pore water (Fig. 3B).

Comparing the different effect assessment tiers for sediment organisms and fludioxonil, it is concluded that the modified equilibrium partitioning approach (Tier-0 RAC_{sed}) and the effect assessments based on sediment-spiked laboratory toxicity tests (Tier-1 and Tier-2 RAC_{sed}) provide sufficient protection when compared with the results of the sediment-spiked outdoor microcosm experiment (Tier-3 RAC_{sed}), irrespective whether these RACs are expressed in terms of total sediment or pore water concentrations (Fig. 3 A&B).

4.4. Concluding remarks

4.4.1. Consistency of the tiered approach

A tiered effect assessment approach is consistent if lower tiers are more conservative than higher tiers. Differences between the RAC_{sed} values derived in Tier-1 and Tier-2, however, appear to be minor (WoE approach) and even inconsistent (SSD approach). The fact that the SSD approach resulted in a lower Tier-2 RAC_{sed} than the Tier-1 RAC_{sed}, may be explained by the observation that the median 28d-EC_{10} values of *A. aquaticus* and *C. dipterum* had a position in the tail of the SSD curve, while these EC_{10} values were surrounded by a high uncertainty, as reflected by wide 95% confidence bands.

4.4.2. Laboratory reared and field-collected test species

In our study, the benthic standard test species reared in the laboratory, viz., *L. variegatus, C. riparius*, *H. azteca*, all showed 28d-EC_x values characterised by a small spread in 95% confidence band in contrast to the benthic test species we collected in the field (including *A. aquaticus* and *C. horaria*). For biocidal fungicides and the SSD approach it is recommended to test 6 different taxonomic orders/families, but laboratory cultures for freshwater benthic species with a more complex life cycle (e.g. univoltine species such as *C. horaria*) are difficult to maintain. To include these taxa in laboratory testing, their collection in the field often is the only option, despite the limited experience in the conduct of sediment-spiked laboratory toxicity test with these field-collected benthic taxa. This experience needs to be expanded and published to facilitate future studies.

4.4.3. Total sediment versus pore water concentration

It is suggested by EFSA (2015) to use both total sediment concentration and pore water concentration as a metric in the sediment risk

Table 4
Proposed Assessment Factor (AF) to be applied to the lowest valid chronic toxicity estimate in a Weight-of-Evidence approach for sediment invertebrates and biocidal fungicides, if a sufficient number of valid toxicity data for the SSD approach is not made available.

AF	Sediment spiked toxicity data
10	2 valid chronic toxicity data for standard benthic test species (*Lumbriculus variegatus and Chironomus* sp.)
9	3 valid chronic toxicity data for 3 different taxonomic orders/families (including *L. variegatus and Chironomus* sp.)
8	4 valid chronic toxicity data for 4 different taxonomic orders/families (including *L. variegatus and Chironomus* sp.)
7	5 valid chronic toxicity data for 5 different taxonomic orders/families (including *L. variegatus and Chironomus* sp.)
6	6 valid chronic toxicity data for 5 different taxonomic orders/families (including *L. variegatus and Chironomus* sp.)
5	6 valid chronic toxicity data for 6 different taxonomic orders/families (including *L. variegatus and Chironomus* sp.)
4	7 valid chronic toxicity data for 6 different taxonomic orders/families (including *L. variegatus and Chironomus* sp.)

assessment for pesticides. Although, it is also recommended in OECD guidelines to measure both the total concentration and pore water concentrations in sediment toxicity tests, it appears that published toxicity values for benthic invertebrates and pesticides are predominantly expressed as total sediment concentrations and not in pore water concentrations (see e.g. the review of Deneer et al., 2013). Expressing the RAC_{sed} in terms of total sediment concentration may be more important if the sediment-dwelling organisms consume sediment particles and oral exposure contributes to toxicity. For other benthic organisms, like nematodes (see e.g. Höss et al., 2019) and rooted aquatic macrophytes, exposure via pore water seems more important. Most likely the relative contribution of oral and dermal exposure in the toxicity of sediment-dwelling organisms is species and substance specific.

Our experiments allow comparison of the RAC_{sed} values derived from different effect assessment tiers in terms of total sediment concentration and sediment pore water concentration. The differences in RAC_{sed} estimates between tiers, overall show the same trend, irrespective of the exposure matrix selected (Fig. 3). So strictly speaking, in case of the fungicide fludioxonil and macroinvertebrates, a sediment ERA based on either total sediment concentration or pore water concentration as metric for the PEC and RAC estimate would suffice. For sediment-dwelling nematodes, however, Höss et al. (2019) concluded that pore water concentrations are a better predictor of fludioxonil toxicity than total concentrations in the sediment.

4.4.4. Tier-2 SSD versus WoE approach

To apply the SSD approach in ERA for pesticides and invertebrates, valid toxicity data for at least 8 taxa are required (EFSA, 2013; 2015). As discussed above, the position of the 28d-EC_{10} values of *A. aquaticus* and *C. horaria* in the tail of the SSD curve (see Fig. 2) may be considered a problem for the interpretation of the HC_5 value derived, since the 28d-EC_{10} estimates for these species are characterised by a wide 95% confidence band.

An alternative approach to derive a Tier-2 RAC_{sed} on basis of the SSD approach could be to select the HC_{50} from the SSD curve constructed with 28d-EC_{10} values and the application of an AF of 10. Note that the spread in 95% confidence band generally is smaller for HC_{50} estimates than for HC_5's (see e.g. Table 3). HC_{50} estimates resemble the geometric mean 28d-EC_{10} of all species tested, while the AF of 10 is the extrapolation factor normally used in Tier-1 chronic risk assessment for pesticides. This approach, and using the HC_{50} values presented for option B in Table 3, would result in an alternative Tier-2 RAC_{sed} estimate of 1.3 mg a. s./kg dry sediment or 14.0 µg/L pore water. These alternative Tier-2 RAC_{sed} values based on HC_{50} estimates have an intermediate position compared to the Tier-2 RAC_{sed} values based on HC_5 estimates and the Tier-2 WoE approach presented in Fig. 3.

Although median HC_{50} estimates in general are surrounded with a lower uncertainty than median HC_5 estimates, the problem remains that the SSD may be constructed with toxicity data of variable quality. This problem may be avoided in selecting toxicity data of sufficient quality only (e.g. 28d-EC_{10} values that have 95% confidence bands with an acceptable spread) and applying the Tier-2 WoE approach described in Table 4. A disadvantage of this Tier-2 WoE approach, however, is that the information of the less reliable data is completely discarded. In case less reliable toxicity data are available (e.g. values characterised by wide confidence bands), it makes sense still to explore the Tier-2 RAC_{sed} assessment based on the SSD approach, selecting the HC_5 and HC_{50} estimates and AFs of respectively 3 of 10, and to use this information to motivate the choices made in the Tier-2 WoE approach.

4.4.5. Further sediment studies required

In our study we aimed to explore the protectiveness of different effect assessment tiers for sediment-dwelling organisms and the fungicide fludioxonil. In the scientific literature, comparable studies on sediment-spiked chronic laboratory toxicity tests with benthic species and pesticides that allowed the application of the SSD and WoE approach, hardly could be found. In the study of Brock et al. (2018) an attempt was undertaken for the lipophilic insecticide lufenuron, but this study focussed on 10d-L(E)C_x values derived from sediment-spiked toxicity tests with a set of benthic arthropods. Further studies with pesticides that differ in fate properties and toxic mode-of-action are necessary to validate the consistency of the tiered approach for sediment organisms.

CRediT authorship contribution statement

Theo C.M. Brock: Conceptualization, Funding acquisition, Methodology, Formal analysis, Project administration, Writing - original draft, Writing - review & editing. **João Romão:** Investigation. **Xiao Yin:** Investigation. **Rima Osman:** Investigation. **Ivo Roessink:** Data curation, Methodology, Resources, Supervision.

Declaration of competing interest

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.

Acknowledgements

This research is a follow-up of TKI project 5200043069, a project co-funded by both the Dutch government and the European Crop Protection Association. The follow-up research presented in this paper is mainly financed by the Dutch Ministry of Agriculture, Nature and Food Quality (project BO-20-0002-001). The authors are indebted to Laura Buijse and Steven Crum for their help in analysing fludioxonil in sediment samples. Jasper van Smeden, Marie-Claire Boerwinkel, Dick Belgers, Maria Oursouzidou and Ioannis Anestis supported the conduct of and biological observation in the laboratory toxicity tests. Mick Hamer critically read a previous version of the paper and made valuable suggestions for improvement. Two anonymous reviewers provided useful comments to improve a previous version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.110504.

Compliance with ethical standards

- The authors declare that they have no conflict of interest
- This article does not contain any studies with human participants or vertebrate animals
- Informed consent was obtained from all individual participants included in the study

References

Aldenberg, T., Jaworska, J.S., 2000. Uncertainty of the hazardous concentration and fraction affected for normal species sensitivity distributions. Ecotoxicol. Environ. Saf. 49, 210–212.

Boyle, R.L., Hoak, M.N., Pettigrove, V.J., Hoffmann, A.A., Long, S.M., 2016. Comparing the impacts of sediment-bound bifenthrin on aquatic macroinvertebrates in laboratory bioassays and field microcosms. Ecotoxicol. Environ. Saf. 133, 489–500.

Brock, T.C.M., Bas, D.A., Belgers, J.D.M., Bibbe, L., Boerwinkel, M.-C., Crum, S.J.H., Diepens, N.J., Kraak, M.H.S., Vonk, J.A., Roessink, I., 2016. Effects of sediment-spiked lufenuron on benthic macroinvertebrates in outdoor microcosms and single-species toxicity tests. Aquat. Toxicol. 177, 464–475.

Brock, T.C.M., Belgers, J.D.M., Boerwinkel, M.-C., Jollie, L., Kraak, M.H.S., Pabo, M.J., Vonk, J.A., Roessink, I., 2018. Toxicity of sediment-bound lufenuron to benthic arthropods in laboratory bioassays. Aquat. Toxicol. 198, 118–128.

Charles, C., Veber, P., Delignette-Muller, M.L., 2018. MOSAIC: a web-interface for statistical analyses in ecotoxicology. Environ. Sci. Pollut. Control Ser. 25, 11295–11302.

Delignette-Muller, M.L., Ruiz, P., Charles, S., Duchemin, W., Lopes, C., Kon-Kam-King, G., Veber, P., 2016. Morse: modelling tools for reproduction and survival data in

ecotoxicology. https://CRAN.R-project.org/package=morse. R package version 2.2.0.
Deneer, J.W., Arts, G.H.P., Brock, T.C.M., 2013. Sediment Toxicity Data for Benthic Organisms and Plant Protection Products. A Literature Review, vol. 2485. Alterra report, Wageningen, The Netherlands, pp. 47.
Diepens, N.J., Arts, G.H.P., Brock, T.C.M., Smidt, H., Van den Brink, P.J., Van den Heuvel-Greve, M.J., Koelmans, A.A., 2014. Sediment toxicity testing of organic chemicals in the context of prospective risk assessment: a review. Crit. Rev. Environ. Sci. Technol. 44, 255–302.
Diepens, N.J., Koelmans, A.A., Baveco, H., Van den Brink, P.J., Van den Heuvel-Greve, M.J., Brock, T.C.M., 2017. Prospective environmental risk assessment for sediment-bound organic chemicals: a proposal for tiered effect assessment. Rev. Environ. Contam. Toxicol. 239, 1–77.
Di Toro, D.M., Zarba, C.S., Hansen, D.J., Berry, W.J., Swarts, R.C., Cowan, C.E., Pavlou, S.P., Allen, H.E., Thomas, N.A., Che Paquin, P.R., 1991. Technical basis for the equilibrium partitioning method for establishing sediment quality criteria. Environ. Toxicol. Chem. 11, 1541–1583.
EFSA (European Food Safety Authority), 2007. Conclusion regarding the peer review of the pesticide risk assessment of the active substance fludioxonil. EFSA Scientific Report 110, 1–85.
EFSA (European Food Safety Authority), 2013. Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters. EFSA Journal 11 (7), 3290.
EFSA (European Food Safety Authority), 2015. Scientific opinion on the effect assessment for pesticides on sediment organisms in edge-of-field surface water. EFSA Journal 13 (7), 4176.
Fleming, R.J., Holmes, D., Nixon, S.J., 1998. Toxicity of permethrin to *Chironomus riparius* in artificial and natural sediment. Environ. Toxicol. Chem. 17, 1332–1337.
Goedkoop, W., Widenfalk, A., Haglund, A.-L., Steger, K., Bertilsson, S., 2005. Microbial characterization of artificial sediment and comparisons with natural sediments – implications for toxicity testing. Environ. Toxicol. Chem. 24, 2725–2733.
Höss, S., Roessink, I., Brock, T.C.M., Traunspurger, W., 2019. Response of the nematode community to the fungicide fludioxonil in sediments of outdoor freshwater microcosms – comparison with a single species toxicity test with *Caenorhabditis elegans*. Sci. Total Environ. 710 (2020), 135627.
Huff Hartz, K.E., Nutile, S.A., Fung, C.Y., Sinche, F.L., Moran, P.W., Van Metre, P.C., Nowell, L.H., Lydy, M.J., 2019. Survey of bioacessible pyrethroid insecticides and sediment toxicity in urban streams of the northeast Unites States. Environ. Pollut. 254, 112931. https://doi.org/10.1016/j.envpol.2019.07.099.
Hunt, L., Bonetto, C., Resh, V.H., Forsin Buss, D., Fanelli, S., Marrochi, N., Lydy, M.J., 2016. Insecticide concentrations in stream sediments of soy production regions of South America. Sci. Total Environ. 547, 114–124.
Maltby, L., Brock, T.C.M., Van den Brink, P.J., 2009. Fungicide risk assessment for aquatic ecosystems: importance of interspecific variation, toxic mode of action, and exposure regime. Environ. Sci. Technol. 43, 7556–7563.
Marinković, M., Verweij, R.A., Nummerdor, G.A., Jonker, M.J., Kraak, M.H.S., Admiraal, W., 2011. Life cycle responses of the midge *Chironomus riparius* to compounds with different modes of action. Environ. Sci. Technol. 45, 1645–1651.
Moermond, C.T.A., Kase, R., Korkaric, M., Ågerstrand, M., 2016. CRED: criteria for reporting and evaluating ecotoxicity data. Environ. Toxicol. Chem. 35, 1297–1309.
Moran, P.W., Nowell, L.H., Kemble, N.E., Mahler, B.J., Waute, I.R., Van Metre, P.C., 2017. Influence of sediment toxicity on macroinvertebrate communities across 99 wadable streams of the Midwestern USA. Sci. Total Environ. 599–600, 1469–1478.
OECD, 2004. Guidelines for Testing of Chemicals – 218: Sediment-Water Chironomid Toxicity Test Using Spiked Sediment. OECD Publishing, Paris, France.
OECD, 2007. Guidelines for the Testing of Chemicals – 225: Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment. OECD Publishing, Paris, France.
OECD, 2010. Guidelines for the Testing of Chemicals – 233: Sedimen-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment. OECD Publishing, Paris, France.
Rico, A., Brock, T.C.M., Daam, M.A., 2019. Is the effect assessment approach for fungicides as laid down in the EFSA Aquatic Guidance Document sufficiently protective for freshwater ecosystems? Environ. Toxicol. Chem. 38, 2279–2293.
Rogers, H.A., Schmidt, T.S., Dabney, B.L., Hladik, M.L., Mahler, B.J., Van Metre, P.C., 2016. Bifenthrin causes trophic cascade and altered insect emergence in mesocosms: implications for small streams. Environ. Sci. Technol. 50, 11974–11983.
Schäfer, R.B., Pettigrove, V., Rose, G., Allinson, G., Wightwick, A., Von der Ohe, P.C., Shimeta, J., Kühne, R., Kefford, B.J., 2011. Effects of pesticides monitored with three sampling methods in 24 sites on macroinvertebrates and microorganisms. Environ. Sci. Technol. 45, 1665–1672.
USEPA, 2000. Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates, second ed. pp. 192 E.600/R-99/064.
Wall, D.H., 2004. Sustaining Biodiversity and Ecosystem Services in Soils and Sediments. Island Press, Washington, DC,USA. https://doi.org/10.1017/S0016756804009185.
Warren, N., Alan, I.J., Carter, J.E., House, W.A., Parker, A., 2003. Pesticides and other micro-organic contaminants in freshwater sedimentary environments – a review. Appl. Geochem. 18, 159–194.
Yin, X.H., Brock, T.C.M., Barone, L.E., Belgers, J.D.M., Boerwinkel, M.-C., Buijse, L., Van Wijngaarden, P.P.A., Hamer, M., Roessink, I., 2018. Exposure and effects of sediment-spiked fludioxonil on macroinvertebrates and zooplankton in outdoor aquatic microcosms. Sci. Total Environ. 610–611, 1222–1238.
Zubrod, J.P., Bundschuh, M., Arts, G., Brühl, C.A., Imfeld, G., Knäbel, A., Payraudeay, S., Rasmussen, J.J., Rohr, J., Scharmüller, A., Smalling, K., Stehle, S., Schulz, R., Schäfer, R., 2019. Fungicides – an overlooked pesticide class? Environ. Sci. Technol. 53, 3347–3365.