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Article details

Barmentlo S.H., Schrama M., Hunting E.R., Heutink R., Bodegom P.M. van, Snoo G.R. de & Vijver M.G. (2018), Assessing combined impacts of agrochemicals: Aquatic macroinvertebrate population responses in outdoor mesocosms, Science of the Total Environment 631-632: 341-347.
Doi: 10.1016/j.scitotenv.2018.03.021

Science of the Total Environment 631–632 (2018) 341–347

Contents lists available at ScienceDirect

Science of the Total Environment

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

Assessing combined impacts of agrochemicals: Aquatic macroinvertebrate population responses in outdoor mesocosms

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HIGHLIGHTS

- Combined effects of agrochemicals were assessed in outdoor mesocosms inoculated with aquatic invertebrate assemblages.
- Environmentally realistic concentrations of binary mixtures showed additive species' responses.
- Tertiary mixtures affected species' responses indescribable from cumulative responses of the single exposures treatments.
- This indicates that in agricultural ditches, non-additive induced shifts in aquatic invertebrate assemblages might occur.

GRAPHICAL ABSTRACT

ARTICLE INFO

Article history:
Received 19 December 2017
Received in revised form 29 January 2018
Accepted 2 March 2018
Available online xxxx

Editor: D. Barcelo

Keywords:
Agrochemicals
Mixtures
Outdoor testing
Aquatic invertebrates
Mesocosm

ABSTRACT

Agricultural ditches host a diverse community of species. These species often are unwarrantedly exposed to fertilizers and a wide-array of pesticides (hereafter: agrochemicals). Standardized ecotoxicological research provides valuable information to predict whether these pesticides possibly pose a threat to the organisms living within these ditches, in particular macro-invertebrates. However, knowledge on how mixtures of these agrochemicals affect macro-invertebrates under realistic abiotic conditions and with population and community complexity is mostly lacking. Therefore we examined here, using a full factorial design, the population responses of macroinvertebrate species assemblages exposed to environmentally relevant concentrations of three commonly used agrochemicals (for 35 days) in an outdoor experiment. The agrochemicals selected were an insecticide (imidacloprid), herbicide (terbuthylazine) and nutrients (NPK), all having a widespread usage and often detected together in watersheds. Effects on species abundance and body length caused by binary mixture combinations could be described from single substance exposure. However, when agrochemicals were applied as tertiary mixtures, as they are commonly found in agricultural waters, species' abundance often deviated from expectations made based on the three single treatments. This indicates that pesticide-mixture induced toxicity to population relevant endpoints are difficult to extrapolate to field conditions. As in agricultural ditches often a multitude (approx. up to 7) of agrochemicals residues are detected, we call other scientist to verify the ecological complexity of non-additive induced shifts in natural aquatic invertebrate populations and aquatic species assemblages.

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https://doi.org/10.1016/j.scitotenv.2018.03.021
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1. Introduction

Agricultural ditches host many different organisms, and their water quality is important for the watersheds as well as the fringing terrestrial fields. Agricultural ditches collect a wide variety of pesticides from adjacent fields due to run off, direct drift and leaching (Nollet and Rathore, 2010) and they generally are the primary receivers of agrochemicals (Vijver et al., 2017). From the monitoring data that takes place in these water bodies (eg. Snoo et al., 2006), it is learned that in surface waters pesticides often co-occur in mixtures (Gilliom et al., 2006) with high collinearity (Vijver and van den Brink, 2014). It is well known that these pesticides can differentially impact various species within the aquatic community. Consequently, it is likely that pesticide exposure adversely impacts biodiversity and ecosystem processes such as primary production (eg. Relyea et al., 2005) and decomposition (eg. Schäfer et al., 2007; Hunting et al., 2016). Thus, obtaining reliable predictions on how these pesticides and their mixtures affect the environment and the organisms living therein is key for maintaining healthy ecosystems.

Following standardized protocols, the impact of single compounds and mixtures are mostly tested in the laboratory with easy-to-culture organisms (e.g. OECD, 2004; Barmentlo et al., 2015; Gessner and Tlili, 2016). These laboratory tests provide valuable information on whether chemicals impose a threat to organisms and are generally indicative for the toxicity of substances. However, within these laboratory approaches, abiotic (fluctuating water chemistry parameters such as changes induced by rain events and wind) and biotic (population and community interactions such as competition and predation) factors are often simplified or even overlooked. It is, however, well-known that abiotic factors such as pH, temperature and other chemistry parameters alter toxicity and bioavailability of chemicals (see examples in Holmstrup et al., 2010; Bundschuh et al., 2016; Barmentlo et al., 2017). Biotic conditions affecting ecological responses can also impact toxicity, for example through increasing predation pressure (Schulz and Dabrowski, 2001) or by modulating inter- and intraspecific competition (Liess, 2002; Kattwinkel and Liess, 2014). The variation in these abiotic and biotic variables is thus likely to alter toxicity under natural conditions compared to the standardized protocols. Exclusion of these variables may lead to uncertainties in the extrapolation of responses to field situations (Heugens et al., 2001; Clements et al., 2012; Halstead et al., 2014). These uncertainties are possibly even higher for mixtures of agrochemicals as combined effects may complicate the overall response (Côté et al., 2016; Gessner and Tlili, 2016).

To test for these uncertainties, this study aims to assess quantitatively the combined effects of multiple agrochemicals from single exposure under realistic conditions to individual macroinvertebrate species. We investigated the effects of single exposure as well as binary and tertiary mixtures of a commonly used insecticide, herbicide and nutrients to different endpoints of 9 functionally distinct aquatic macroinvertebrates species. In order to test these species under more (a)biotic context, we investigated them in assemblages for 35 days in an outdoor mesocosm experiment.

2. Material and methods

2.1. Species selection

The species assembly chosen (Table 1) consisted of aquatic macrofauna species that are often found in European aquatic ecosystems, particularly in semi-stagnant water bodies such as ditches (Verdonschot et al., 2011; Ieromina et al., 2015; see Appendix Table A1 for additional information on the species). The different test species and their abundancies (Table 1) reflected broadly the feeding mode trait distribution (eg. predator, grazer etc.; retrieved from www.freshwaterecology.info; Schmidt-Kloiber and Hering, 2012) as found in dune ditch systems in order to mimic a natural ditch food web (Ieromina et al., 2015).

Daphnia magna Straus were obtained from laboratory cultures of Leiden University (Leiden, The Netherlands). *Lymnaea stagnalis* Linnaeus were obtained from cultures from the Vrije Universiteit Amsterdam (Amsterdam, The Netherlands). Algae, fungi and microbial communities were collected from ditch water by filtering water over a 150 μm mesh. The sediment-dwelling species *Chironomus riparius* Meigen and *Tubifex* sp. Lamarck were purchased from VitalFish (Boskoop, The Netherlands). All other species were collected in March 2016 from water columns or sediments of ditches located in peaty nature reserves by sweeping nets. Organisms were kept at 4 °C for one day to acclimate prior experimental usage.

2.2. Experimental setup

In March – April 2016, a mesocosm experiment of 48 mesocosms was conducted in the botanical garden of Leiden (Leiden University, The Netherlands). In this setting, several abiotic variables were expected and observed to (co-)vary, including average air temperature (gradually increased from 5 °C to 15 °C), solar irradiance (481–2234 J/cm^2 per day), rain fall (0–10.1 mm/day), wind velocity (2.2–11.7 m/s), air-pressure (997–1030 hPa). Information on water quality parameters is provided in Section 3.3. Mesocosms consisted out of 65 L poly-ethylene tubs closed by 50% shadow cloth nets to prevent migration of the animals. A sediment layer of 8 cm depth was added to each mesocosm. The sediment was prepared from fine-grained, ignited quartz sand as mineral substrate (12.5 kg, grain size: 0.1–0.5 mm), ground dry hay (0.5 kg) which was pre-soaked and then mixed. The water column was prepared by 36 L of copper-free tap water and 4 L of filtered (planktonic net, mesh size 150 μm) ditch water in order to inoculate the mesocosms with natural micro communities (algae/bacteria/fungi).

The micro community was allowed to equilibrate for seven days prior to non-predacious macrofauna species (Table 1) were added. All animals were slowly cooled (1 °C/h, using an incubator) to the water temperature of the mesocosms while mixing in water from the mesocosm to avoid a temperature or medium shock. One day later, the top-predator *Notonecta glauca* Linnaeus was added. We observed all mesocosms to contain an additional copepod species *Cyclops* sp. at the end of the experiment. The nauplius larvae of *Cyclops* sp. Müller are 150–200 μm in size and therefore likely passed the sieve (mesh size 150 μm) when ditch water had been added to the mesocosms during microbial inoculation. *Cyclops* sp. is not expected to disrupt the simplified food web as it is common in most aquatic habitats that are susceptible to agricultural run-off (Kulkarni et al., 2013). To provide oxygen and to homogenize the water columns, mesocosms were gently aerated with air pumps throughout the duration of the experiment.

A full factorial design ($n = 6$) of imidacloprid (two levels; present and absent), terbuthylazine (two levels; present and absent) and nutrients (two levels: oligotrophic and eutrophic) was applied in a randomized fashion, resulting in eight different treatments (see below for all concentrations). The treatment in which pesticides were absent and nutrients were maintained at oligotrophic levels served as control treatment. In nutrient enriched mesocosms, we added 6.16 mL of liquid plant fertilizer (232 mg N: 133 mg P: 232 mg K – 7:4:7 combined with micro-elements) in order to approach nutrient concentrations that have been shown to stimulate fresh water algal growth (Ieromina et al., 2014). Imidacloprid and terbuthylazine were selected in this experiment as being representative for a large group, namely the neonicotinoids (neurotoxins) and triazines (photosynthetic inhibitors). Both pesticides commonly exceed the current water quality criteria for surface water concentrations in many European (Leiden University and Rijkswaterstaat-WVL, 2016; Vijver et al., 2017) and United States waters (USGS National Water-Quality Assessment (NAWQA) Program, 2017). The insecticide imidacloprid (99.7% purity, CAS Number: 138261-41-3) and herbicide terbuthylazine (99.4% purity, CAS Number: 5915-41-3) were purchased from Sigma-Aldrich (Zwijndrecht, The

Table 1
Known toxicity values for the insecticide (imidacloprid) and herbicide (terbuthylazine) per test species. Nutrients are not expected to have any direct effects on the test species and are therefore not included in this table. The most sensitive endpoint and corresponding toxicity values are shown for the different test species as obtained from literature.

		Lowest toxicity value (μg/L)	
Species	Number added to each mesocosm	Imidacloprid	Terbuthylazine
Asellus aquaticus	25	28d LC_{10} = 1.35[1]	NA
Chironomus riparius	50	4d NOEC (growth) = 0.74[2]	2d NOEC (swimming) = 200[3]
Cloeon dipterum	15	28d LC_{10} = 0.041[1]	NA
Culex pipiens	12	NA	NA
Daphnia magna	78	21d NOEC = 2000[4]	2d EC_{50} (immobility) = 21,200[5]
Lymnaea stagnalis	5	NA	NA
Notonecta glauca	2	96 h EC_{50} (mobility) = 18.2[1]	NA
Sigara striata	12	NA	NA
Tubifex sp.	220	1d LC_{50} = 320[6]	NA

EC = effect concentration, LC = lethal concentration, NOEC = no effect concentration.
[1] Roessink et al., 2013 (note: toxicity data shown is for *Notonecta* spp).
[2] Azevedo-pereira et al., 2011.
[3] Pérez et al., 2013.
[4] Ieromina et al., 2014.
[5] EPA: Office of Pesticides Program (OPP), 1992.
[6] Gerhardt, 2009.

Netherlands). Nominal concentrations of imidacloprid and terbuthylazine were 0 (no addition), 4 μg/L and 0 (no addition) and 3.5 μg/L respectively. These experimental concentrations were based on concentrations found in Dutch surface waters in the year 2014 (Pesticide Atlas, 2016; Snoo et al., 2006) in order to conduct the current experiment under environmentally relevant concentrations. Both imidacloprid and terbuthylazine were dissolved in demineralized water in glass bottles. All substances were distributed equally over the water surface level of each mesocosm to stimulate homogenization. Substances were added at the start ($t = 0$, t in days) of the experiment and half-way through ($t = 18$) in order to maintain experimental concentrations. Excess water due to rainfall was carefully (not extracting any animals) removed one day prior ($t = 17$) to the second treatment application. After 35 days, the experiment was terminated.

2.3. Abiotic endpoints

Water quality in the mesocosms was determined twice a week by recording pH, dissolved oxygen (DO), temperature and conductivity using a portable hq 40d electronic multi-parameter meter (Hach Ltd., Colorado, US). Imidacloprid, terbuthylazine and nutrient concentrations were sampled weekly with additional sampling points at $t = 18$ (2 h after the second treatment application, as described in 3.2) and $t = 21$. Samples were collected by sampling the water in the middle of a mesocosm 5 cm below the surface. Terbuthylazine and imidacloprid concentrations were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS; Agilent Technologies; see Roessink et al., 2013 for the detailed procedure). Dissolved inorganic nitrogen (DIN: extractable soil ammonium (NH_4^+) and nitrate/nitrite (NO_3^-/NO_2)) and PO_4^{2-} were determined colorimetrically using an auto-analyzer (Seal AA3; SEAL Analytical, UK).

2.4. Biotic endpoints

2.4.1. Biomass and numbers determination

In order to determine algae densities, water samples (15 mL) were collected from each mesocosm 1 h prior, one day after treatment addition and at the end of the experiment. Of each sample, 200 μL was pipetted on a Bürker-Türk (0.25 μm raster), spread out and algal cells were counted subsequently.

Periphyton samples were collected at the end of the experiment ($t = 35$) by scraping a 10*20 cm plot from the north side of the mesocosm. These samples were then dried for 48 h at 60 °C and weighed on a BP210S balance (Sartorius AG) in order to quantify the biomass.

After 35 days, animals were extracted from the mesocosms by filtering the water and sediment over a 2 mm filter to extract the larger animals. These animals were collected by hand and stored at 4 °C. After this step, water was filtered again over a fine filter (106 μm) in order to extract the zooplankton species *D. magna* and *Cyclops* sp. from the water column. Animals were then transferred to the lab and counted in order to determine the effect of the agrochemicals on the abundance per species.

2.4.2. Body length determination

After counting, ten individuals of each species per mesocosm were randomly selected and photographed with an eScope DP-M17 USB-microscope camera to determine the effect of the agrochemicals on body length. Only species were in an early life stage before the start experiment were analyzed on body length, being *Asellus aquaticus* Linnaeus, *Cloeon dipterum* Linnaeus, *D. magna* and L. *stagnalis*. *A. aquaticus* and L. *stagnalis* were photographed from a dorsal perspective. *A. aquaticus*' body length was measured from the tip of the head to end of the pleotelson. *L. stagnalis* shell length was determined by measuring from apex to aperture. *D. magna* and *C. dipterum* were photographed from a dorsal and lateral perspective. We selected egg-carrying *D. magna* to separate these from juveniles and ensure sufficient growth. We measured from these animals from rostral end to the attachment of the tail. Dorsal *C. dipterum* photographs were measured from rostral end to the attachment of the tail, whereas the lateral images were analyzed at the dorsal side from mouth to the caudal end of the abdomen. Body length was determined with ImageJ (version 1.48f; set scale of 20 mm).

2.5. Statistical analyses

Single and combined effects of the agrochemicals on the number of macroinvertebrates after 5 weeks of exposure were evaluated by analyzing the abundance of all species separately by means of a factorial ANOVA (type-II sum of squares). The factors tested were imidacloprid (2 levels; addition/no addition), terbuthylazine (2 levels; addition/no addition), nutrients (2 levels; addition/no addition) and all possible interactions of these factors. To further investigate possible interaction effects, we investigated the number of animals per species residing in the mesocosms per treatment via one-way ANOVA (*lm* function) followed by Tukey's post-hoc test. The agrochemicals were entered in the model as a factor (8 levels: control, imidacloprid, terbuthylazine, nutrients, imidacloprid + nutrients, terbuthylazine + nutrients, imidacloprid + terbuthylazine, imidacloprid + terbuthylazine + nutrients). Possible impacts of the treatments on body length of the animals

were analyzed via linear mixed-effect models (*lme* function), in a design similar to that for the number of animals. As multiple individuals had been measured per mesocosm, a random factor was included in all models: the body length per animal was nested within its corresponding mesocosm. Normality of the linear mixed-effect model (random) variables and residuals were evaluated through Quantile-Quantile plots (QQ-plots). Homogeneity of variances was evaluated for all variables with Levene's test. If model assumptions were violated, data was either log10 or square root transformed in order to improve model fit. The data, residuals and random variables of all models followed a normal distribution, except for L. *stagnalis* and *N. glauca* abundancies. Data transformation did not improve normality of the data. However, these species showed no response to agrochemical addition (all main effects tested with kruskall-wallis $p > 0.05$). Statistical analyses were performed with R (version 3.3.0; R Core Team, 2016).

3. Results

3.1. Control performance

At the end of the experiment, all species except for *Culex pipiens* Linnaeus were found in the replicated controls. *C. pipiens* had disappeared in all mesocosms due to intense predation by *N. glauca* (personal observation) and was therefore excluded from further analysis. We also did not observe any large increases in one taxon in the controls during the duration of test period (visual inspection). Abundance of L. *stagnalis* and *N. glauca* was 92% (SE 4.5%) and 90% (SE 9.1%) of the initial abundance, respectively. Abundance for the other species was lower; abundance was 16% (SE 5.4%) for *A. aquaticus*, 21.7% (SE 8.9%) for *Sigara striata* Linnaeus and 33.3% (SE 12.3%) for *C. dipterum*. The species *T. tubifex* and *C. riparius* were collected at the end of the experiment, but abundance was so low that it did not allow for any further statistical comparison. Reproduction was observed for *D. magna*, *Cyclops* sp. and *L. stagnalis*, showing that the individuals reached sexual adulthood within the experimental period. At the end of the experiment, the control treatment showed densities of periphyton to be 31 μg/cm^2 (SE 0.017 and TWA of free-floating algae to be 4.5 cells/cm^2 (SE 1.4) (See Appendix, Fig. A1).

3.2. Exposure conditions of the treatments

Mesocosms that had received imidacloprid applications had a time-weighted average (TWA; 35 days) concentration of 2.9 μg imidacloprid/L with average peak concentrations of 3.2 ± 0.2 μg/L ($t = 0$) and 4.2 ± 0.1 μg/L ($t = 18$) after first and second application, respectively (see Appendix, Fig. A2). Treatments that had received solely imidacloprid (TWA concentration: 2.8 μg/L) and mesocosms that had received imidacloprid application as part of the mixture showed experimental concentrations diverging <0.2 μg/L (TWA concentration: 3.0 μg/L). Mesocosms that had received terbuthylazine applications had a TWA concentration (35 days) of 2.0 μg/L with average peak concentrations of 2.2 ± 0.5 μg/L ($t = 0$) and 3.1 ± 0.2 μg/L ($t = 18$) after first and second application, respectively. There were no traces of either imidacloprid or terbuthylazine in mesocosms that did not receive applications of these compounds (TWA < detection limit for both compounds).

Mesocosms to which nutrients had been added showed a factor of 2.4 higher actual TWA total nitrogen concentrations (4.21 ± 0.13 mg/L) compared to mesocosms that had not received additional nutrients (1.76 ± 0.10 mg/L). Likewise, total phosphorus TWA concentrations was increased by a factor of 4.4 in mesocosms that had received additional nutrients (1.27 ± 0.06 mg/L) compared to mesocosms that had not received additional nutrients (0.29 ± 0.09 mg/L).

Periphyton dry weight was highest in treatments that received either terbuthylazine addition ($F_{1,40} = 4.47$, $p = 0.041$) or nutrient addition, although the latter could not be confirmed statistically ($F_{1,40} = 3.666$, $p = 0.063$) (see Appendix, Fig. A1). The TWA of free-floating algae densities increased significantly by nutrient addition ($F_{1,40} = 22.1$, $p < 0.001$) with a significant interaction between all agrochemicals (terbuthylazine, imidacloprid and nutrients; $F_{1,40} = 4.55$, $p = 0.039$) (see Appendix, Fig. A1).

There was no deviation in water chemistry parameters between mesocosms at the start of the experiment ($t = 0$, water temperature, pH, oxygen concentration and water conductivity; $p > 0.05$ for all comparisons). The average water temperature was not affected by any of the treatments but did increase significantly over time ($F_{1,40} = 89.5$, $p < 0.001$) (Appendix, Fig. A3A). Both pH and oxygen concentration were positively affected by an interaction between time and nutrients ($F_{1,16} = 22.0$, $p < 0.001$; $F_{1,16} = 9.36$, $p = 0.002$) (Appendix, Fig. A3B and A3C respectively). Water conductivity increased significantly by time, nutrient addition and herbicide addition ($F_{1,16} = 19.6$, $p < 0.001$; $F_{1,16} = 5.36$, $p = 0.022$; $F_{1,16} = 6.92$, $p = 0.009$) (Appendix, Fig. A3D).

3.3. Effects of the agrochemicals on species' abundance

Imidacloprid negatively affected the number of surviving *C. dipterum* ($F_{1,40} = 32.7$, $p = 0.004$). The number of surviving *C. dipterum* individuals was significantly reduced and almost non-existent in treatments with imidacloprid additions compared to the control (control – imidacloprid, $p = 0.07$/imidacloprid * nutrients, $p < 0.05$/imidacloprid * terbuthylazine, $p < 0.05$) except for the tertiary mixture, where no significant difference from the control was observed (Fig. 1C). Imidacloprid also significantly decreased the abundance of *S. striata* ($F_{1,40} = 4.23$, $p = 0.046$) (Table 2).

Nutrient additions increased the abundance of *D. magna* ($F_{1,40} = 19.3$, $p < 0.001$) with a positive interaction between nutrients and terbuthylazine ($F_{1,40} = 8.59$, $p = 0.003$). The number of *D. magna* was lowest in the single terbuthylazine treatment and the mixture of terbuthylazine and imidacloprid, although this number did not significantly deviate from the control ($p > 0.05$) (Fig. 1A). However, the *D. magna* abundance in these two treatments was significantly lower than the terbuthylazine and nutrient mixture treatment ($p < 0.05$). The tertiary mixture treatment (nutrient addition, imidacloprid, terbuthylazine) showed significantly more *D. magna* individuals than all other treatments ($p < 0.05$) (Fig. 1A).

Comparable to *D. magna*, nutrient additions also significantly increased the abundance of *Cyclops* sp. ($F_{1,40} = 8.95$, $p = 0.003$; Table 2). Equal to *D. magna*, the number of *Cyclops* sp. was lowest in the single terbuthylazine treatment and in the mixture of terbuthylazine and imidacloprid (Fig. 1B). The results on abundance in those treatments deviated significantly from the tertiary mixture treatment that showed the highest number of *Cyclops* sp. ($p < 0.05$).

The number of *A. aquaticus*, *N. glauca* and L. *stagnalis* was not significantly affected by either imidacloprid, terbuthylazine or nutrients additions.

3.4. Effects of the agrochemicals on species' body length

The body length of *A. aquaticus* was significantly reduced by imidacloprid addition ($F_{1,40} = 5.56$, $p = 0.018$) and its mixture with nutrient addition ($F_{1,40} = 3.84$, $p = 0.050$). The body length of *C. dipterum* and L. *stagnalis* was positively affected by nutrient additions ($F_{1,40} = 7.40$, $p < 0.001$ and $F_{1,40} = 4.32$, $p = 0.038$ respectively) while imidacloprid reduced the overall body size of *C. dipterum* ($F_{1,40} = 14.6$, $p < 0.001$). No significant effects between individual treatments compared to the control ($p > 0.05$) were observed for the four tested species (Fig. 2). However, the body length of *C. dipterum* was lowered in most treatments where imidacloprid had been added. Due to the low number of surviving animals, this could not be confirmed statistically. In the tertiary mixture (also containing imidacloprid), the body length of *C. dipterum* was significantly reduced when compared to nutrient addition and the binary mixture of nutrients and terbuthylazine

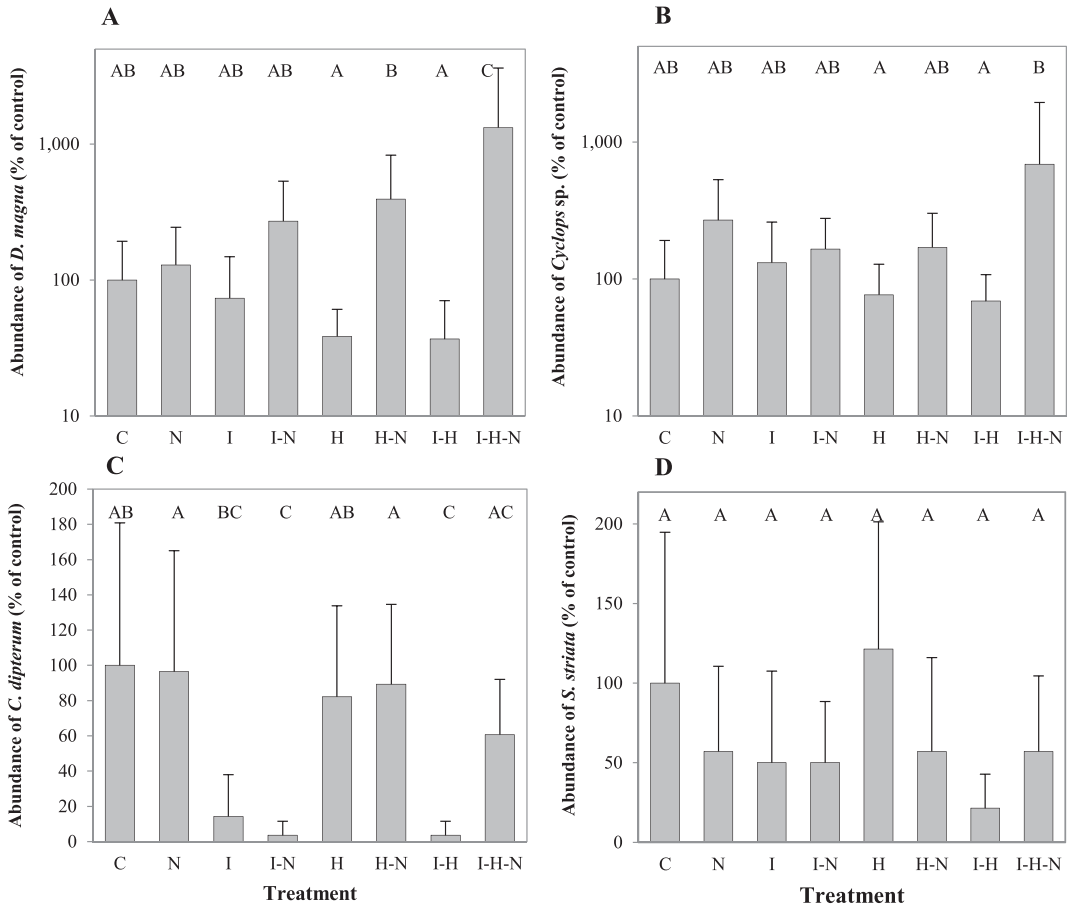

Fig. 1. Abundance of A) *D. magna* B) *Cyclops* sp. C) *C. dipterum* and D) *S. striata*. Average number of animals are shown as the percentage of the control (+SD). Different letters (A, B, C) indicate significant differences (One-way ANOVA + Tukey's post hoc) between treatments at significance level $p = 0.05$.

($p < 0.05$ for both comparisons). For none of the other species, significant differences between treatments were observed.

No significant effects from either imidacloprid, terbuthylazine nor nutrients additions were found on the body length of *D. magna*.

4. Discussion

This study aimed to investigate the combined effects of a commonly used insecticide, herbicide and nutrients on aquatic invertebrate species within an assemblage under realistic exposure conditions. Our results indicate clear effects of all three agrochemicals (nutrients, imidacloprid, and terbuthylazine) on different components of the invertebrate assemblage. The single effect of the agrochemicals mostly coincided with expectations of responses based on known ecotoxicological data. However, tertiary mixtures that are typically found in surface waters often showed effects on species' abundance that deviated from the single stressor effects, pointing at complicated shifts in species' populations and structures.

Table 2
The single and combined effects of the different agrochemicals on abundance and body length of the test species as assessed via factorial ANOVA. N: nutrients, I: insecticide = imidacloprid, H: herbicide = terbuthylazine, '-': indicates mixtures. Significant effects ($p \leq 0.05$) of the treatments are shown in bold.

Variable	Species	Treatment						
		H	I	N	H-I	H-N	I-N	H-I-N
Abundance	*A. aquaticus*	0.576	0.381	0.936	0.266	0.266	0.523	0.381
	C. dipterum	0.310	**<0.001**	0.132	0.197	0.065	0.427	0.096
	Cyclops sp.	0.867	0.503	**0.003**	0.303	0.165	0.588	0.097
	D. magna	0.467	0.283	**<0.001**	0.597	**0.003**	0.105	0.862
	L. stagnalis	0.220	0.757	0.356	0.757	0.356	0.220	0.220
	S. striata	1.000	**0.046**	0.335	0.578	0.853	0.068	0.459
	N. glauca	0.317	0.137	0.317	0.317	0.615	0.317	0.615
Body length	*A. aquaticus*	0.671	**0.018**	0.330	0.786	0.871	**0.050**	0.592
	C. dipterum	0.898	**<0.001**	**<0.001**	0.949	0.830	0.515	0.199
	D. magna	0.878	0.794	0.217	0.412	0.271	0.904	0.787
	L. stagnalis	0.839	0.151	**0.038**	0.383	0.859	0.413	0.164

4.1. Single stressor effects in the outdoor setting

Responses of the invertebrate species to the effects of single substances were largely in line with expectations. For example, nutrient additions stimulated zooplankton species' abundance, likely caused by the observed increase of free-floating algae densities through nutrient additions and a resulting increase in reproduction and/or survival of zooplankton species (e.g. Ieromina et al., 2014). Similarly, nutrients stimulated the growth of *C. dipterum* and L. *stagnalis* which would likely be a result of increased food source availability (here: periphyton) but this could not be confirmed statistically. Unexpectedly, the herbicide terbuthylazine as a single treatment did not induce effects on herbivorous species even though one might expect direct effects on primary producers and trophic levels that are directly dependent on primary production. Based on toxicity data of Sbrilli et al. (2005; 3 day NOEC for population growth of *Pseudokirchneriella subcapitata* = 2 μg/L), we hypothesized that terbuthylazine would inhibit algal population growth, subsequently limiting the resources available to filter feeders. We suggest that this lack of effects is due to absence of terbuthylazine induced toxicity or that algae cell numbers did not decline as more resilient algal species were dominating, which is a hypothesis that needs validation.

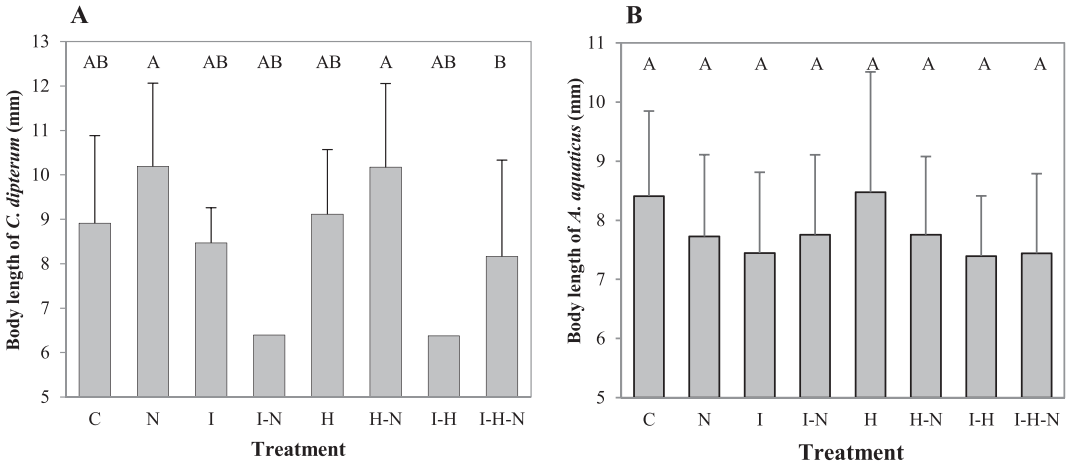

Fig. 2. Average body length (+SD) of A) *C. dipterum* and B) *A. aquaticus*. Different letters (A, B, C) indicate significant differences (One-way ANOVA + Tukey's post hoc) between treatments at significance level $p = 0.05$. Absent standard deviations are due to the limited surviving test animals within that treatment. C: control, N: nutrients, I: insecticide = imidacloprid, H: herbicide = terbuthylazine, '-': indicates mixtures.

In contrast, we found pronounced negative effects of imidacloprid on the abundance of both *C. dipterum* and *S. striata* and on the growth of *C. dipterum* and *A. aquaticus*. These effects on *C. dipterum* were expected as Van den Brink et al. (2016) showed that the 28 day EC_{10} (10% effect concentration) for mobility of *C. dipterum* was 0.041 μg/L (Table 1), which is a seventy-fold lower exposure concentration than the average experimental concentration in the present study. Toxicity data for *S. striata* were not available in literature, likely due to the fact that it is not a standard test species for ecotoxicological testing. Results of our study show that this species is potentially at risk at levels of imidacloprid concentrations currently occurring in surface waters (Pesticide Atlas, 2016; USGS NAWQA Program, 2017). Toxicity data for *A. aquaticus* was available, but abundancy was not reduced after exposure to imidacloprid. This was in contrast to data provided by Roessink et al. (2013; 28d LC_{10} = 1.35 μg/L). However, the reduced body size of *A. aquaticus* did show that this species was affected at the present imidacloprid concentration. In general, the single effects of the agrochemicals were conform expectations of responses based on known ecotoxicological data.

4.2. Combined effects strongly deviate from expectations of single effects

Overall, effects on the aquatic species exposed to mixtures were much more variable and counterintuitive. This was particularly illustrated by the response of *C. dipterum* to the various combinations of agrochemical mixtures. Exposure of the mayfly larvae to imidacloprid and binary mixtures of imidacloprid with either nutrients or terbuthylazine resulted in extreme low abundance (3.6% of control abundance for both mixtures). However, abundance of *C. dipterum* after exposure to the tertiary mixture (imidacloprid, terbuthylazine and nutrients) no longer deviated from the control treatment. This illustrates that the abundance was higher than expected based on the single and binary combination treatments. *C. dipterum* also exhibited reduced growth due to imidacloprid exposure which was nullified by the presence of both terbuthylazine and nutrients. As imidacloprid stunted and nutrients increased *C. dipterum* growth, the observed mixture effect was thus non-additive (see Fig. 1B in Côté et al., 2016) which was only expressed when mediated with terbuthylazine (which showed no single response). These results for both the abundance and growth of *C. dipterum* clearly illustrate that for more complex mixtures of agrochemicals, which are typically found in surface waters (Schreiner et al., 2016), laboratory results do not accurately predict ecotoxicity.

Unexpected effects of the mixtures were also obtained for both zooplankton species (*D. magna* and *Cyclops* sp.). While the abundance of both zooplankton species increased by nutrient applications as expected, pesticide addition did not lower their abundance. This result was most pronounced in the tertiary mixture treatment in which consistently the highest zooplankton densities were observed with a significant interaction between terbuthylazine and nutrients for *D. magna* and a marginal significant interaction of the tertiary mixture to *Cyclops* sp.. These results were particularly unexpected because previous studies have indicated that NOEC values of imidacloprid to *D. magna* are 667 fold higher (21d NOEC = 2000 μg/L; Ieromina et al., 2014; Table 1) than the experimental concentrations in the present study, thus suggesting no effect. We expected that herbicide application would indirectly negatively affect the reproductive output of primary consumer zooplankton species by altering the algal food quality (Halstead et al., 2014; Bessa da Silva et al., 2016). These predicted effects of herbicides and nutrients would classify as an antagonistic mechanism (Côté et al., 2016). However, the actual results showed that impacts were only expressed in mixtures (e.g. herbicides and nutrients to *D. magna*) and not when exposed to a single substance: either herbicides or nutrients. This illustrates that the effects of these agrochemicals in the natural environment can be much more complex than additive or antagonistic effects. We speculate that imidacloprid (through mortality of other species hence lower competition for food resources) and nutrient exposure (hence increased biomass of food sources) leads to increased resource availability for *D. magna*. It has long been established that *Daphnia* species' reproduction is altered when fed with different species of algae (Infante and Litt, 1985). The herbicide terbuthylazine, like most herbicides, differentially affects algae species (Hawxby et al., 1977), thus the herbicide can possibly be related to shifts in algal species composition declining the less energetically favorable algal species for *D. magna* while additional nutrient input increased the more favorable algal species.

Overall, our (sub)lethal, effects after long term exposure may indicate shifts in species' population sizes and their interactions (e.g. food web structure) due to agrochemicals thereby giving risks to cascading shifts in different ecosystem processes.

5. Conclusions

We investigated the combined effects of different combinations of three types of agrochemicals to 9 different aquatic macroinvertebrates species. Using environmentally realistic concentrations, our results showed that the ecotoxicological responses of binary mixtures for all species at population relevant endpoints were in line with those from single exposure data. Tertiary mixtures showed effects on species' abundance and body lengths that deviated from the cumulative single exposures. As in agricultural ditches often a multitude (approx. up to 7) of

agrochemicals residues are detected, we call other scientist to verify the ecological complexity of non-additive induced shifts in natural aquatic invertebrate populations.

Acknowledgements

Snails from the breeding facility of Vrije Universiteit Amsterdam (Amsterdam, The Netherlands) were kindly provided by Dr. J.M. Koene of the Department of Ecological Science. Determination of imidacloprid concentrations was performed by Laura Buijse from Wageningen Environmental Research. We like to thank Rody Blom, Arjen van Putten and Erik Gertenaar for practical assistance and colleagues of the conservation biology department of Leiden University for their help with collecting the data. S. Henrik Barmentlo, Ellard R. Hunting and Martina G. Vijver were funded by NWO-ASPASIA 015.009.009 and NWO-VIDI 864.13.010 granted to Martina G. Vijver.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.03.021.

References

Azevedo-pereira, H.M., Lemos, M.F., Soares, A.M., 2011. Behaviour and growth of *Chironomus riparius* Meigen (Diptera: Chironomidae) under imidacloprid pulse and constant exposure scenarios. Water Air Soil Pollut. 219:215–224. https://doi.org/10.1007/s11270-010-0700-x.

Barmentlo, S.H., Stel, J.M., Van Doorn, M., Eschauzier, C., De Voogt, P., Kraak, M.H.S., 2015. Acute and chronic toxicity of short chained perfluoroalkyl substances to *Daphnia magna*. Environ. Pollut. 198:47–53. https://doi.org/10.1016/j.envpol.2014.12.025.

Barmentlo, S.H., Van Gestel, C.A., Álvarez-Rogel, J., González-Alcaraz, M.N., 2017. Influence of climate change on the multi-generation toxicity to *Enchytraeus crypticus* of soils polluted by metal/metalloid mining wastes. Environ. Pollut. 222:101–108. https://doi.org/10.1016/j.envpol.2016.12.078.

Bessa da Silva, M., Abrantes, N., Rocha-santos, T.A.P., Duarte, A.C., Freitas, A.C., Gomes, A.M., Carvalho, A.P., Marques, J.C., Gonçalves, F., Pereira, R., 2016. Effects of dietary exposure to herbicide and of the nutritive quality of contaminated food on the reproductive output of *Daphnia magna*. Aquat. Toxicol. 179:1–7. https://doi.org/10.1016/j.aquatox.2016.08.008.

Bundschuh, M., Weyers, A., Ebeling, M., Elsaesser, D., Schulz, R., 2016. Narrow pH range of surface water bodies receiving pesticide input in Europe. Bull. Environ. Contam. Toxicol. 96:3–8. https://doi.org/10.1007/s00128-015-1665-7.

Clements, W.H., Hickey, C.W., Kidd, K.a., 2012. How do aquatic communities respond to contaminants? It depends on the ecological context. Environ. Toxicol. Chem. 31: 1932–1940. https://doi.org/10.1002/etc.1937.

Côté, I.M., Darling, E.S., Brown, C.J., 2016. Interactions among ecosystem stressors and their importance in conservation. Proc. R. Soc. B Biol. Sci. 283, 20152592. https://doi.org/10.1098/rspb.2015.2592.

Environmental Protection Agency (EPA), National Water Quality Monitoring Council (NWQMC), 2017. National Water Quality Monitoring Council. Water Quality Portal. Collaboration of United States Geological Survey (USGS) https://www.waterqualitydata.us, Accessed date: 22 September 2016.

EPA: Office of Pesticides Program (OPP), D, 1992. Pesticide Ecotoxicity Database. (Available from:). http://www.epa.gov/pesticides/science/efed_databas.

Gerhardt, A., 2009. Screening the toxicity of Ni, Cd, Cu, ivermectin, and imidacloprid in a short-term automated behavioral toxicity test with *Tubifex tubifex* (Müller 1774) (Oligochaeta). Hum. Ecol. Risk Assess. Int. J. 15:27–40. https://doi.org/10.1080/10807030802614983.

Gessner, M.O., Tlili, A., 2016. Fostering integration of freshwater ecology with ecotoxicology. Freshw. Biol. 61:1991–2001. https://doi.org/10.1111/fwb.12852.

Gilliom, R., Barbash, J., Crawford, C., Hamilton, P., Martin, J., Nakagaki, N., Al, E., 2006. The Quality of our Nation's Waters- Pesticides in the Nation's Streams and Ground Water, 1992–2001. U.S. Geological Survey Circular 1291, Reston, VA, USA.

Halstead, N.T., McMahon, T.A., Johnson, S.A., Raffel, T.R., Romansic, J.M., Crumrine, P.W., Rohr, J.R., 2014. Community ecology theory predicts the effects of agrochemical mixtures on aquatic biodiversity and ecosystem properties. Ecol. Lett. 17:932–941. https://doi.org/10.1111/ele.12295.

Hawxby, K., Tubea, B., Ownby, J., Basler, E., 1977. Effects of various classes of herbicides on four species of algae. Pestic. Biochem. Physiol. 7, 203–209.

Heugens, E.H.W., Hendriks, A.J., Dekker, T., van Straalen, N.M., Admiraal, W., 2001. A review of the effects of multiple stressors on aquatic organisms and analysis of uncertainty factors for use in risk assessment. Crit. Rev. Toxicol. 31:247–284. https://doi.org/10.1080/20014091111695.

Holmstrup, M., Bindesbøl, A.M., Oostingh, G.J., Duschl, A., Scheil, V., Köhler, H.R., Loureiro, S., Soares, A.M.V.M., Ferreira, A.L.G., Kienle, C., Gerhardt, A., Laskowski, R., Kramarz, P.E., Bayley, M., Svendsen, C., Spurgeon, D.J., 2010. Interactions between effects of environmental chemicals and natural stressors: a review. Sci. Total Environ. 408, 3746–3762.

Hunting, E.R., Vonk, J.A., Musters, C.J., Kraak, M.H., Vijver, M.G., 2016. Effects of agricultural practices on organic matter degradation in ditches. Sci. Rep. 6:1–9. https://doi.org/10.1038/srep21474.

Ieromina, O., Peijnenburg, W.J.G.M., De Soo, G., Müller, J., Knepper, T.P., Vijver, M.G., 2014. Impact of imidacloprid on *Daphnia magna* under different food quality regimes. Environ. Toxicol. Chem. 33:621–631. https://doi.org/10.1002/etc.2472.

Ieromina, O., Peijnenburg, W.J.G.M., Musters, C.J.M., Vijver, M.G., 2015. The effect of pesticides on the composition of aquatic macrofauna communities in field ditches. Basic Appl. Ecol. 17:125–133. https://doi.org/10.1016/j.baae.2015.08.002.

Infante, A., Litt, A.H., 1985. Differences between two species of *Daphnia* in the use of 10 species of algae in Lake Washington. Limnol. Oceanogr. 30, 1053–1059.

Kattwinkel, M., Liess, M., 2014. Competition matters: species interactions prolong the long-term effects of pulsed toxicant stress on populations. Environ. Toxicol. Chem. 33:1458–1465. https://doi.org/10.1002/etc.2500.

Kulkarni, D., Gergs, A., Hommen, U., Ratte, H.T., Preuss, T.G., 2013. A plea for the use of copepods in freshwater ecotoxicology. Environ. Sci. Pollut. Res. 20:75–85. https://doi.org/10.1007/s11356-012-1117-4.

Leiden University (CML) and Rijkswaterstaat-WVL, 2016. Pesticide Atlas, version 2.0. www.bestrijdingsmiddelenatlas.nl, Accessed date: 2 February 2016.

Liess, M., 2002. Population response to toxicants is altered by intraspecific interaction. Environ. Toxicol. Chem. 21:138–142. https://doi.org/10.1002/etc.5620210120.

Nollet, L.M.L., Rathore, H.S. (Eds.), 2010. Handbook of Pesticides: Methods of Pesticide Residues Analysis. CSC Press Taylor & Francis Group.

OECD, 2004. OECD Guideline for Testing of Chemicals - *Daphnia* sp., Acute Immobilization Test 202 (Paris, France).

Pérez, J., Monteiro, M.S., Quintaneiro, C., Soares, A.M.V.M., Loureiro, S., 2013. Characterization of cholinesterases in *Chironomus riparius* and the effects of three herbicides on chlorpyrifos toxicity. Aquat. Toxicol. 144-145:296–302. https://doi.org/10.1016/j.aquatox.2013.10.014.

R Core Team, 2016. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria (ISBN 3-900051-07-0). http://www.R-project.org/.

Relyea, R.A., Schoeppner, N.M., Hoverman, J.T., 2005. Pesticides and amphibians: the importance of community context. Ecol. Appl. 15:1125–1134. https://doi.org/10.1890/04-0559.

Roessink, I., Merga, L.B., Zweers, H.J., Van den Brink, P.J., 2013. The neonicotinoid imidacloprid shows high chronic toxicity to mayfly nymphs. Environ. Toxicol. Chem. 32:1096–1100. https://doi.org/10.1002/etc.2201.

Sbrilli, G., Bimbi, B., Cioni, F., Pagliai, L., Luchi, F., Lanciotti, E., 2005. Surface and ground waters characterization in Tuscany (Italy) by using algal bioassay and pesticide determinations: comparative evaluation of the results and hazard assessment of the pesticides impact on primary productivity. Chemosphere 58:571–578. https://doi.org/10.1016/j.chemosphere.2004.07.012.

Schäfer, R.B., Caquet, T., Siimes, K., Mueller, R., Lagadic, L., Liess, M., 2007. Effects of pesticides on community structure and ecosystem functions in agricultural streams of three biogeographical regions in Europe. Sci. Total Environ. 382:272–285. https://doi.org/10.1016/j.scitotenv.2007.04.040.

Schmidt-Kloiber, A., Hering, D., 2012. The taxa and autecology database for freshwater organisms. Version 5.0. (Availabe from). http://www.freshwaterecology.info.

Schreiner, V.C., Szöcs, E., Bhowmik, A.K., Vijver, M.G., Schäfer, R.B., 2016. Pesticide mixtures in streams of several European countries and the USA. Sci. Total Environ. 573: 680–689. https://doi.org/10.1016/j.scitotenv.2016.08.163.

Schulz, R., Dabrowski, J.M., 2001. Combined effects of predatory fish and sublethal pesticide contamination on the behavior and mortality of mayfly nymphs. Environ. Toxicol. Chem. 20:2537–2543. https://doi.org/10.1002/etc.5620201120.

Snoo, G.R.D.E., Tamis, W.L.M., Vijver, M.G., Musters, C., Van't Zelfde, M., 2006. Risk mapping of pesticides: the Dutch atlas of pesticide concentrations in surface waters: www.pesticidesatlas.nl. J. Appl. Biol. Sci. 71 (21), 49–58.

Van den Brink, P.J., Van Smeden, J.M., Bekele, R.S., Dierick, W., De Gelder, D., Noteboom, M., Roessink, I., 2016. Acute and chronic toxicity of neonicotinoids to nymphs of a mayfly species and some notes on seasonal differences. Environ. Toxicol. Chem. 35: 128–133. https://doi.org/10.1002/etc.3152.

Verdonschot, R.C.M., Keizer-vlek, H.E., Verdonschot, P.F.M., 2011. Biodiversity value of agricultural drainage ditches: a comparative analysis of the aquatic invertebrate fauna of ditches and small lakes. Aquat. Conserv. Mar. Freshwat. Ecosyst. 21:715–727. https://doi.org/10.1002/aqc.1220.

Vijver, M.G., van den Brink, P.J., 2014. Macro-invertebrate decline in surface water polluted with imidacloprid: a rebuttal and some new analyses. PLoS One 9, e89837. https://doi.org/10.1371/journal.pone.0089837.

Vijver, M.G., Hunting, E.R., Nederstigt, T.A.P., Tamis, W.L.M., van den Brink, P.J., van Bodegom, P.M., 2017. Postregistration monitoring of pesticides is urgently required to protect ecosystems. Environ. Toxicol. Chem. 36:860–865. https://doi.org/10.1002/etc.3721.