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Neonicotinoids in nature: The effects on aquatic invertebrates and their role in ecosystems

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Henrik Barmantlo

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S. Henrik Barmentlo

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Chapter 1

General introduction

1.1 Freshwater biodiversity in a changing world

To date, there are 1.5 million identified animal species known to mankind (Chapman, 2009). This is only a fraction of the total number of species living on our planet with estimates roughly ranging from 5 million to 8.3 million species (Raven and Yeates, 2007; Mora et al., 2011; Costello et al., 2013; Pimm et al., 2015;). While there are clear uncertainties in the total number of species, a known fact is that invertebrates dominate life on earth in both species richness and sheer abundance. Invertebrate life is estimated to comprise 97% of all animal species (May, 1988). They established roughly three billion years before the vertebrates and generally reproduce quickly and adapt easily. Consequently, invertebrates with their multitude of lifeforms are the taxa that hamper our estimations of the number of animal species on earth.

All animal life is dependent on water and freshwaters contain roughly 10% of all species, among which 1/3 of all vertebrate species (Strayer and Dudgeon, 2010). Yet, freshwaters cover less than 1% of the Earth's surface (Dudgeon et al., 2006). Freshwater invertebrate taxa, being mostly insects (60% of all freshwater animal species) and crustaceans (10%), together comprise over 126,000 described species (Balian et al., 2008). These invertebrates are responsible for ecosystem functioning of the aquatic, but also somewhat the terrestrial realm. For example, they are important for aquatic food web stability, decomposition, nutrient cycling, water flow, water quality, disease regulation and many other services (Prather et al., 2013). They are also an important food source for other animals (including humans) and even some plants (Prather et al., 2013).

Due to anthropogenic interference, biodiversity (including invertebrates) is currently declining worldwide at an unprecedented rate (Díaz et al., 2019). Within total biodiversity, freshwater species are among the most endangered overall as declines are far greater than within terrestrial or marine biodiversity (Dudgeon et al., 2006; Harrison et al., 2018). Populations of freshwater species have declined since 1970 by an average of 83% (according to the Freshwater Index, based on vertebrates; (WWF, 2018). The major drivers of the biodiversity decline are 1) climate change, 2) invasive species, 3) overexploitation, 4) habitat destruction and fragmentation and 5) pollution (Dudgeon et al., 2006; Strayer and

Dudgeon, 2010; Collier et al., 2016; Díaz et al., 2019; Sánchez-Bayo and Wyckhuys, 2019; Wagner, 2020). The latter two drivers of biodiversity are predominantly associated to agricultural practices (and other types of land conversion) which are evermore increasing; food crop production has intensified up to 300% since the 1970s. To date, well over a third of the earth's land surface and roughly 75% of freshwater resources is used for agricultural purposes (Díaz et al., 2019).

It is clear that with such accelerating increases, agriculture has its impact on biodiversity, albeit simply for the sheer amount of habitat conversion. However, we lack knowledge to what extent agricultural pollution, through pesticide and fertilizers use, has contributed to this decline.

1.2 The rise of neonicotinoids

Biologically derived pesticides are already used in agriculture from the early days onwards; the Romans were already known to use extracts of Lupine flowers and wild cucumber to combat insect pests such as grasshoppers and soldier beetles (Smith and Secoy, 1975). However, synthetic pesticides started to be massively used since the time that agriculture became a large scale intensive production system. One of the most common examples of such uses originates from the mid-20th century involves organochlorine insecticides 'DDT' (Dichlorodiphenyl-trichloroethane). Almost two decades later, Rachel Carson published her book 'Silent Spring' that documented the large-scale damage to ecosystems caused by these insecticides (Carson, 1962). The main reasons for the severity of effects were the organochlorine's persistent accumulative nature as well as that the compounds accumulated in higher trophic levels and spread to ecosystems worldwide where they continued to exhibit toxic pressure (Carson, 1962). After these findings, several organochlorine pesticides were banned in the early 1970s and restrictions on organic chemicals with persistent natures (i.e. a persistent organic pollutant or 'POP') were later formalized by the United Nations in the 'Stockholm Convention on Persistent Organic Pollutants' (United Nations, 2001).

In the early 1990s, a new class of insecticides entered the market that was initially hailed as the solution to the environmentally problematic properties of earlier insecticides (Sánchez-Bayo et al., 2016). This class of insecticides was produced with a focus on *non*-bioaccumulative and non-persistent properties and underwent more rigorous safety screening before usage. This class was the neonicotinoids (Jeschke and Nauen, 2008). Since the initial introduction of neonicotinoids, they have been replacing other insecticides such as the organochlorines, but also pyrethroids, organophosphates, carbamates and others that had been used to control insect pests (Nauen and Denholm, 2005; Jeschke and Nauen, 2008). To date, they are the most sold class of insecticides in the world as they are praised for their relatively swift degradability (compared to earlier compounds) and specific mode of action (MoA; Jeschke et al., 2011).

Neonicotinoids work by antagonistically binding to nicotinic acetylcholine receptors (nAChR) in insects, which in turn causes “continuous excitation of the neuronal membranes, producing discharges leading to paralyses and cell energy exhaustion” (from: Simon-Delso et al., 2015). This bond and the chemical structure of the compounds is similar to that of nicotine, hence the name ‘neonicotinoid’, which means ‘new nicotine-like insecticides’. The bond to the nicotinic receptor is most effective in invertebrate nervous systems (as they generally have higher numbers of receptors with high neonicotinoid affinity). Indeed, the effectiveness to agricultural pest invertebrates is broad as it includes aphids, whiteflies, leafhoppers, beetles, weevils and others (Jeschke et al., 2011). They are also effective in nonagricultural usage, for example in termite control (Jeschke et al., 2011). Perhaps the most beneficial property of all is that neonicotinoids can be used as a systemic insecticide; meaning that they can be taken up by the crop and distribute throughout the plant, thus protecting the plant against all kinds of insect damage. The neonicotinoids can be applied as a seed coating which minimizes the drift to other non-target areas as otherwise happens when pesticides are sprayed (EASAC, 2015). Nonetheless, it has been shown that about 80 to 98% of neonicotinoids applied as seed coating end up in soil and soil water (Goulson, 2013). It has also been shown that this class of pesticides have

high leaching and runoff potential from the crop fields. As for all pesticides, neonicotinoids are thus not only found within and on the crop but also in fringing ecosystems. Given their high usage, neonicotinoid are now found in freshwaters globally (for example: Morrissey et al., 2015; Hladik et al., 2018; Leiden University and Rijkswaterstaat-WVL, 2018; Casado et al., 2019; Chen et al., 2019;).

1.3 Risk assessment of neonicotinoids

Methods to collect data fitting environmental risk assessments of neonicotinoids, and other pesticides, generally rely highly on standardization. For this reason there are numerous of standardized guidelines provided by 'The Organisation for Economic Co-operation and Development' (OECD) and 'International Organization for Standardization' (ISO) so that scientists all over the world can compare their results following standardized criteria (Moermond et al., 2016). Most of these guidelines describe bioassays that are performed with a single test species and a single chemical in a simple setup; usually a small glass container in which organisms are fed *ad libitum* (i.e. 'at one's pleasure'), well-described medium, strict temperature and light settings (for example: OECD, 2012). These experiments provide data that can be used as an input for several models (such as 'species sensitivity distributions', SSD) upon which potential environmental hazards can be estimated (De Zwart and Posthuma, 2005).

Like all pesticides nowadays, neonicotinoid insecticides have also experienced rigorous ecotoxicological testing. As suggested by the main mode of action, being that the nicotine neuronal pathway of insects is inhibited (see section 1.2), it has been assumed for a long period of time that neonicotinoids mainly act on insects (Simon-Delso et al., 2015). Data as used in the EPA ECOTOX database (<https://cfpub.epa.gov/ecotox/>) and published in literature has indeed shown that the direct toxicity of neonicotinoids towards vertebrates such as amphibians and fish is indeed lower than to invertebrates (Vijver and van den Brink, 2014). This toxicity is thusly severe that the European Academies of Sciences (EASAC, 2015) concluded in 2015 that the preventive usage of imidacloprid year by year, has severe negative effects on non-target biodiversity in agricultural areas. In particular, this referred to honeybees and many different insects

such as wild bees, bumblebees, butterflies and hoverflies that are vulnerable to low doses of neonicotinoids. For agriculture systems, this eventually may create dramatic cascading effects because these insects play an important role in the pollination of crops.

For freshwater species, a recent review by Morrissey et al. (2015) showed that ecotoxicological data is strongly centered around one neonicotinoid, imidacloprid (66% of reviewed studies), and one test species, the crustacean *Daphnia magna* (16% of reviewed studies). *D. magna* is repeatedly shown to be especially insensitive to neonicotinoids as laboratory surveys showed a range of 4,100 to over 1,000,000 µg/L where 50% toxic effect is observed (lethal or other effects, LC₅₀ and EC₅₀, respectively), which is at least a factor 80 out of range of maximal measured surface water concentrations (see section 1.4). Other invertebrates, especially insects, are much more sensitive; Roessink et al. (2013) showed high toxicity of neonicotinoids to mayflies with EC₅₀ (immobilization) values as low as 0.1 µg/L. Chironomid species are also reported to be highly sensitive with toxicity values in the same order of magnitude as for mayflies (Langer-Jaesrich et al., 2010). The toxicity of neonicotinoids to different invertebrate species thus shows a large range of dosing. Some invertebrates are not sensitive while others are considered to be extremely sensitive. Such differences might have consequences for communities that can end up imbalanced as some species are removed by toxic pressure while others are not. More ecologically complex studies with standardized assemblages of multiple species (i.e. mesocosm studies) have shown that several invertebrates, especially the aforementioned sensitive species groups, are indeed also affected within an environment with higher ecological complexity (for example: Beketov et al., 2008; Berghahn et al., 2012; Hayasaka et al., 2012), indicating a risk to freshwater biodiversity (Stehle and Schulz, 2015).

1.4 Ditches in the Netherlands; a case study of biodiversity impacted by agriculture

Freshwater biodiversity is rooted in Dutch history as 26% of its total surface is below sea-level (Rijkswaterstaat, 2018) and the Netherlands is therefore a water-rich country. In order to prevent the country against floods, the Dutch have historically been pumping out this water with a mosaic of drainage ditches and windmills (van Dam, 2009). The ditch system provides irrigation but also drainage to arable land via the removal of excess water. To date, a total length of over 300,000 km ditch can be found in the Netherlands (Highler, 1994). Water quantity is constantly monitored and water levels are maintained by pumping water in or out (depending on evaporation and precipitation), excess vegetation is removed to keep the depths controlled and ditches are dredged to prevent obstruction of water flow.

Through their function, ditches are in close vicinity to the arable landscape and the first surface water body to receive agrochemicals residues used in croplands. Several neonicotinoids (amongst other pesticides) are frequently detected in these surface waters by monitoring activities of the Dutch water boards (de Snoo et al., 2006; Leiden University and Rijkswaterstaat-WVL, 2018). For two out of five neonicotinoids measured, concentrations levels are more often than not above environmental quality criteria (the 'JG-MKN/MTR' norm) when detected (Table 1.1). The other neonicotinoids that do not as commonly exceed the norm also have higher environmental quality criteria; they differ up to at least a factor 1500 between the least and most strict norm (Table 1.1). The observed range of neonicotinoid concentrations also overlaps with toxicity values as found in ecotoxicological experiments (see 1.3, Figure 1.1). Most notable are imidacloprid, thiamethoxam and thiacloprid with surface water concentrations observed above 1 µg/L and up to 54 µg/L in the period 2013 to 2017 (Figure 1.1). Given the short half-life of neonicotinoids in water (generally less than one or a few days; Table 1.1), the chance of detecting maximal emissions by grab sampling is relatively low and peak concentrations can therefore be higher than currently measured. As there is overlap between toxicity values and surface water concentrations, we can expect that fitness of test species is

affected in the aquatic environment because of the residues of neonicotinoid leaching from the agricultural fields. Field surveys on freshwater macrofaunal communities have indeed shown that neonicotinoids and other pesticides are explanatory factors for the presence and/or abundance of several invertebrate species (Ieromina, 2015; Musters et al., 2019; Waite et al., 2019). The relative importance of a single neonicotinoid, and other pesticides compared to other stressors like phosphates, explaining the presence of invertebrates is often low.

Table 1.1. Overview of neonicotinoid detections in Dutch surface waters in relation to environmental norms during the period 2013-2017. Data were retrieved from the Dutch Pesticide Atlas (accessed 22-11-2018).

	Aceta- miprid	Clothia- dinin	Imida- cloprid	Thia- cloprid	Thiame- thoxam
Total no. measurements	11,368	2,276	15,903	12,691	11,732
Detections	445	30	5,019	801	1,705
Norm* (ng/L)	100	14,000	8.3	10	140
Detections above norm	31	0	3529	502	342
% of detections that exceed norm	7%	0%	70%	63%	20%
Water $t_{1/2E}$ (days) in Spring	16.5 ¹	0.5 ¹	0.4 ¹	14.3 ¹ / 3.3-3.6 ²	0.3 ¹

Data for the neonicotinoids Dinotefuran and Nitenpyram were not available. *norm for ecological quality (JG-MKN / MTR). DL = detection limit, $t_{1/2E}$ = aqueous half-life. ¹ Lu et al, 2015, ² This thesis, Chapters 2 and 3.

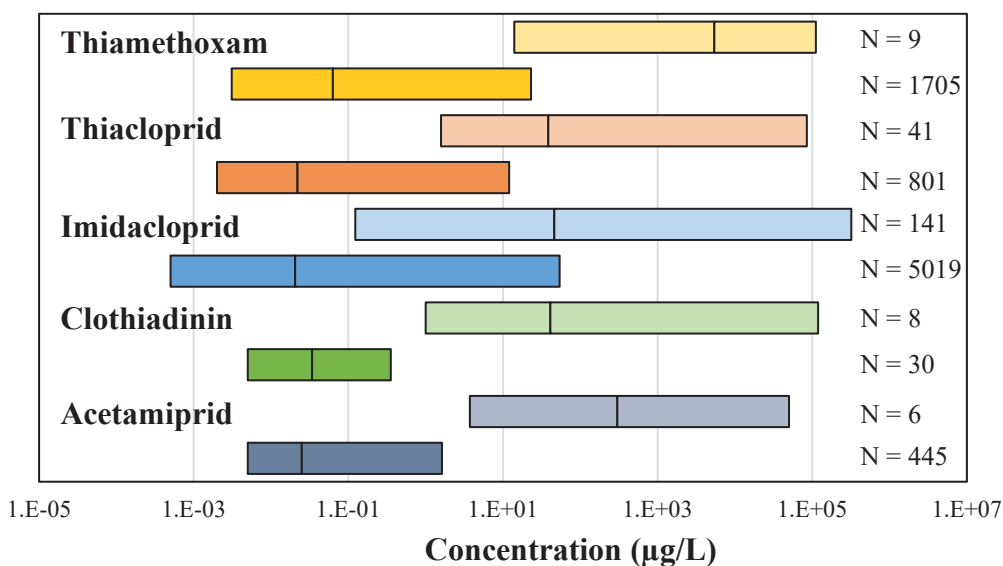


Figure 1.1. Range of neonicotinoid toxicity values (both acute [178 values] and chronic [36 values] EC and LC₅₀) versus the range of Dutch neonicotinoid surface water concentrations from 2013 to 2017. Light colors show toxicity values while darker counterparts show surface water concentrations per neonicotinoid. Vertical lines in each bar represent the median value. Toxicity data were derived from (Morrissey et al., 2015) and surface water concentrations were retrieved from the Dutch Pesticide Atlas (2018).

1.5 Environmental complexity hampers toxicity predictions

There are, next to toxic pressure, numerous other (abiotic) drivers of aquatic fauna such as pH, dissolved organic matter (DOM) and temperature. These drivers can affect toxicity exhibited by a pesticide or even its persistence (Clements et al., 2012). For example, higher temperatures have been shown to enhance imidacloprid uptake by aquatic invertebrates, leading to higher toxicity (Camp and Buchwalter, 2016). Pesticides themselves can also alter each other's toxicity. Both in soil or water, pesticides are often found in concord with some mixtures consisting of even 70 different compounds (Vijver and van den Brink, 2014; Stehle and Schulz, 2015; Schreiner et al., 2016; Casado et al., 2019). Each of these can theoretically lead to a difference in the toxic pressure exhibited by another. For example, Sgolastra et al. (2017) showed that a fungicide (propiconazole) can increase the toxicity of the neonicotinoid

clothianidin to bees, probably by inhibiting detoxification. This is known as a synergism; a more than additive response (Côté et al., 2016). Multiple substances can also reduce each other's toxicity, leading to a less than additive response, i.e. an antagonism (Côté et al., 2016). These are not necessarily pesticides. The agricultural chemical (hereafter: 'agrochemical') that accompanies a neonicotinoid most often are fertilizers as these are used in almost every type of agriculture in order to increase crop yield. Nitrogen fertilizers are estimated to support 40-50% of the world's human population (Erisman et al., 2008). Fertilizers, being primarily nitrogen, phosphorus and potassium that are combined with several microelements, provide the usually limiting building blocks of crops. Like pesticides, fertilizers also leach or rinse from the land into the neighboring surface waters (Erisman et al., 2008), where they can stimulate growth of aquatic plants and algae, leading to eutrophication. This increased primary production can increase consumer numbers (such as algae filter feeders and periphyton grazers) and in turn their predators, i.e., a bottom-up effect (Davis et al., 2010). Species interactions may thus be affected. The changes in species interactions through fertilizer addition can be complex. A similar point can be made for pesticides; Halstead et al. (2014) showed that effects of (mixtures of) pesticides may be estimated using community ecology theory. For instance, insecticides may negatively impact predators and herbivores which indirectly can cause an increase in resources. These predictions of indirect effects of both pesticides and fertilizers are relatively easily tested in closed mesocosm environments with often simple assemblages of test species. In these systems, 'recovery' is also studied with relative ease as one simply investigates when the impacted species increase in numbers until it once again reflects the control situation (for example, Beketov et al., 2008). In the actual environment, however, the removal or suppression of one or more species can have larger consequences as these systems are open and thus (re)colonization can take place. Through niche creation or niche occupation, the impacted communities can be more or less suitable for other species, which potentially alters biodiversity and its functioning in the long-term. Communities can thus experience a pressure-induced species turnover where more tolerant species increase in presence (as discussed in 1.3). As a result, while aiming to identify the effects of

pesticides in a field survey, one might find communities that are already more resilient. An indication for this is provided by Musters et al. (2019) who showed that aquatic communities next to agricultural fields are more homogeneous (i.e. they more closely resemble one another) than communities in nature reserves, meaning that species turnover may have already occurred as communities assembled according to the agricultural pressure. This potentially follows the ongoing worldwide pattern of changing biodiversity while species richness does not seem to change (Blowes et al., 2019).

1.6 Integrating ecology into ecotoxicology

Extrapolating single species bioassays data (or even more ecologically complex mesocosm toxicity data) to the actual field situation is notoriously difficult, hence safety factors are applied to cope with the uncertainty (Raimondo et al., 2008). It is equally difficult to identify the true effect of a single pesticide in the field due to a myriad of interacting pressures and/or legacy effects. Within risk assessment, environmental complexity is largely overlooked while it is a clear driver of toxicity (Clements et al., 2012). This is simply because standardized testing of all different environmental variables in combination with all different chemical stressors is an unmanageable task. This gets even worse if one additionally considers the alterations in biotic interactions between species that can also be affected by the neonicotinoids (Gessner and Tlili, 2016) and that may have long-term consequences for biodiversity. Differentiating between the effect of a neonicotinoid and other (interacting) factors on biodiversity would therefore require a new experimental setup that 1) includes full ecological complexity and 2) standardizes this same complexity.

1.7 The outdoor test-facility Living Lab

To study the effects of agrochemicals on natural ecological communities and species interactions, a new outdoor research facility was conceptualized (Figure 1.2). The facility consisted of 38 newly dug individual ditches (being the first recipients of agrochemicals, see 1.4). This setup aimed to differ from the classical mesocosm setup in the sense that the ditch communities and species interactions would form fully

naturally, while communities in mesocosm studies are usually designed in ponds with caught or reared species (OECD Mesocosms). In addition, mesocosm experiments often focus on specific components of a food web, such as zooplankton or benthic assemblages (for example see Pestana et al., 2009; Lobson et al., 2018) rather than full-scale biodiversity in freshwater systems such as ditches. At the same time, the facility aimed to maintain a standardized and experimental character, where abiotic and biotic conditions were comparable among individual ditches. The outdoor test facility was named the 'Living Lab' where 'Living' refers to the natural formation of ecological communities and 'Lab' refers to the standardized and replicated setup as found in a laboratory environment. Taken all the above aims into account within the creation of this facility, three main aspects were held in close consideration:

- 1) Minimal anthropogenic interference
- 2) Natural and similar habitats
- 3) Natural community formation through unmanaged colonization

Minimal anthropogenic interference (1) was realized by selecting a site with both land and water being distant from agricultural practices; an uncultivated grass field of over 30 years with a small lake (roughly 65*90 m, acting as a water level compensation reservoir) that is interconnected by ditches and canals to the Old Rhine river shed. The field (at the Leiden Bio Science Park in Leiden, the Netherlands and owned by Leiden University) was mown yearly and grasses were removed, adding to the observed low (oligotrophic) nutrient levels in the neighboring lake as shown on Figure 1.2. Ditches were dug to the surface water level by removing 1.5 to 1.8m of the field's topsoil. This removed potential pollution that occurred over the past ~2000 years as validated by the findings of Roman currency in the soil at this depth. In Roman times, there were no *synthetic* pesticides and fertilizers.

Natural and similar habitats (2) were realized by first shaping each ditch to be a replicate of each other. Each ditch has a dimension of 10:0.8:0.3 m (Length:Width:Depth) and a volume of 1,750 L. All ditches are characterized by mostly clayey sediments and water provided by the adjoining lake. The ditches are protected from waves from the lake by a wooden barrier which more closely mimics actual drainage ditches. Ditch banks (alternately having a width of 0.6 and 1.2 m between ditches) were covered by organically grown grass (to avoid pesticide and fertilizer inputs) supplemented with a mixture of grass and clover seeds. In addition, fish and birds were repelled by netting (5 mm mesh size, placed behind the wooden barrier) and brightly colored wires (above each ditch), respectively.

Natural community formation through unmanaged colonization (3) was realized by large source populations from the adjoining lake (see Figure 1.2) that continuously colonized the ditches. This lake was already present before the realization of the ditches and yielded full ecological complexity as it contained diverse plants, fish, invertebrates and others. As the lake was much larger than a single ditch or all ditches combined, it provided a strong inflow of species that colonized the ditches in a similar manner. A row of *Typha latifolia* (broadleaf cattail) was planted at the end of each ditch to stimulate this colonization. Furthermore, the ditches at the most outer ends (left and right) acted as buffer communities to avoid edge effects for the inner 36 ditches. To date, over 450 bacterial, 140 algal, 150 faunal and 8 plant species have been identified in the waters of the site.



Figure 1.2. Schematic visualization of the 'Living Lab' concept (© Els Kolfshoten). Note that, in contrast to this early design, ditches are actually directly connected to the lake.

1.8 Aims of this thesis

Using the natural but experimental setup of the outdoor facility Living Lab, this thesis aimed to identify the potential hazardous effects of neonicotinoids not by elimination, but by standardization of natural interacting factors. The overall aim of this thesis was to identify and unravel the impact of neonicotinoid insecticides as well as fertilizers on freshwater life, from individuals to ecosystems. The neonicotinoid and fertilizers represent agrochemical combinations often used and monitored within the field setting. This was done by performing ecotoxicological experiments in the Living Lab to study the following objectives:

- 1) To understand the importance of fertilizer and food on neonicotinoid toxicity to aquatic invertebrates.
- 2) To quantify whether single species bioassays yield results similar to toxicity tests that incorporate more ecological context.
- 3) To quantify the risk of current neonicotinoid surface water concentrations to invertebrates in terms of individuals, populations, communities and the ecosystem processes they fulfill.

1.9 Thesis outline

In Chapter 2, an *in situ* cage experiment in the Living Lab ditches was performed to investigate if fertilizers affect the toxicity of field-relevant concentrations of a model neonicotinoid (Obj. 1 & 3). Four different invertebrate species were exposed to thiacloprid, fertilizer and a mixture of both to study the agrochemical's effects on survival, growth, reproduction, consumption and emergence. These results were subsequently compared to laboratory toxicity data as obtained from literature (Obj. 2).

In Chapter 3, the potential effects of food on thiacloprid toxicity were further investigated using a predator species rather than the herbivorous species as in Chapter 2 (Obj. 1). The predator selected was the damselfly *Ischnura elegans*, the most common dragonfly in a large part of Europe. A dragonfly was selected because toxicity data was lacking as these are not standard test species. Like in Chapter 2, caged individuals were studied on

several endpoints: survival, growth, molting, mobility, consumption and emergence. In addition, the emergence of natural populations exposed to the same neonicotinoid concentrations was investigated. The experiments were again performed at field relevant concentrations and the data was then compared to Dutch national trends of dragonflies (Obj. 3).

In Chapter 4, the effects of more complex agrochemical mixtures on neonicotinoid toxicity were investigated. A full factorial mesocosm experiment with a neonicotinoid (imidacloprid), herbicide (terbuthylazine) and fertilizers was conducted to assess their single and combined effects to invertebrate populations of a species assemblage (Obj. 1 & 3). These data were compared to results from bioassays as found in literature to investigate if single chemical and single species toxicity results remain accurate in more complex agrochemical mixtures (Obj. 2).

In Chapter 5, the potential role of neonicotinoid as an actor of the ongoing insect decline was experimentally investigated (Obj. 3). The effects of field-relevant concentrations of thiacloprid on naturally assembled insect populations were assessed using emergence traps that were placed over the Living Lab ditches. Insects were caught and identified to identify potential neonicotinoid-driven declines in abundance, biomass and diversity.

In Chapter 6, both field-relevant concentrations of a neonicotinoid, fertilizer and their mixture were questioned to be single and joint drivers of naturally assembled aquatic communities (Obj. 1 and 3). Invertebrate communities in the Living Lab ditches were exposed to the agrochemicals and subsequently monitored on invertebrate abundance, species richness and structure. Community structure was investigated by examining potential changes in beta dispersion and dissimilarity compared to control (undisturbed) communities.

In Chapter 7, it was investigated if network-based statistical approaches can be used to identify the effects of a stressor, a neonicotinoid, to communities. In addition, it was investigated whether disruptions in the networks can also lead to a loss in ecosystem functioning (Obj. 3). Living Lab invertebrate communities were exposed to a range of field-relevant

thiacloprid concentrations and then investigated on species co-occurrences relative to the control and randomly generated communities. Next, the role of the invertebrates in ecosystem functionality was assessed by transforming their abundance to reflect feeding trait biomass. This data was subsequently linked to several measured ecosystem processes (primary production and decomposition) using piecewise structural equation modelling.

Finally, in Chapter 8, the results are discussed in light of what ecological context can learn us to investigate the effects of neonicotinoids, and other agrochemicals, in the actual environment.

Chapter 2

Thiacloprid-induced toxicity influenced by nutrients:

Evidence from in situ bioassays in experimental ditches

Environmental toxicology and chemistry (2018) 37(7), 1907-1915

S. Henrik Barmentlo, Elinor M. Parmentier, Geert R. de Snoo
and Martina G. Vijver

Highlights

- 1) This study aimed to show the combined effects of nutrient enrichment and the thiacloprid-induced toxicity to four aquatic invertebrates using a caged experiment in outdoor experimental ditches.
- 2) We show thiacloprid-induced toxicity at environmentally relevant concentrations for different endpoints of two crustaceans and for one insect species out of the two tested insect species tested.
- 3) For *D. magna* we observed thiacloprid-induced toxicity expressed as LOEC for growth and reproduction at a 2456 fold lower concentrations than laboratory derived LOEC values.
- 4) Thiacloprid-induced toxicity was often not observed under nutrient enriched conditions, likely ascribed to compensatory feeding.
- 5) The common laboratory approach does not test for neonicotinoid-induced toxicity under low food (quality) conditions and may therefore underestimate the toxicity in the natural situation.

Abstract

Many studies show that neonicotinoid insecticides cause toxicity to aquatic invertebrates. Some studies report that insecticide toxicity may differ in combination with other agrochemicals under realistic field conditions. To explore such altered toxicity further, we aimed to determine the single and combined effects of environmentally relevant levels of the neonicotinoid thiacloprid and nutrients to different endpoints of four aquatic invertebrate species. Animals were exposed to these agrochemicals using a caged experiment within experimental ditches. We observed thiacloprid-induced toxicity for two crustacean *D. magna* and *A. aquaticus* and for one out of two tested insect species *C. dipterum*. We observed no toxic effects for *C. riparius* at the time weighed average test concentration of 0.51 µg thiacloprid/L. For *D. magna*, the observed toxicity expressed as LOEC (Lowest Observed Effect Concentration) on growth and reproduction was present at thiacloprid concentrations that were a 2456 fold lower than laboratory derived LOEC values. This shows that these species, when exposed under natural conditions, may exhibit neonicotinoid-induced toxic stress. Contrary to the low nutrient treatment, such toxicity was often not observed under nutrient enriched conditions. This was likely due to the increased primary production that allowed for compensatory feeding. These findings warrant for the inclusion of different feeding regimes in laboratory experiments in order to retrieve the best estimates of neonicotinoid-induced toxicity in the natural environment.

Keywords: agrochemicals, crustaceans, fertilizer, insects, multiple stressors, neonicotinoids

2.1 Introduction

The field of ecotoxicology has a long history of attempting to extrapolate results between laboratory and field. Field surveys of indigenous biota are used to estimate overall ecological water quality as impacted by all stressors present at the sampling site. Relating these ecological monitoring data to environmental chemical monitoring data is having many challenges and gives at best a statistical-based indication of stress (Vijver and van den Brink, 2014; Hallmann et al., 2017). This can be explained by the many confounding factors present that may alter toxicity or species fitness, for example nutrient loadings (Alexander et al., 2013), competition (Liess, 2002; Kattwinkel and Liess, 2014) and predation (Schulz and Dabrowski, 2001). In comparison, a laboratory approach to study toxicity shows direct effects of chemical compounds to an organism with high experimental control of possible confounding factors. However this approach lacks ecological realism as it standardizes or does not replicate additional natural stressors such as the confounding variables mentioned before (Burton et al., 2005; Crane et al., 2007). An approach to combine the influence of natural stressors with experimental control is through the use of caged experiments: a method to study toxicity under increased ecological realism while being less subjected to sample collection (Crane et al., 2007).

Caged experiments allow for testing additional anthropogenic alterations to the environment that do not necessarily induce stress but can indirectly affect species' fitness. An example thereof is increased reproductive output of zooplankton species due to increased nutrient levels (Ieromina et al., 2014), through increased primary production (Jak et al., 1998). Such elevated nutrient levels are commonly found in agricultural drainage ditches because of fertilizer run-off, drift or leakage (Janse and Puijenbroek, 1998). These emissions often include other agrochemicals such as neonicotinoids; insecticides that are well-known to cause toxicity to aquatic invertebrate species and communities (Beketov et al., 2008; Roessink et al., 2013; Van den Brink et al., 2016; Vijver et al., 2017). Alexander et al. (2013) showed that nutrient enriched conditions can

'mask' the toxicity of insecticides to invertebrate communities. However, experimental data whether nutrients can reduce neonicotinoid toxicity of different single invertebrate species' fitness, and if fitness is reduced at a similar rate, is lacking.

This data gap on combined effects is not surprising due to the difficulty of experimental approaches capable of dealing with the indirect effects of nutrients and also monitoring population responses in a similar fashion as a laboratory experiment. To reduce this data gap one needs an experimental setup including multiple trophic levels while still controlling the confounding factors mentioned before. In order to reduce the data gap and to determine if neonicotinoids and nutrients potentially affect a single species fitness, we aim to determine the single and combined effects of environmentally relevant levels of the neonicotinoid thiacloprid and nutrients on the fitness of four aquatic invertebrate species. Our overarching hypothesis is that thiacloprid-induced toxicity will be reduced under nutrient enriched conditions. Baas and Kooijman (2015) showed that somatic maintenance - being a parameter in the dynamic energy budget (DEB) theory - correlates with species sensitivity to toxicants. This was confirmed for 50 different animal species to chemical effects on survival was investigated for 4 different pesticides. To this end, we test the effects of these agrochemicals to three different endpoints of two crustacean (proposed less-susceptible organisms for neonicotinoid insecticides) and two insect species (proposed more-susceptible species) using a cage setup in ecologically and chemically similar experimental ditches.

2.2 Materials and Methods

Test species and culture conditions

Test species used in this study were juveniles of two crustacean species; *Asellus aquaticus* Linnaeus (<2 weeks old) and *Daphnia magna* Straus (<24 hours old), and two insect species; *Cloeon dipterum* Linnaeus (4-5

instar nymphs) and *Chironomus riparius* Meigen (<9 days old). All species were indigenous at the site of the in situ experiment.

The crustacean species *A. aquaticus* and *D. magna* originated from longstanding cultures present in the laboratory of Leiden University (Leiden, The Netherlands). *A. aquaticus* was cultured at room temperature on fine-grained, ignited quartz sand in 50% demineralized water and 50% filtered (106 µm sieve) ditch water and fed with 'decomposition and consumption tablets' (Decotabs; consisting out of 2% agar, 6% finely ground hay and demineralized water; Kampfraath et al., 2012). *D. magna* was reared at 18 °C with a light:dark cycle of 16:8 in Elendt m4 medium and fed triweekly with fresh algae (*Pseudokirchneriella subcapitata*; OECD, 2004). At intervals of about four months, the sensitivity of the *D. magna* culture was investigated with the reference toxicant K₂Cr₂O₇. These tests showed that the sensitivity of the daphnid culture was well within limits as set by the OECD guideline (24h 50% effective concentration for mobility = 0.6 – 2.1 mg/L K₂Cr₂O₇) (OECD, 2004a).

The insect species were either caught from a non-agricultural site (*C. dipterum*; < 500m away from the test site) or originated from longstanding cultures from the University of Amsterdam (*C. riparius*; Amsterdam, The Netherlands). *C. riparius* was reared at 22 °C with a light:dark cycle of 16:8 (OECD, 2011) in Dutch Standard Water (DSW; 200 mg/L CaCl₂·2H₂O, 180 mg/L MgSO₄·7H₂O, 100 mg/L NaHCO₃ and 20 mg/L KHCO₃) on fine-grained, ignited quartz sand and fed triweekly with fish flakes (20:1, Trouvit:Tetraphyll).

Exposure conditions

The caged experiments were performed in outdoor experimental ditches at the 'Living Lab' facility of Leiden University, Oegstgeest, The Netherlands. The site is not located in any close proximity to agricultural practices and is adjacent to grassland. The 36 ditches used in this study have a length of 10 m, a width of 0.8 m at the surface level and 0.4 m width at the bottom at a depth of 0.3 m. The ditches are adjoining to a water level compensation reservoir that is characterized by low levels of soluble

nutrients and is connected to the watershed of the Old Rhine. At the end of April 2017, after leaving the ditches connected to the reservoir for six months to form natural ecological conditions (fauna, flora etc.), each ditch was hydrologically isolated from the reservoir to avoid cross-contamination of the added agrochemicals (thiacloprid and nutrients, see below) between ditches. We isolated the ditches by placing a 1000*500*2 mm acrylic plate firmly into the ditch banks and 15 cm deep into the sediment at the end of every ditch. After isolation, half of the ditches were continuously enriched with nutrients by hanging three sachets containing slow-releasing fertilizer granulates (75 g Osmocote per sachet; N:P:K = 15:9:11 combined with microelements, Dordrecht, The Netherlands) into each ditch equally divided over the ditch length. Two weeks later, following a block design, nine ditches that did and did not receive additional nutrients were spiked with an environmentally relevant nominal concentration of thiacloprid. As in our experiment, thiacloprid is subject to processes such as degradation and adsorption and we do not refresh exposure 'media' (here: ditchwater); we provide our exposure concentration (see below for the method of measuring thiacloprid concentrations) as a time weighed average (TWA), calculated following equation 1:

$$C_{TWA} = \frac{\sum_{n=1}^N \left(\Delta t_n \frac{c_{n-1} + c_n}{2} \right)}{\sum_{n=1}^N \Delta t_n} \text{ eq. 1}$$

Where Δt is the time interval between measurements, c is the concentration (in ng/L) at time interval n (in days) and N the total number of intervals. Our nominal time weighed average concentration (TWA) was 400 ng/L thiacloprid (99.9% purity; purchased from Sigma-Aldrich, Zwijndrecht, The Netherlands) over the period of one month. We based this concentration on thiacloprid concentrations residing in surface waters of the Netherlands over a five-year period (2011-2015, 11.751 observations, Pesticide Atlas, 2016). Of all observations, 608 unique observations showed a concentration of thiacloprid above the detection limit (DL = 10 or 20 ng/L in most cases), with a maximum observed concentration of 12,000 ng/L (for details see Supplement Figure 2.S1). Including both the nutrient and thiacloprid additions, the experiment

entailed a full factorial design of four different treatments with nine replicates per treatment: i) control (no added substances), ii) thiacloprid addition, iii) nutrient addition and iv) thiacloprid and nutrient addition. In order to maintain experimental concentrations, two weeks after the first thiacloprid spike, a second spike was performed in equal nominal concentrations. Spiking was conducted by diluting a 25 mg/L stock concentration of thiacloprid in a glass bottle with 10 L of ditch water originating of the respective ditch the thiacloprid was added to. Subsequently, the bottle was mounted with a watering can head and the mixture was spread evenly over the ditch to simulate spray drift and to stimulate homogenization of the compound.

Once a week, the experimental ditches were monitored on pH, dissolved oxygen (DO), temperature and conductivity using a portable hq 40d electronic multi-parameter meter (Hach Ltd, Colorado, US). In addition, water samples were collected 5 cm below the surface level in the middle of each ditch each day in the first week after the first thiacloprid spike and thereafter twice per week. One hour after the first as well as after the second spike, we analyzed all collected samples on thiacloprid concentration. Subsequent samples were randomly selected blocks within the block design. These samples were then analyzed on their thiacloprid concentration at Wageningen University (Wageningen, The Netherlands) using liquid chromatography-tandem mass spectrometry (LC-MS/MS; Agilent Technologies; see Roessink et al., 2013 for the detailed procedure). From these data, we calculated the average DT50 and DT90 for both spikes individually. Biweekly, dissolved nitrate (NO_3^-) and phosphate (PO_4^{2-}) concentrations were determined colorimetrically using a Nova 60 spectroquant® photometer (Merck, Amsterdam, The Netherlands). Finally, right before nutrient addition, four days after nutrient addition and three days prior to the end of the experiment, we collected, extracted and analyzed water samples to determine chlorophyll A concentrations using the method as described by Arar and Collins (1997) on a F900 fluorescence spectrometer (Edinburgh Instruments, Livingston, United Kingdom).

Experimental setup

Three days prior to the first thiacloprid spike, test species were entered in enclosures and placed into each ditch in order to let the animals acclimatize to the present water conditions. *D. magna* acclimatized for only one day prior to the thiacloprid spike to prevent substantial unexposed growth. Before inoculation, animals were allowed to slowly acclimatize to the ditch temperature (which was 20 °C at the time of inoculation) to prevent heat shock. After the acclimatization period the animals were, if possible, checked on survival (>98% for *C. dipterum* and *D. magna*). For the test species *A. aquaticus* and *C. dipterum*, 150mL high-density polyethylene enclosures with a 3.75 cm opening on one side covered with mesh (mesh size 700 µm) were prepared. Equal enclosures were prepared for *D. magna* except for that we used a mesh size of 150 µm in order to prevent escaping neonates. *C. riparius* enclosures were prepared out of 250 mL glass jars containing 60 g of fine-grained, ignited quartz sand (grain size: 0.1–0.5 mm) as sediment and closed with 150 µm mesh.

Each 150 mL enclosure held five juveniles per test species and was placed horizontally in the ditch by sticking a ring of PVC steel line wire into the sediment. The *C. riparius* enclosures held ten juveniles and were dug roughly 5 cm vertically into the sediment. Each ditch received one enclosure of *A. aquaticus*, *C. dipterum* and *D. magna* and two enclosures of *C. riparius*. Right before placing the enclosures, we fed each cultured species according to their culture conditions (see 2.1). For *C. dipterum* we provided 0.1 mg wet weight of live periphyton of the respective ditch the enclosure was inoculated in. Generally, enclosures were retrieved 21 days after the first thiacloprid spike. The first of the two *C. riparius*' enclosures, however, was retrieved after 14 days of exposure to measure several larval endpoints (explained below) before emergence.

Test species endpoints

Survival of *C. dipterum* and *D. magna* species was recorded every three days by bringing the enclosures to the surface, carefully opening them and counting the mobile animals. Animals were considered immobile if they did not respond within 15 s of gentle stimulation by stirring (OECD, 2004a). During inspection, we removed and counted the number of juveniles produced by *D. magna* and the number of emerged *C. dipterum* individuals to score reproduction and emergence respectively. The emerged *C. dipterum* individuals were easily collected as they were trapped in a small air pocket within the enclosure (<5% of enclosure total volume). Survival of *A. aquaticus* and *C. riparius* was estimated after enclosure retrieval, as it proved unfeasible to accurately estimate survival of these species due to water clarity and *C. riparius*' nature to bury itself into the sediment. These animals were therefore collected by filtering the contents of the enclosure over a sieve (106 µm) and survival was scored subsequently.

After 18 days of exposure, emergence was scored for *C. riparius* by placing an emergence trap (60x60 cm) directly above the remaining enclosure that was connected to a 1 mm mesh tunnel leading directly into the trap. We did not use the same method to score emergence for *C. riparius* and for *C. dipterum* due to high tendency of degradation of *C. riparius*.

Directly prior to inoculation of the test species, 20 animals per species were photographed using an eScope DP-M17 USB-microscope camera. After 14 days of exposure for *C. riparius* (due to very fast growth in the nutrient addition treatments) and 21 days for the remaining species, enclosures were retrieved, surviving animals were collected, shock preserved in 96% EtOH for 15 s and stored in 80% EtOH to be photographed a week later. Image J (version 1.48f) was then used to determine the initial and final body length of each species. *A. aquaticus* was measured from the tip of the head to end of the pleotelson, *C. riparius* from the tip to the head to the anal tubules, and *C. dipterum* and *D. magna* from rostral end to the attachment of the tail. Subsequently, for each species, daily growth was calculated by subtracting the final body length with the initial body length and dividing by the number of days the

organism was exposed. Finally, in order to determine differences in the consumption of *A. aquaticus*, we loaded each enclosure with one Decotab (0.3 g wet weight consisting of 2% agar and 6% finely ground hay). We also loaded three test vessels with one Decotab, but no animals, per treatment to correct for possible microbial decomposition. After test vessel retrieval, we dried the Decotabs for 3 days at 60 °C and weighed them subsequently using a microbalance.

Statistical analyses

Chlorophyll A concentration and the endpoints survival, consumption, emergence and reproduction were analyzed by means of Factorial ANOVA (type 3 SS) in order to explore the effects of thiacloprid addition (two levels: yes/no) and nutrient addition (two levels: yes/no) and their possible combined effects. Similarly, we explored the possible effects of thiacloprid and nutrients on the water chemistry parameters water temperature, pH, DO concentration and conductivity. To further investigate possible differences between treatments (four levels: control, thiacloprid, nutrients, thiacloprid & nutrients), we performed One-way ANOVAs followed by Tukey post-hoc tests. As we measured multiple individuals per ditch to determine the daily growth rate, we used linear mixed effects models (function 'lme' of package 'nlme') and nested individuals in their respective experimental ditch to account for possible effects of the ditch. For each model, we tested for homogeneity of variances using Levene's tests and for normality of the random factor and model residuals using Quantile-Quantile plots. If either of these assumptions were not met, data were either log10 or square root transformed to improve their fit. As data transformation did not improve the normality of the survival data for *C. riparius*, we tested these data with Kruskal-Wallis tests. Statistical analyses were performed using R-studio, version 1.0.153 (R version 3.4.1; R Core Team, 2017).

2.3 Results

The average ditch temperature was between 18 °C (SD 0.33) and 24 °C (SD 0.54) during the exposure period of the animals and did not differ between treatments ($p > 0.05$). Nutrient addition significantly increased the pH over time with a TWA of 0.27 (SD 0.21) during the exposure period ($F_{1,29} = 18.7$, $p < 0.001$). Nutrient addition also significantly increased DO concentrations at $t=7$ ($F_{1,32} = 46.9$, $p < 0.001$) compared to the treatments that did not receive additional nutrients. In addition, long-term monitoring (9 months; data not shown) of the experimental ditches never showed DO concentrations below 9.5 mg/L, meaning there is no oxygen deficiency. Unexpectedly, nutrient addition also significantly decreased conductivity interacting with time with an average of 57 $\mu\text{S}/\text{cm}$ (SD 34) during the exposure period ($F_{1,29} = 5.56$, $p = 0.020$), likely ascribed to the depletion of bicarbonate (HCO_3^-) because of increased algal photosynthesis (see below). There was no observed effect of thiacloprid on the tested water chemistry parameters. The actual TWA (3 weeks) exposure concentration of thiacloprid was 0.51 $\mu\text{g}/\text{L}$ (SD 0.08). The average DT50 and DT90 (calculated for both spikes individually and averaged subsequently) of thiacloprid in water was 3.3 days (SD 0.1) and 11.1 days (SD 0.4) respectively. Our observed DT50 and DT90 values were comparable as those summarized by the European Chemicals Agency (ECHA, 2015), who reported DT50 and DT90 values of 2.9 and 9.7 days in a pond setup. There was no effect of nutrient enrichment on thiacloprid concentration at a given time point ($p > 0.05$). Ditches without added thiacloprid showed no presence of the compound (all values below detection limit). As expected, both time weighed averages of nitrate and phosphate concentrations significantly increased by nutrient addition ($F_{1,32} = 10.6$, $p = 0.002$; $F_{1,32} = 28.8$, $p < 0.001$, respectively) over the course of the experiment. On average, TWA concentrations of nitrate and phosphate increased by a factor of 1.5 (SD 0.5) and 2.2 (SD 0.9) respectively. There was no effect of thiacloprid on the measured nutrient concentrations ($p > 0.05$ for all comparisons). All time weighed averages of these exposure conditions are provided in Table 2.1.

Chlorophyll A concentrations did not differ between treatments before the addition of the agrochemicals ($p > 0.05$) (see Supplement, Table 2.S1).

Four days after nutrient addition, chlorophyll A concentrations in the water significantly increased in nutrient enriched ditches by a factor 1.9 (SD 0.9) on average ($p < 0.001$) compared to ditches that had not received additional nutrients. Three days before enclosure retrieval, chlorophyll A concentrations were significantly lowered by thiacloprid addition ($F_{1,32} = 13.7$, $p < 0.001$) and was significantly lowest in the mixture treatment compared to the control and nutrient treatment ($p = 0.019$ and $p = 0.011$ respectively) but not the thiacloprid treatment ($p > 0.05$) respectively.

After 21 days of exposure, thiacloprid or nutrients did not significantly affect the survival or growth of the crustacean species *A. aquaticus* (Table 2.2). Survival of *A. aquaticus* was 31% (SD 23) in the control treatment (Figure 2.1A) and these animals grew on average 0.12 mm per day (SD 0.06, Figure 2.1B). In contrast to the absence of any treatment effect on these endpoints, consumption significantly decreased by thiacloprid addition ($F_{1,27} = 5.33$, $p = 0.029$; Table 2.2). Decotab consumption was 73% lower in the thiacloprid treatment and 45% in the thiacloprid and nutrient mixture compared to the control treatment (Figure 2.1C), however this difference was not confirmed statistically by the One-way ANOVA analysis ($p = 0.08$ and $p = 0.09$ respectively). We recognize that Decotab consumption is likely affected by the number of animals within the enclosure, which was severely reduced in all treatments irrespective of the treatment. However, as we have no knowledge on when mortality occurred (and thus feeding of an individual stopped), we chose to not standardize Decotab consumption to the number of animals remaining in the enclosure at $t=21$. We believe this to be a valid method as the survival between treatments was not statistically different at $t=21$. For the crustacean species *D. magna*, survival was 59% (SD 37) in the control treatment and we did not detect any differences between treatments after 21 days of exposure ($p > 0.05$; Figure 2.1D). However, both growth ($F_{1,61} = 6.00$, $p = 0.017$) and reproduction ($F_{1,26} = 16.5$, $p < 0.001$) were significantly reduced by thiacloprid addition (Table 2.2). Growth was significantly reduced in the thiacloprid treatment compared to the control ($p = 0.028$) and nutrient treatment ($p = 0.027$ respectively) but not compared to the thiacloprid and nutrient mixture ($p > 0.05$; Figure 2.1E). At the end of the experiment, *D. magna* females within the control

treatment produced an average of 58 (SD 11) neonates per female while not being fed by the researchers, meaning only food was consumed as they could find in the experimental ditches. The average reproduction of 58 neonates approached the recommended 60 neonates (under *ad libitum* food conditions in the laboratory) as stated by the OECD guideline (OECD, 2012). Within the thiacloprid treatment, reproduction was significantly lowered compared to all other treatments ($p < 0.05$) with an average reduction of 51% (SD 19) compared to the control (Figure 2.1F). In addition, we observed and collected ephippia (resting eggs) in all treatments but these were not found in the control. In total, 1 ephippium was found in the nutrient treatment, 14 in the thiacloprid treatment and 3 in the mixture treatment. A presence/absence analysis (glm) showed that the number of *D. magna* enclosures where ephippia were observed was significantly higher in the thiacloprid treatment compared to the control ($p = 0.002$) and nutrient treatment ($p = 0.010$). We also found a marginally higher number of enclosures with ephippia in the thiacloprid treatment compared to the mixture treatment ($p = 0.061$).

In contrast to the crustacean species, the agrochemicals did affect the survival of both insect species. The survival of the insect *C. dipterum* was significantly decreased by thiacloprid ($F_{1,32} = 10.9$, $p = 0.002$). We also observed a marginally significant interaction effect of thiacloprid and nutrients ($F_{1,32} = 3.68$, $p = 0.064$). Survival in the thiacloprid treatment was significantly reduced by 51 to 56% (SD 24-26) compared to the other treatments ($p < 0.05$ for all comparisons; Figure 2.1G). Survival of the mixture treatment did not differ from either the control or the nutrient treatment ($p > 0.05$). Toxicity of thiacloprid was not observed for the growth and emergence of *C. dipterum*, however, these endpoints were significantly increased by nutrient addition ($F_{1,30} = 6.49$, $p = 0.016$ and $F_{1,32} = 11.8$, $p = 0.002$ respectively). Growth significantly increased in the nutrient and mixture treatment compared to the control treatment ($p \leq 0.05$ for both comparisons) and compared to the thiacloprid treatment ($p < 0.001$ for both comparisons; Figure 1H). After 21 days of exposure, on average 12% (SD 21) of animals within the control treatment emerged (Figure 2.1I). Emergence in the thiacloprid treatment was lower, on average 2% (SD 6), represented by a single emerged individual; but we

could not confirm this statistically ($p > 0.05$). The nutrient treatment showed a significantly higher emergence of 41% (SD 23), compared to both the control and thiacloprid treatment ($p = 0.008$ and $p < 0.001$ respectively). Emergence was also increased in the mixture treatment, 20% (SD 16), but only deviated statistically from the thiacloprid treatment ($p = 0.033$; Figure 2.1I; Supplement Figure 2.S2).

There was no observed effect of thiacloprid on the different endpoints of the insect species *C. riparius*. However, nutrients showed significant effects on both survival ($\chi^2_{1,38} = 4.67$, $p = 0.030$) and growth ($F_{1,32} = 4.06$, $p = 0.052$; Table 2.2). Average survival in the control and thiacloprid treatment was 70% (SD 24) and 69% (SD 26) respectively and increased to 88% (SD 17) and 90% (SD 20) for the nutrient and mixture treatment, although no significant differences between individual treatments were observed ($p > 0.05$; Figure 2.1J). Growth was highest in the mixture treatment and deviated statistically from both the control ($p = 0.004$) and thiacloprid treatment ($p < 0.001$), but not from the nutrient treatment ($p > 0.05$; Figure 2.1K). Similarly, growth was increased in the nutrient treatment, but only differed significantly from the thiacloprid treatment ($p = 0.017$) and not the control ($p = 0.096$). While many *C. riparius* individuals appeared nearly ready to emerge after retrieval of the first enclosure, we only recorded low number of emergence (on average $\leq 7\%$ per treatment; Figure 2.1L). This was due to unfortunate escape of emerged individuals as the emergence traps were blown away. We therefore excluded these data from further analysis.

Table 2.1. Time weighed averages ($\pm$ SE; 21 days) of the water chemistry parameters per treatment during the exposure period^a.

Parameter	Treatment			
	C	T	N	TN
Conductivity (μ S/cm)	783 ($\pm$ 11.6)	760 ($\pm$ 8.08)	727 ($\pm$ 8.36)	702 ($\pm$ 13.3)
Oxygen (mg/L)	12.4 ($\pm$ 0.62)	12.1 ($\pm$ 0.37)	13.8 ($\pm$ 0.59)	14.2 ($\pm$ 0.31)
Nitrate (mg/L)	1.28 ($\pm$ 0.18)	1.04 ($\pm$ 0.15)	1.81 ($\pm$ 0.23)	1.70 ($\pm$ 0.17)
pH	8.07 ($\pm$ 0.03)	8.03 ($\pm$ 0.02)	8.35 ($\pm$ 0.08)	8.31 ($\pm$ 0.06)
Phosphate (mg/L)	0.17 ($\pm$ 0.06)	0.13 ($\pm$ 0.03)	0.38 ($\pm$ 0.06)	0.27 ($\pm$ 0.03)
Temperature ($^{\circ}$ C)	21.9 ($\pm$ 0.01)	21.9 ($\pm$ 0.01)	22.0 ($\pm$ 0.02)	21.9 ($\pm$ 0.01)
Thiacloprid (μ g/L)	< DL	0.49 ($\pm$ 0.03)	< DL	0.53 ($\pm$ 0.03)

^a For nitrate and phosphate time weighed averages are given since the start of enrichment (35 days). 'C' = control, 'T' = thiacloprid, 'N' = nutrients, 'TN' = thiacloprid and nutrients.

<DL: below detection limit (100 ng/L).

Table 2.2. The overall effects of thiacloprid and nutrient addition on the different endpoints of the test species.

Species	Endpoint	Treatment		
		T	N	TN
<i>A. aquaticus</i>	Survival	0.442	0.251	0.415
	Growth	0.324	0.785	0.361
	Consumption	0.029	0.644	0.340
<i>D. magna</i>	Survival	0.635	0.645	0.844
	Growth	0.017	0.530	0.179
	Reproduction	< 0.001	0.710	0.092
<i>C. dipterum</i>	Survival	0.002	0.708	0.064
	Growth	0.691	0.016	0.339
	Emergence	0.234	0.002	0.703
<i>C. riparius</i>	Survival	0.771	0.031	n.d.
	Growth	0.549	0.052	0.422
	Emergence	n.d.	n.d.	n.d.

Shown are the p-values of the comparisons assessed with (nested) Factorial ANOVA or Kruskal-Wallis. 'T' = thiacloprid addition, 'N' = nutrient addition, 'TN' = thiacloprid and nutrient addition, 'n.d.' = not determined. Significant effects ($p \leq 0.05$) are indicated in bold.

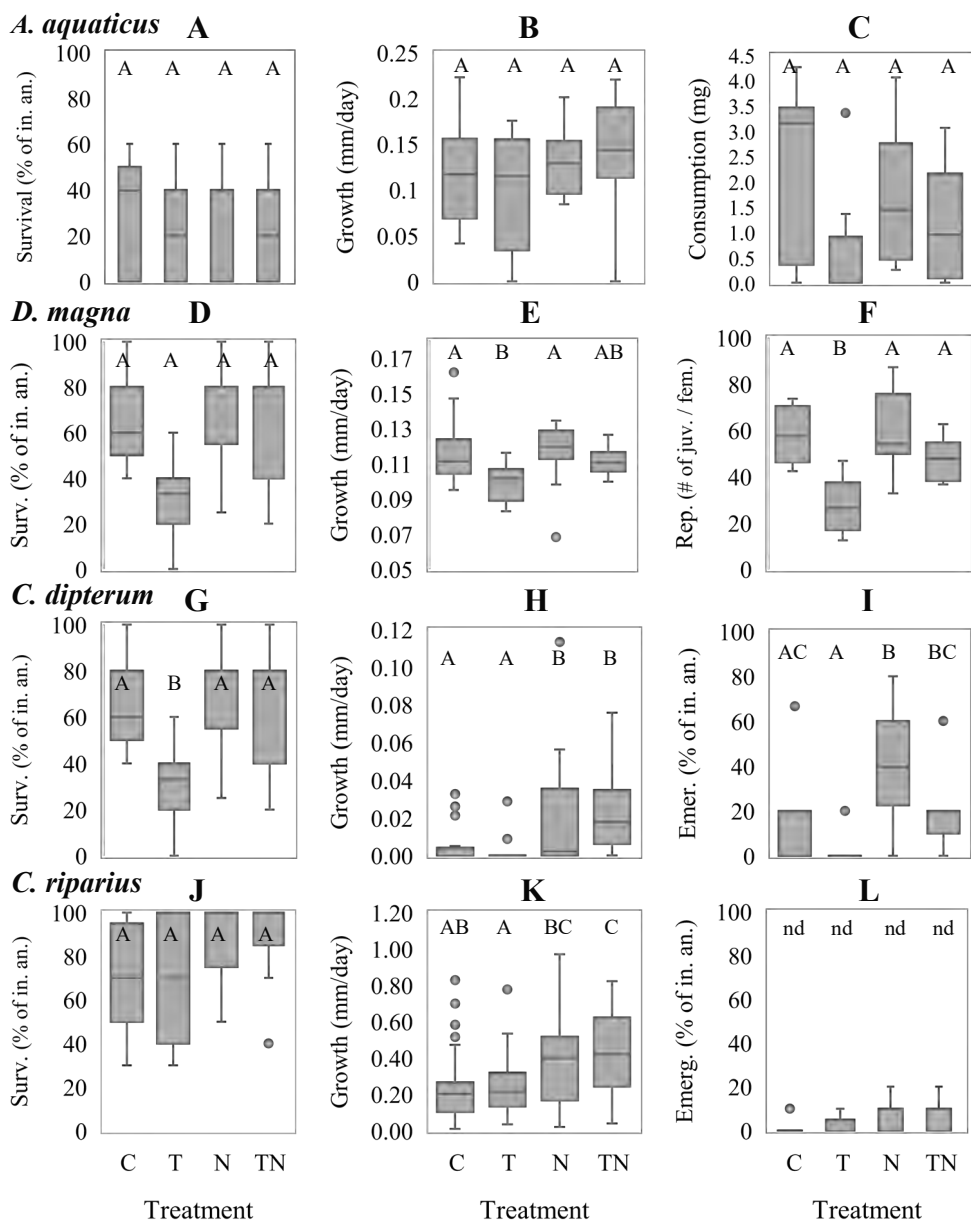


Figure 2.1. Box and Whisker plots of the survival (percentage of initial animals), growth (mm/day) and consumption (mg) / cumulative emergence (% of initial animals) / cumulative reproduction (number of juveniles per female) for *A. aquaticus* (A, B, C), *D. magna* (D, E, F), *C. dipterum* (G, H, I) and *C. riparius* (J, K, L) after 21 days of exposure to thiacloprid and/or nutrient addition (14 days for *C. riparius* survival and growth). Different letters ('A', 'B', 'C') indicate significant differences (One-way ANOVA + Tukey's post-hoc) between treatments at significance level $p \leq 0.05$. 'C' = control, 'T' = thiacloprid, 'N' = nutrients, 'TN' = thiacloprid and nutrients, 'Surv.' = survival, 'in. an.' = initial animals, 'Rep.' = reproduction,

2.4 Discussion

This study aimed to quantify the possible interactions between nutrients and thiacloprid on specific endpoints of four aquatic invertebrate species under semi-field conditions. We showed toxicity of thiacloprid to different endpoints for two crustacean and one insect species. We observed no toxicity to the insect species *C. riparius*. Overall insect fitness increased due to nutrient enrichment for the endpoints survival, growth and emergence (dependent on species). More importantly, we observed reduced thiacloprid toxicity under nutrient enriched conditions.

Both crustacean species were significantly impacted by exposure to thiacloprid. Thiacloprid exposure significantly reduced (45-73% compared to the control) the consumption of *A. aquaticus* but not its survival nor growth. To our knowledge, chronic toxicity data for *A. aquaticus* is currently lacking, except for post-exposure toxicity effects provided by Beketov and Liess (2008) who reported an 18d LOEC for survival of 287 µg/L, a concentration that is out of limits for environmental relevance. Thus, this study is the first to show that the consumption of *A. aquaticus* may be significantly impacted at thiacloprid levels currently residing in surface waters. Although we observed no effects on either survival or growth, longer-term experiments might show reduced fitness because of a thiacloprid-induced lack of nutrition for this species. More substantial toxicity data for *D. magna* was available in literature; Pavlaki et al. (2011) report 21d LOECs of 1250 µg/L for both reproduction and body length while Beketov and Liess (2008) report a post-exposure 21d LOEC for survival of 4740 µg/L. As expected, we observed no effect of thiacloprid on the survival of *D. magna*. However, we did observe significant thiacloprid-induced toxicity for both growth (15% reduction compared to the control) and reproduction (51% reduction compared to the control) at environmentally relevant concentrations that are a 2456 fold lower than the reported LOEC values by Pavlaki et al. (2011). In addition, within the thiacloprid treatment we observed significant ephippium production, which is behavior of *D. magna* to endure unfavorable environmental conditions (Ebert, 2005). These toxic effects observed for *D. magna* can be direct or indirect toxicity or a combination thereof. Direct toxicity would mean that thiacloprid would

directly reduce the fitness of *D. magna*, but such toxicity at our experimental concentration would be absent when animals are exposed under stable lab conditions (e.g. the study of Pavlaki et al., 2011). This can be explained by the fact that the experimental ditch is a multi-stress environment, which may add to observed toxicity (Clements et al., 2012). Indirect effects would mean that the aquatic invertebrate communities residing in the ditches possibly depressed algae concentrations (food quantity) and/or altered the algal community composition (food quality). Our observed toxic effects of thiacloprid (being direct or indirect) were absent under nutrient enriched condition. This indicates the importance of nutrients that led to increased food availability and/or quality for *D. magna*, enabling the species to cope with neonicotinoid-induced toxicity. We strongly suspect that this lack of toxicity is a result of compensatory feeding (Alexander et al., 2007; Goedkoop et al., 2010) on free-floating algae as chlorophyll A concentrations were lowest in the mixture treatment. Such chlorophyll A depressing effects have been suggested earlier by Alexander et al. (2013). While high nutrient loadings within agricultural ditches are a common ecosystem property (Janse and Puijenbroek, 1998), *D. magna* populations residing in environments with lower nutrient loadings may be currently at risk when exposed to thiacloprid. Such apparent risks are likely to be missed by common laboratory approaches as they rely on static test conditions with regular high-quality feeding regimes (e.g. OECD, 2012). These tests are missing the mechanism that poor food quality can reduce the fecundity of *Daphnia* (Van Donk et al., 1997) which may add to toxicity. This is illustrated by Ieromina et al. (2014) who showed that poor food quality as measured on P-contents of algae indeed increased the sensitivity of nontarget grazing species to the neonicotinoid insecticide imidacloprid. Thus, the common laboratory tests might severely underestimate neonicotinoid-induced toxicity in the long term.

The insect species *C. dipterum* showed strongly reduced survival within the thiacloprid treatment (53% compared to the control) conform previous results on the severe toxicity of neonicotinoids to this mayfly species (Roessink et al., 2013; Van den Brink et al., 2016). However, when *C. dipterum* was exposed to thiacloprid under nutrient enriched

conditions, this toxicity was not detected. Such absence of toxicity can be, comparable to the argument made for *D. magna*, due to stimulated primary production which allows for compensatory feeding (Alexander et al., 2007; Goedkoop et al., 2010). Nutrient enrichment stimulated the growth and emergence of *C. dipterum*, thus completing its aquatic life stage more quickly. We found no significant effects of thiacloprid on these endpoints. However, emergence within the thiacloprid treatment was represented by a single emerged individual while all other treatments showed increasing emergence over time (Supplement Figure 2.S2). Thus, it is likely that effects of thiacloprid on emergence would become apparent with a longer test duration. Similar to *C. dipterum*, nutrient enrichment also increased the growth of *C. riparius* as well as its survival. However, we found no effects of thiacloprid on either of these measured endpoints. This was unexpected as Langer-Jaesrich et al. (2010) report a 17d LOEC for survival of 0.5 µg/L, which is equal to the time weighed average water concentration used in the present study. A possible explanation for the absence of toxicity is that we did not actively spike thiacloprid into the sediment (as we simulated spray drift), thus lowering the actual exposure compared to Langer-Jaesrich et al. (2010) who did spike the, comparable, sediment (OECD, 2004b).

Survival within our control treatment for each test species was never one-hundred percent. This is a clear indication that there were additional environmental stressors present within the test setup. This was expected as we tested the species in experimental ditches that yielded full ecosystem complexity (although we excluded biotic processes such as predation in our cage setup). There can be numerous environmental factors that might have reduced our control survival (weather patterns, fluctuating water chemistry, etc.). Such additional stress probably added to toxicity, which was likely the case for *D. magna* as we observed far lower toxicity values compared to laboratory studies (Pavlaki et al., 2011). This is in accordance with many aquatic toxicologists that showed altered toxicity of chemicals when additional environmental factors were assessed (Clements et al., 2012). Overall, we showed toxicity induced by environmentally relevant concentrations of the neonicotinoid thiacloprid to three aquatic invertebrate species. For the insect species *C. riparius*, we

observed no toxic effects on the measured endpoints. Sensitivity of the organisms to thiacloprid often deviated from laboratory derived toxicity values, especially for *D. magna*.

2.5 Conclusions

We showed the importance of nutrient enrichment (and the resulting increase in primary production) for coping with neonicotinoid-induced toxicity. It is likely that such neonicotinoid-induced toxicity is often not observed in laboratory experiments due to high-quality feeding regimes that allow for compensatory feeding. Similarly to laboratory-derived data, this might explain that this toxicity is not observed in agricultural ditches as the common high nutrient loadings allow for compensatory feeding as well. Thus, of all the natural stressors and abiotic fluctuations that are abundant in the field, *ad libitum* feeding as often done in the laboratory conform guidelines can explain much of the discrepancies between laboratory and field derived ecotoxicity data observed in oligotrophic conditions. This warrants for the inclusion of different feeding regimes in laboratory experiments in order to retrieve the best estimates of neonicotinoid-induced toxicity in the natural environment. Often many water quality management actions focus either on a reduction of nutrient or a reduction of pesticides emissions for the protection of biodiversity. As our data show that the relative pressure of neonicotinoids to aquatic biodiversity may become relatively more pronounced when nutrient emissions are reduced, we plea that the greatest protection of aquatic biodiversity is achieved by reducing the emissions of both agrochemicals.

Acknowledgements

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Data accessibility

Additional data is available in the supplement and the basic data can be obtained by the first author via e-mail (s.h.barmentlo@cml.leidenuniv.nl).

Supplement

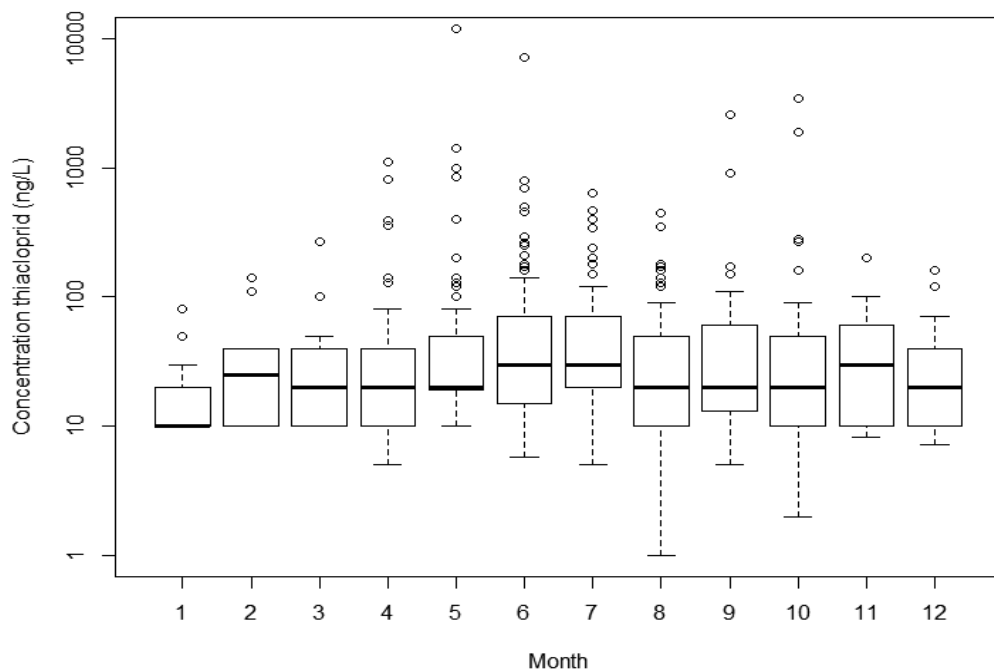


Figure 2.S1. Box and Whisker plot of above detection limit thiacloprid concentrations (ng/L, 608 observations) measured in Dutch surface waters from 2011 to 2015 per month of the year. For visualization purposes, we only show the data per month and not year as well. The detection limit of thiacloprid was 10 or 20 ng/L in most cases. These data were gather by Dutch Water Boards and derived from the Dutch Pesticide Atlas (Pesticide Atlas, 2016).

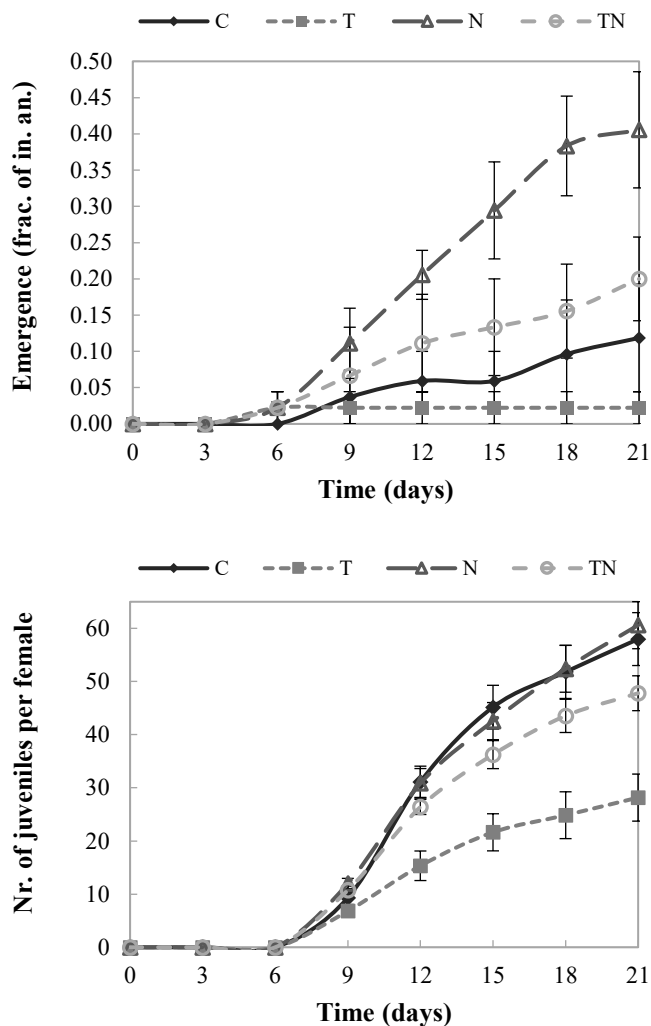


Figure S2. Cumulative emergence (average fraction of initial animals $\pm$ SE, $n = 9$) for *C. dipterum* (left Figure) and reproduction (average number of juveniles per female $\pm$ SE, $n = 9$) for *D. magna* (right Figure) over time. Nutrients were added on $t=-14$ and thiocloprid was spiked on $t=0$ and $t=14$. 'C' = control, 'T' = thiocloprid, 'N' = nutrients, 'TN' = thiocloprid and nutrients, 'in. an.' = initial animals.

Table 2.S1. Average chlorophyll A concentrations ($\pm$ SE, n = 9). 'C' = control, 'T' = thiacloprid, 'N' = nutrients, 'TN' = thiacloprid and nutrients. t=0: time of first thiacloprid spike. Nutrients were added on t=-14.

Time (days)	Treatment			
	C	T	N	TN
-18	3.43 ($\pm$ 0.10)	3.17 ($\pm$ 0.14)	3.16 ($\pm$ 0.10)	3.06 ($\pm$ 0.08)
-10	1.22 ($\pm$ 0.41)	1.20 ($\pm$ 0.27)	2.22 ($\pm$ 0.42)	2.30 ($\pm$ 0.30)
18	3.94 ($\pm$ 0.76)	1.81 ($\pm$ 0.63)	6.26 ($\pm$ 2.21)	1.10 ($\pm$ 0.24)

Chapter 3

Environmental levels of neonicotinoids reduce prey consumption, mobility and emergence of the damselfly *Ischnura elegans*

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Abstract

1. Freshwaters are among the most endangered ecosystems in the world as a result of anthropogenic interference such as pollution. Pollution in the form of neonicotinoids has been intensively studied, but data thus far is often conflicted by contrasting responses between laboratory and field experiments. In addition, toxicity data is scarce and contradictory for insects such as Odonates (dragonflies and damselflies) and a potential risk to them may therefore be overlooked.
2. We investigate the potential risk of neonicotinoids to Odonates by exposing nymphs of the blue-tailed damselfly *Ischnura elegans* to environmentally relevant concentrations of the neonicotinoid thiacloprid. We consider *I. elegans* as an indicator species for other Odonates as it is an abundant, widespread and eurytopic species. We analyze the effects of thiacloprid on multiple endpoints (survival, consumption, growth, molting, mobility and emergence), using cage-experiments as well as controlled field observations in naturally colonized experimental ditches. In addition, we assess sensitivity by either feeding the damselfly nymphs with lab-cultured prey or by letting them feed freely on natural aquatic invertebrates.
3. All sublethal endpoints of *I. elegans* are affected to some degree, and strongly depend on the food offered; free-feeding nymphs are more sensitive than culture-fed nymphs. Environmental relevant concentrations of thiacloprid strongly reduce the emergence of *I. elegans* and this effect is more substantial in the natural populations compared to the caged damselflies. This is likely explained by exclusion of additional biotic pressures such as predation in the caged experiment.
4. *Policy implications.* Literature reports that one out of seven Odonates is threatened and 24% of the species have declining populations. Our observations show that current risks of neonicotinoids to Odonates are underestimated in laboratory experiments as the toxicity is governed by multiple biotic factors such as food quantity/quality and predation. Given the widespread

abundance of blue-tailed damselfly *Ischnura elegans*, the observed sensitivity to neonicotinoids and current population trends of this species, these results indicate neonicotinoids play a central role in the Odonate decline in general.

Keywords: Biomonitoring, Freshwater, Insecticide, Insect decline, Neonicotinoids, Odonata, Zygoptera

3.1 Introduction

Pesticides have been indicated as a potential pollution threat to freshwater ecosystems (Tilman et al., 2001; Beketov et al., 2013), one of the most endangered ecosystems in the world (Dudgeon et al., 2006). Currently, neonicotinoid insecticides receive much of the attention as their usage have increased sharply over the past two decades (Simon-Delso et al., 2015) and they are now the most commonly used insecticides globally (Jeschke et al., 2011).

Neonicotinoids combat insect pest species by binding to the nicotinic acetylcholine receptors (nAChR), which induces continuous excitation of the neuronal membranes leading to paralysis (Simon-Delso et al., 2015). Like most agricultural chemicals, neonicotinoids are often found in surface waters due to processes such as run-off, drift and leaching (Morrissey et al., 2015). The effects of neonicotinoids on non-target freshwater organisms have been studied intensively in the laboratory (for example Roessink et al., 2013 and Raby et al., 2018), but the data is actually scarce for many groups, such as Odonates (dragonflies and damselflies). Consequently, scientists that focused on the risk of neonicotinoids to Odonates (e.g. Morrissey et al., 2015; Vijver et al., 2017) largely had to interpolate their assessment on only two bioassays (Beketov and Liess, 2008; Jinguji et al., 2013). These studies showed that the survival and emergence of the dragonfly genus *Sympetrum* was negatively affected by concentrations that are higher than those typically found in natural environments. This contrasts with the study of van Dijk et al. (2013) who indicated possible effects of current environmental levels of neonicotinoids on the abundance of the damselfly *Ischnura elegans*. Nevertheless, the low amount of data available and the contrasting findings indicate that the risk of neonicotinoids to Odonates is currently poorly understood. On top of this, recent findings show that the toxicity of neonicotinoids to aquatic invertebrates is food quantity or quality dependent (Alexander et al., 2013; Ieromina et al., 2014; Barmantlo et al., 2018). High food quality/quantity, as offered in standard laboratory experiments, may allow for higher energy expenditure to cope with the toxic stress (the Dynamic-Energy budget theory, see Nisbet et al., 2010). However, in the field, a plethora of (indirect) effects on the aquatic

communities can limit food quality/quantity and consequently species sensitivity in a laboratory setup can differ from that in the field (Barmentlo et al., 2018).

Approximately one out of seven Odonates is threatened with extinction in Europe and currently 24% of the species have declining populations (Kalkman et al., 2010). Since conservation science of Odonates is limited in general (Bried and Samways, 2015) and toxicity data are scarce and contradictory for this group, a potential hazard of neonicotinoids to Odonates could be overlooked. Therefore, we aimed to investigate if environmentally relevant concentrations of the neonicotinoid thiacloprid could affect a widespread, eurytopic, Odonate: the blue-tailed damselfly *I. elegans*. We investigated the effects of thiacloprid on several endpoints (survival, consumption, growth, molting, mobility and emergence) using an environmentally realistic setup in naturally colonized ditches. Additionally, we investigated toxicity by either feeding the damselfly nymphs with laboratory-cultured prey to have culture-fed specimens versus nymphs that hunted natural aquatic invertebrates to account for the potential indirect effects affecting the food quantity/quality that in turn can alter toxicity. Finally, we compared our toxicity results by monitoring the emergence of natural populations of *I. elegans* that were exposed to the same spikes of thiacloprid.

3.2 Materials and Methods

Test species

We selected *I. elegans* (Zygoptera: Coenagrionidae) for multiple reasons. First and most importantly, it is the most common damselfly in large parts of Europe (Boudot and Kalkman, 2015), including the Netherlands (Van Swaay et al., 2018). Because of its high abundance and widespread distribution, *I. elegans* has the potential to be used as an indicator species for damselflies in general. Second, *I. elegans* is eurytopic (i.e., it can adapt to a wide range of ecological conditions) and found in a range of lowland fresh and brackish waters; it is often the dominant damselfly in highly anthropogenic habitats (Brockhaus et al., 2015). As such, this species is likely to be found in the vicinity of anthropogenic disturbances such as neonicotinoid runoff from the agricultural landscape. Third, because of its preference for slow flowing or standing water and the fact that they can be identified at an early instar, it is a suitable test species for caged bioassays. Fourth and finally, *I. elegans* typically lives in the water up to nearly a year and has a very long emergence period in the Netherlands, from late April till late September (NVL, 2002). Therefore, *I. elegans* has high ecological relevance for both the aquatic and terrestrial food webs as it is an active opportunistic hunter and also because it is common prey for invertebrates, fish, birds and bats (Corbet, 1962).

Experimental setup

Test location. The experiment was conducted at the outdoor research facility of Leiden University: 'the Living Lab' (The Netherlands). The site consists of 36 experimental ditches (1750 L per ditch) with a length of 10 m, a width of 0.8 m and a depth of 0.3 m that are connected to an adjoining lake that provides a natural input of flora and fauna (see Supplement Figure 3.S1 for an photographic overview). After a 5-month colonization period and prior to the start of the experiment, these ditches were hydrologically isolated from the lake by placing a 1000*500*2 mm acrylic plate firmly into the ditch banks and 15 cm deep into the sediment at the end of every ditch.

In situ conditions. The damselfly nymphs used in the *in situ* experiment were caught from the adjoining lake of the experimental site by sweeping with a dipping net (mesh size 0.5 mm). The selected nymphs had an average length of 8 mm (SD 1.6 mm). Each nymph was placed in a cylindrical cage ($\varnothing$: 5.5 cm, height: 6.8 cm), in an experimental ditch. Inoculation was performed 3 days prior to the start of the treatments (see section 2.3) so that the nymphs could acclimatize to the cage. We also placed a thin twig in each cage for the nymphs to rest and hold onto. All cages contained one nymph and each experimental ditch received two cages. These two cages were nearly identical; high-density polyethylene cages with a 3.75 cm opening on one side (in the lid) covered with mesh. One of the cages was closed with a larger mesh size of 1000 μm and the other with a finer mesh size of 150 μm . Smaller invertebrates, such as Daphniidae, Cyclopidae and Diptera larvae, living in the experimental ditches could disperse freely in and out of the cages with the larger mesh, but not the finer. We did not actively feed the nymphs in the cages with the larger mesh. Instead, these nymphs fed on the natural invertebrates living in the experimental that freely dispersed into the cage. Nymphs in the cages with fine mesh were fed daily with < 3 day old daphnids (*Daphnia magna*) retrieved from a longstanding culture. These two food conditions will hereafter be referred to as either the 'Free-feeding' or the 'Fed' treatment, respectively. During the acclimatization period, we fed the nymphs of the 'Fed' treatment 5 daphnids per day. At the start of the experiment (the first thiacloprid spike, see section 2.3), we started feeding the nymphs 10 daphnids daily and increased this number by 5 daphnids every week to fill the need of the growing nymphs, maintaining so-called *ad libitum* food conditions. However, if uneaten daphnids were observed, we subtracted the number of uneaten daphnids from the number of daphnids that we fed.

Exposure conditions

After the initial acclimatization period of the nymphs, thiacloprid was spiked in the experimental ditches. Thiacloprid in Dutch surface waters is increasingly detected starting in April-May (see Barmantlo et al., 2018) with the highest numbers in June and July. This closely equals the season when *I. elegans* emerge. In May, we exposed the nymphs to four nominal

concentrations of thiacloprid; 0 (control), 0.1, 1 and 10 $\mu\text{g/L}$ with nine replicate ditches per concentration. These concentrations have all been measured in Dutch surface waters in the period 2013-2017, with the lower concentrations being observed more commonly (see Table 3.1, Vijver et al., 2008; Leiden University and Rijkswaterstaat-WVL, 2018). However, the octanol-water coefficient for thiacloprid is low ($\log K_{ow} = 1.26$ for thiacloprid, USEPA, 2003) and as such thiacloprid is removed rapidly of the aqueous phase and transported to the sediment. Consequently the chance of detecting the maximum concentrations (1.8% of all detects, as shown in Table 3.1) by grab sampling is low. The findings reported in Table 3.1 are thus likely an underestimate of the maximal concentrations in surface waters but do give guidance to determine environmentally relevant test concentrations. Our experimental concentrations are not exclusive to the Netherlands but are also commonly observed in other waterbodies (Morrissey et al., 2015; Sánchez-Bayo, Goka and Hayasaka, 2016), thus we consider them environmentally relevant.

Stock solutions were prepared by dissolving thiacloprid (99.9% purity, purchased from Sigma-Aldrich) in demineralized water. These stocks were then diluted in a 10 L bottle with water originating from the ditch to which the thiacloprid was added and carefully poured into each ditch while spreading evenly. As > 95% of the initial thiacloprid concentrations degrades after 2 weeks in our test system ($DT_{90} = 11.1$ days; shown by Barmantlo et al., 2018), a second spike was applied after two weeks in order to maintain experimental concentrations for one month. Water quality was monitored on a weekly basis by measuring water temperature, pH, oxygen concentration and conductivity using a portable hq 40d electronic multiparameter meter (Hach). We also monitored thiacloprid concentrations over time of exposure by collecting water samples 5 cm below the surface level of each experimental ditch daily during the first week after a spike and three times a week thereafter. These samples were then analyzed using liquid chromatography–tandem mass spectrometry (Agilent Technologies; see Roessink et al., 2013 for the detailed procedure).

Table 3.1. Thiacloprid concentrations in Dutch surface waters over the period 2013-2017. In total 12,691 unique samples (time * location combinations over 746 locations) were analyzed of which thiacloprid was detected in 801 samples. Data was retrieved from the Dutch Pesticide Atlas (Leiden University and Rijkswaterstaat-WVL, 2018).

Concentration thiacloprid (ng/L)	No. of thiacloprid detects	% of total no. of detect samples
< 10	299	37.3
10 - 100	424	52.9
100 - 1,000	64	8.0
>1,000	14	1.8

Note that the detection limit was often approximately 10 ng/L.

Damselfly performance

Caged individuals. All nymphs were monitored daily on survival, consumption and molting. Nymphs of the 'Free-feeding' treatment (Figure. 3.1B) were also monitored for emergence while the 'Fed' treatment (Figure 3.1A) was terminated after six weeks of inoculation. Food consumption was monitored in the 'Fed' treatment by counting the number of eaten daphnids that were offered the previous day. For the 'Free-feeding' treatment, we counted all invertebrates that were present in the cage and determined them as prey or non-prey (as according to Thompson 1978). Non-edible species (such as snails) were removed from the cages. Molting was monitored by collecting and counting the shed chitin molts in the cages. When animals were nearly ready to emerge - indicated by the presence of fully developed and non-transparent wing-sheaths, 50% of the water in the cage was removed. The twig was replaced with a broader shoot of dry *Typha latifolia*, being a naturally preferred substrate for emergence of *I. elegans*. Cages were returned to the experimental ditch at a slight downward angle so that the mesh remained completely covered with water, but an air pocket was created in the enclosure where the damselflies could emerge on the shoot of *T. latifolia*. Emergence was monitored daily for 5 weeks since the start of emergence. In addition to these daily observations, growth of the nymphs was determined on a weekly basis until the first emergence by temporarily and carefully removing the nymphs from their cages and photographing them on a Petri dish using an eScope DP-M17 USB-microscope camera. Body length was then determined from the top of the head to the start of the caudal gills using ImageJ (version 1.45S). Fleeing behavior of the nymphs was tested on a weekly basis; swimming responsiveness was recorded from three weeks after the first spike of thiacloprid which was from the controls sixth instar onwards (Brochard and van der Ploeg, 2014). Swimming responsiveness was tested as activity occurring within 15 s of gentle stimulation using a plastic pipette, following the OECD guidance for e.g. daphnids (OECD, 2012).

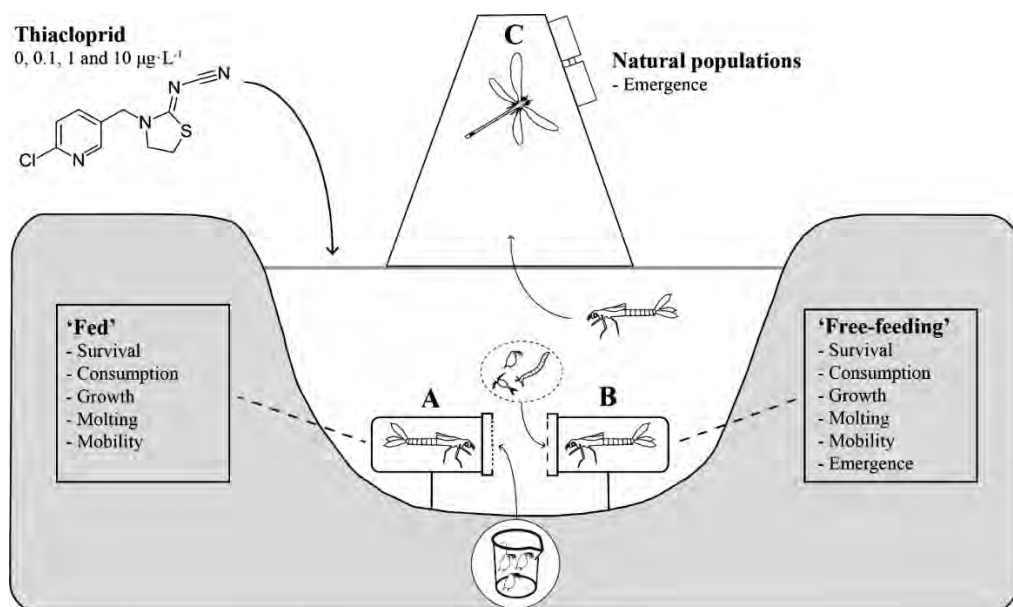


Figure 3.1. Graphical overview of the experimental setup. Shown is a lateral cross section of an experimental ditch with experiment A) caged bioassay with *D. magna* culture-fed nymphs, B) caged bioassay with nymphs living of the natural food supply and C) monitoring of emerged *I. elegans* of naturally colonized populations, exposed to 4 different concentrations of thiacloprid (0, 0.1, 1 and 10 $\mu\text{g}/\text{L}$). Note that invertebrates are not scaled to the equipment.

Natural populations. To quantify the results from our controlled *in situ* setup to actual natural populations of *I. elegans*, natural emergence was monitored by placing one emergence trap above the middle of each ditch (Figure 3.1C). The traps consisted of a 60:60:74 (length:width:height) pyramid-form stainless steel frame fitted with No-See-Um netting (300 holes per cm^2) and the invertebrate collection system as according to Cadmus et al. (2016). The emergence traps were tightly secured to bamboo sticks that were placed horizontally in the ditch banks to hold them in place during the entire experimental period. The emergence traps were installed 3 cm below the water surface level so no emerging insects could escape the trap (or vice versa). Naturally emerging insects were subsequently continuously caught in 100 mL of 80% EtOH and collected from the trap two times per week (every Monday and Thursday) for the

duration of three months. All emerged *I. elegans* were counted subsequently.

Statistical analyses

We tested for the effect of thiacloprid on all different endpoints and the emergence of natural populations of *I. elegans* by using linear mixed-effect models (*lme*, package 'nlme') with the actual concentration of thiacloprid, time and their possible interaction as explanatory variables. As we remeasured the same individuals, we nested the nymphs within the respective ditch they resided in. We used a similar model for the emergence of the caged nymphs, but accounted for the binomial distribution of the data (*glmer*, package 'lme4'). For the models investigating growth, molting and emergence we added the initial length per nymph, and all possible interactions, as this could potentially affect the growth rate and total number of molts. We assessed normality of the model and random variable residuals using QQ-plots and tested for sphericity using the function *ezANOVA* (package 'ez'). Prior to these analyses, we removed one individual from the data due to an accidental release. To further investigate the responsiveness of the nymphs in respect to mobility (swimming), we used Dose-response metrics (function *drm*, package 'drc'). To find the Lowest Observed Effect Concentration (LOEC) of thiacloprid on this responsiveness, we used Dunnet's post-hoc test when a significant effect of thiacloprid was observed. All statistical analyses were performed using R (version 3.5.0; R Core Team, 2019).

3.3 Results

Treatments. Actual concentrations closely followed the nominally applied concentrations (see Table 3.2). Thiacloprid was rapidly removed from the aqueous phase as DT50 and DT90 values, calculated by first order kinetics, were 3.6 and 12.0 days respectively. These values are very similar to those of the study of Barmentlo et al. (2018; DT50 = 3.3 and DT90 = 11.1 days), who also followed the degradation of thiacloprid in the same experimental ditches. Thiacloprid slightly decreased the time-weighted average (TWA) of conductivity by $21 \mu\text{S cm}^{-1}$ at the highest test concentration ($R^2 = 0.21$, $F_{1,34} = 8.9$, $p = 0.005$). Both the TWA of pH and oxygen concentrations increased slightly with increasing concentrations of thiacloprid as well ($R^2 = 0.52$, $F_{1,34} = 36.7$, $p < 0.001$ and $R^2 = 0.20$, $F_{1,34} = 8.3$, $p = 0.007$ respectively, see Supplement Table 3.S1). These alterations in physicochemical characteristics were likely the result of altered primary production as also observed earlier in our experiments (see Barmentlo et al., 2018). The average water temperature was 22.4°C during the experimental period and was not influenced by thiacloprid ($p > 0.05$; see Supplement Table 3.S1). In addition, oxygen concentrations were never below 9.65 mg/L , so no oxygen deficiency occurred.

Effects on survival. Survival was 100% in all cases apart from one death individual in the 'Fed' as well as in the 'Free-feeding' treatment. Thus no significant effects of thiacloprid on survival were observed.

Table 3.2. Average thiacloprid concentrations ($\pm$ SE, n=9) of the two spikes and the time-weighted average (TWA) concentration per treatment.

Nominal conc. thiacloprid ($\mu\text{g/L}$)	Actual conc. thiacloprid ($\mu\text{g/L}$) First spike	Actual conc. thiacloprid ($\mu\text{g/L}$) Second spike	TWA conc. thiacloprid first month ($\mu\text{g/L}$)
0.0	< DL	< DL	< DL
0.1	0.093 ($\pm$ 0.003)	0.075 ($\pm$ 0.003)	0.030 ($\pm$ 0.016)
1.0	0.894 ($\pm$ 0.013)	0.802 ($\pm$ 0.015)	0.284 ($\pm$ 0.024)
10.0	9.662 ($\pm$ 0.215)	9.726 ($\pm$ 0.248)	3.300 ($\pm$ 0.309)

DL = Detection Limit: 0.012 $\mu\text{g/L}$. 'conc.' = concentration.

Effects on consumption. The food consumption by the nymphs was clearly affected by thiacloprid. Nymphs that were fed consumed significantly less daphnids with increasing concentrations and this effect interacted with time of exposure (marginal $R^2 = 0.43$, $F_{1,34} = 7.0$, $p < 0.001$); effects were stronger during the first week of the experiment (Figure 3.2A). On the final day, the sum of unconsumed daphnids increased relative to the control on average by 210, 225 and 469% for the actual spike concentrations 0.08, 0.85 and 9.86 $\mu\text{g/L}$ respectively ($R^2 = 0.27$, $F_{1,33} = 12.6$, $p = 0.001$). In contrast to the effect on food consumption, the total available number of prey (predominantly waterfleas and copepods, but also fly and midge larvae, mayflies, aquatic mites, caddisflies and ostracods) in the 'Free-feeding' cages did not follow a dose-response metric, but was significantly affected by thiacloprid (marginal $R^2 = 0.41$, $F_{1,34} = 474.7$, $p < 0.001$); we observed a decrease in potential prey by 24% at 0.08 $\mu\text{g/L}$ and an increase by 67% at 9.86 $\mu\text{g/L}$ thiacloprid compared to the control (Figure 3.2B).

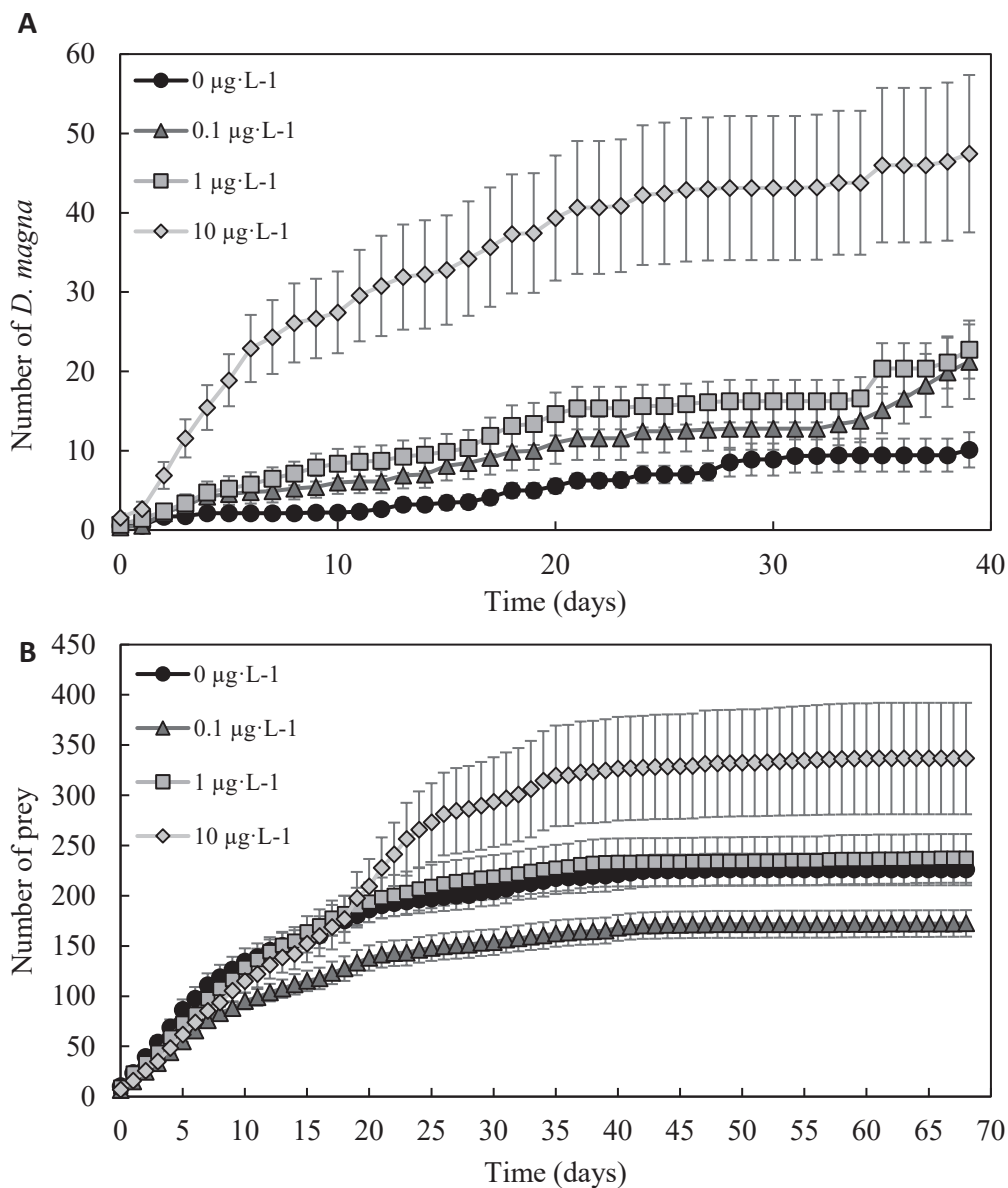


Figure 3.2. Average cumulative number ($\pm\text{SE}$, $n = 8-9$) per day of A) unconsumed *D. magna* in the ‘Fed’ treatment and B) unconsumed potential prey in the ‘Free-feeding’ treatment. Spiking of thiacloprid was conducted at $t=0$ and $t=14$.

Effects on growth and molting. We observed no effect of thiacloprid on the growth of nymphs in the 'Fed' treatment ($p > 0.05$, Figure 3.3A). However, growth of the nymphs in the 'Free-feeding' treatment was significantly delayed by increasing concentrations of thiacloprid interacting with time and the initial length per nymphs (marginal $R^2 = 0.71$, $F_{1,32} = 4.0$, $p = 0.047$, Figure 3.3B). The cumulative growth was slowest three weeks after the start of the treatments (one week after the second spike), by 28% at 9.86 $\mu\text{g/L}$ thiacloprid. There was no significant difference in total growth at the end of the measurements ($p > 0.05$). Similar to the results for growth, we observed no effect of thiacloprid on the cumulative number of molts of the culture-fed nymphs ($p > 0.05$, see Supplement Figure 3.S2A), while molting of nymphs in the 'Free-feeding' treatment was significantly delayed (interacting with the initial length; marginal $R^2 = 0.61$, $F_{1,32} = 19.4$, $p < 0.001$, see Supplement Figure 3.S2B). There was no significant difference in the total number of molts at the end of the measurements ($p > 0.05$).

Effects on mobility. Swimming ability of the nymphs declined in both feeding treatments with increasing concentrations (Figure 3.4A,B). In nearly all cases, the animals that started swimming did so within 1-2 s after gentle stimulation (observation not shown). The LOEC of nymphs within the 'Free-feeding' treatment was 0.84 $\mu\text{g/L}$ (Dunnett's test: $t = -3.1$, $p = 0.010$), which was close to our obtained 50% Effect Concentration (EC_{50}) value of 1.04 $\mu\text{g/L}$ (95% CI: 0.38 - 1.71). After this initial decline, the swimming ability increased again over time (marginal $R^2 = 0.40$, $z = 2.2$, $p = 0.031$, Figure 3.4A,B). The decline was weaker in the culture-fed nymphs, for which maximally 44% reduction in swimming ability at the highest test concentration was observed versus 100% in the 'Free-feeding' treatment. In addition, the EC_{50} within the 'Fed' treatment was higher than the highest spike concentration ($> 9.7 \mu\text{g/L}$) and no statistically significant LOEC could be determined.

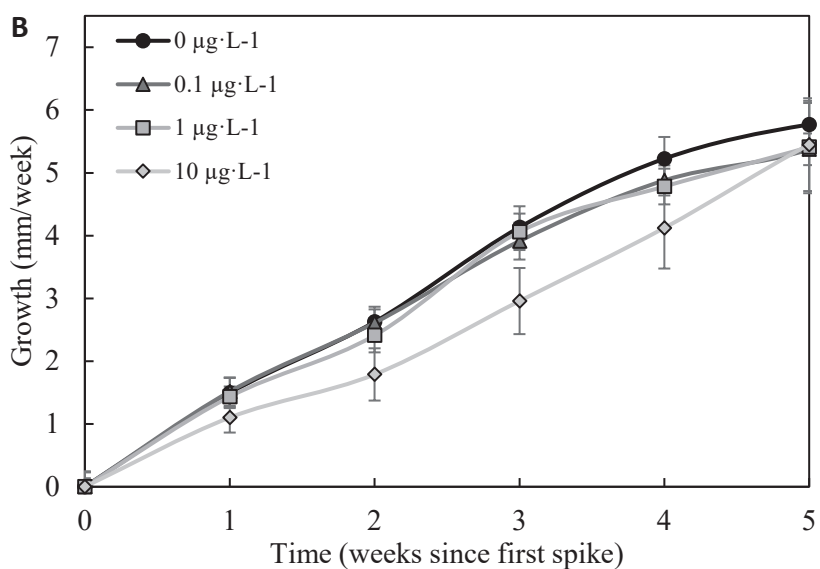
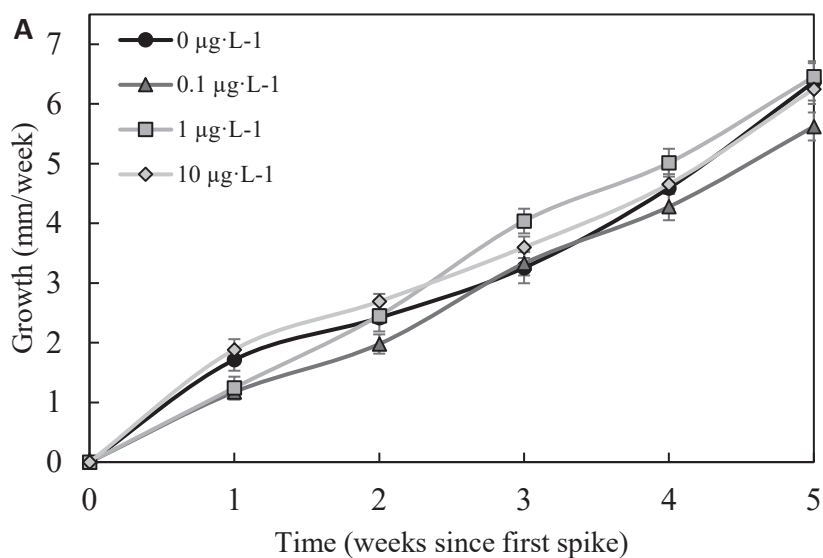


Figure 3.3. Average cumulative growth of *I. elegans* ($\pm$ SE, $n = 8-9$) of A) the 'Fed' treatment and B) the 'Free-feeding' treatment.

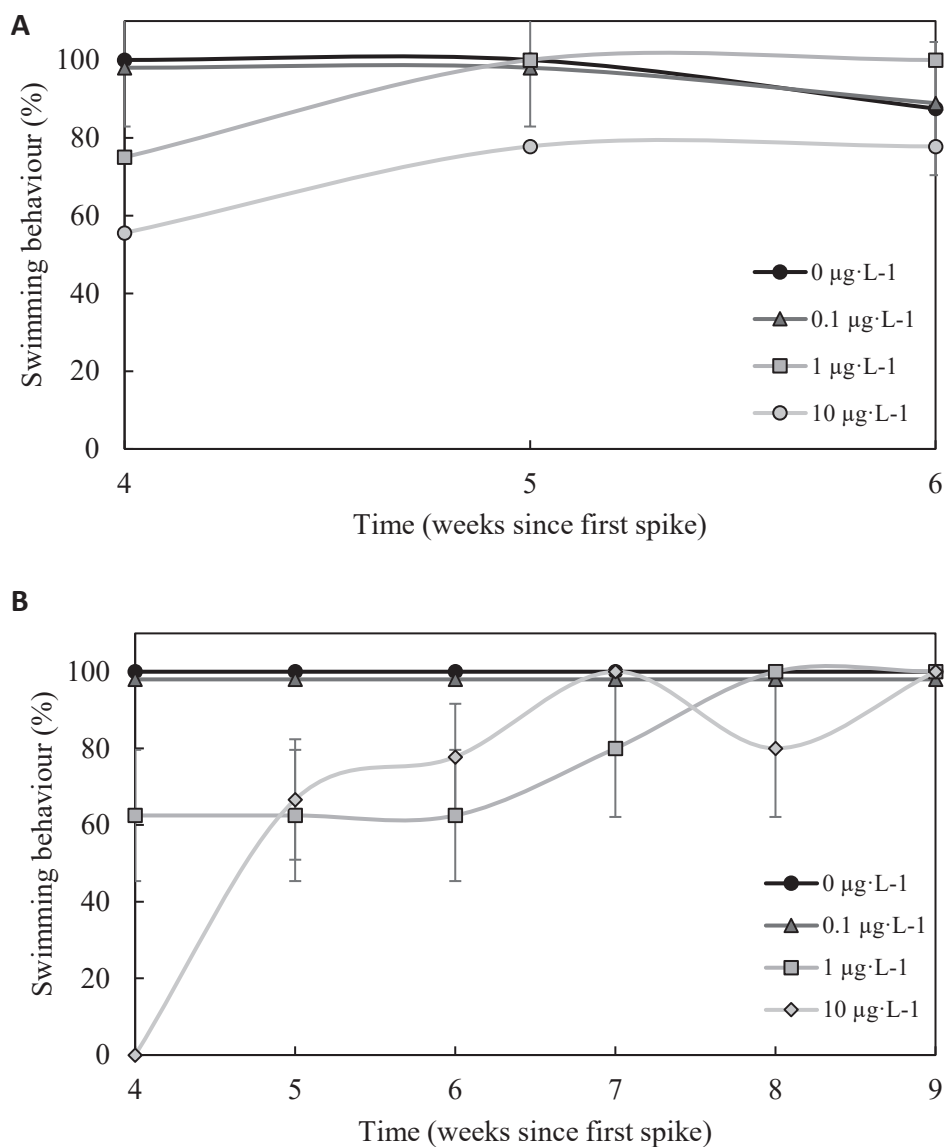


Figure 3.4. Average responsiveness (yes/no) of *I. elegans* ($\pm$ SE) to swim on stimulation per concentration of thiacloprid ($\mu\text{g}/\text{L}$) over time. A) 'Fed' and B) 'Free-feeding' treatment. Note that for B) concentration 0 and 0.1 $\mu\text{g}/\text{L}$ SE is always 0 as responsiveness was 100%.

Effects on emergence. At the end of the experiment, 89% of all caged damselflies within the control treatment emerged (Figure 3.5A). Only one individual within the control did not emerge as this individual lost two of its caudal appendages during the experiment. These appendages were regrown during the course of the experiment but resulted in a delayed emergence. Exposure to thiacloprid significantly reduced the cumulative emergence of damselflies within the 'Free-feeding' treatment (marginal $R^2 = 0.66$, Chi-squared = 7.9, df = 3, $p = 0.047$). At the end of the experiment, 75% of individuals emerged at 0.85 $\mu\text{g/L}$ exposure concentration and 56% at 9.86 $\mu\text{g/L}$ and these concentrations no longer deviated statistically from the control ($p > 0.05$). For the lowest spike concentration of 0.08 $\mu\text{g/L}$, only 11% emergence was observed ($z = -2.8$, $p = 0.015$; Figure 3.5A). We observed no effect of thiacloprid on the time until first emergence of individuals ($p > 0.05$). Thiacloprid significantly decreased the number of emerged damselflies of the natural populations residing in the ditches having the same thiacloprid concentrations as in the cages (marginal $R^2 = 0.34$, $F_{1,34} = 50.8$, $p < 0.001$; Figure 3.5B). In contrast to the caged damselflies, emergence decreased steadily with increasing thiacloprid concentrations (i.e., a dose-response manner). At the final day of measuring ($t=55$), total emergence was reduced by 39%, 55% and 65% for the actual spike concentrations 0.08, 0.85 and 9.86 $\mu\text{g/L}$ respectively.

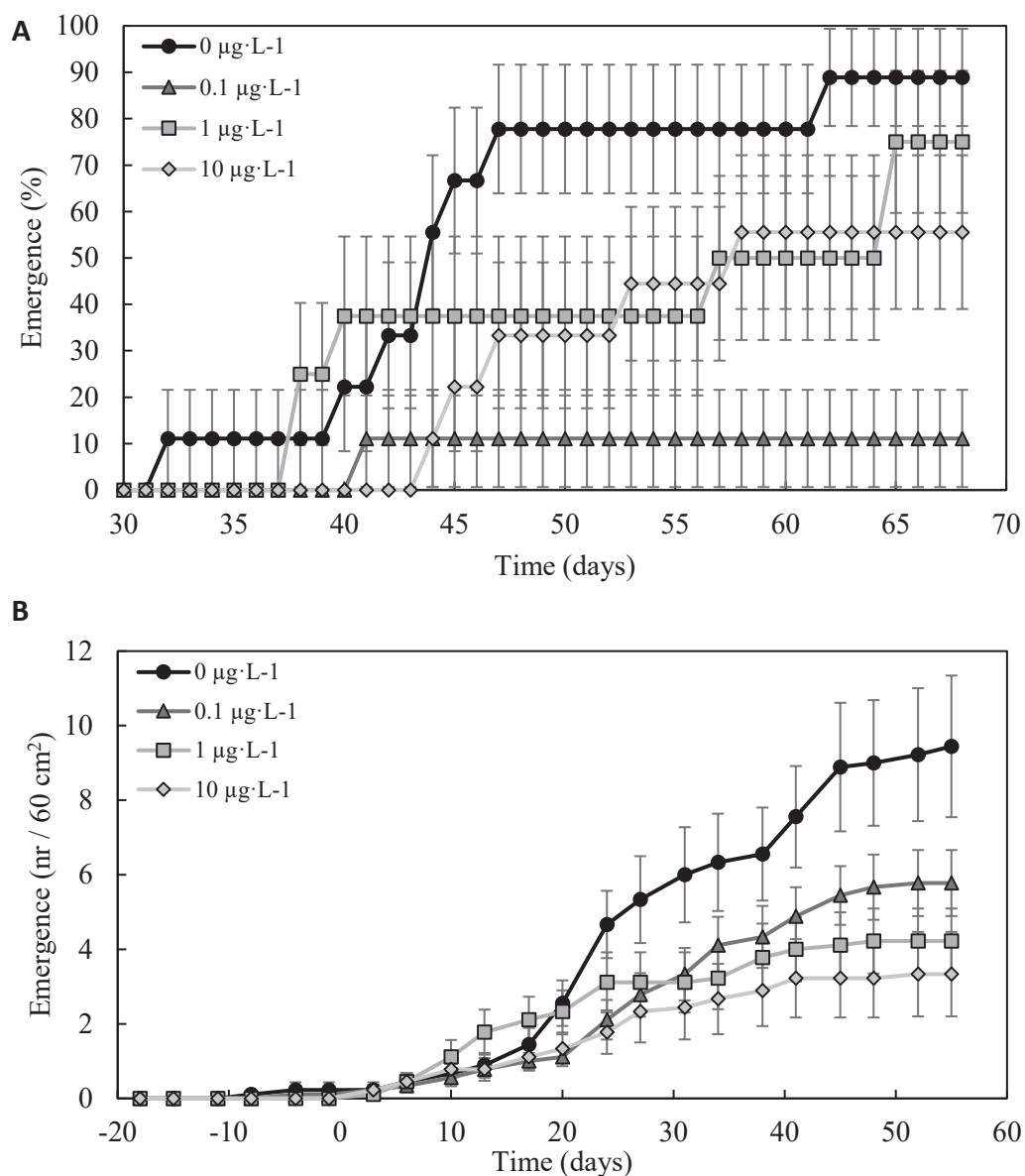


Figure 3.5. Average cumulative emergence of *I. elegans* ($\pm\text{SE}$, $n = 8-9$) per concentration of thiacloprid. A) *In situ* 'Free-feeding' treatment, caged experiment and B) natural collected individuals from the emergence traps. Note that for A) x-axis was set to $t=30$ for clarification purposes and no individuals emerged before this time and for B) we caught 99% of the generations' emergence during the exposure period.

3.4 Discussion

Many alarming reports are currently published on the decline of insects (e.g. Hallmann et al., 2017) with (agricultural) pollution often being recognized as one of the predominant drivers in intensively used environments (Dudgeon et al., 2006). This also holds true for neonicotinoids as it has been acknowledged that they are causing environmental risks higher than lab-based studies previously indicated (Goulson, 2013; EASAC, 2015; Vijver et al., 2017; Barmantlo et al., 2018). We have tested the effects of environmentally occurring thiacloprid concentrations on the Odonate *I. elegans* systematically using various endpoints, allowing to understand the mechanistic pathway of toxicity. If such a eurytopic and widespread species is affected or even in decline, this is indicative that other Odonates are potentially threatened as well. We found clear effects of environmentally relevant neonicotinoid concentrations on both caged *I. elegans* and natural populations. All sublethal parameters selected were affected by thiacloprid to some degree, but the severity of effects was stronger in caged individuals feeding on natural food supplies compared to fed individuals. The severity of effects increased even more so when considering the emergence of natural populations, which decreased strongly due to neonicotinoid exposure in a dose-dependent manner.

In contrast to the observed strong effects for sublethal parameters, survival was not impacted in this study, which is consistent with current literature that indicate lethal effects of neonicotinoids on other Odonates at levels that are at least a 3-fold higher than our highest experimental concentration (Beketov and Liess, 2008; Jinguji et al., 2013; Sugita, Agemori and Goka, 2018). Sublethal effects of thiacloprid on food and prey consumption were found at concentrations as low as the monthly average thiacloprid concentration of 0.03 µg/L (spike concentration 0.08 µg/L); 'Fed' nymphs consumed significantly less daphnids with increasing concentrations. In addition, 'Free-feeding' nymphs had more natural prey leftover at the highest test concentration. These observations are underpinned by the mode of action of neonicotinoids to invertebrates, namely by blocking the nicotinic acetylcholine receptors within the nervous system (Simon-Delso et al., 2015). This can also explain the

significant delays in both growth and molting rate of the nymphs living of the natural food supplies. As well as that, these effects can also explain the reduced swimming performance of the nymphs. The observed strong decline in swimming ability can also be attributed to a physiological response; *I. elegans* nymphs are excellent swimmers, but are only able to do so starting from the sixth instar (Brochard and van der Ploeg, 2014). As all nymphs could eventually swim, it is likely that the delay in molting subsequently led to a delay in reaching the sixth instar, making the nymphs temporally incapable of swimming. Irrespective of the causal mechanism, swimming behavior of *I. elegans* and many other Odonate species is generally used to avoid predators such as fish or other invertebrates (Corbet, 1962). The temporarily reduced swimming capacity can thus have obvious negative effects for natural populations because the nymphs are more susceptible to predation. Fitness reduction throughout the life cycle of the damselflies can also explain the observed delay in emergence time in the 'Free-feeding' treatment. All our tested endpoints are connected through either energy transfer or a physiological response and hence the combined sublethal effects offer a mechanistic explanation for the delay in emergence of the damselflies.

Nymphs feeding on the natural food supplies consistently exhibited more toxic pressure than nymphs that were fed. This is shown by the number of endpoints that were affected; namely all sublethal endpoints for the 'Free-feeding' versus two for the 'Fed' nymphs. For example, growth rate was reduced only within the 'Free-feeding' treatment. The severity of effects for endpoints that were affected in both treatments also differed markedly; swimming ability was more affected in the 'Free-feeding' treatment. Such differences in neonicotinoid-induced toxicity due to differences in food quantity/quality has been observed earlier and explained as a compensatory feeding response to cope with toxic stress (see Alexander et al., 2013; Ieromina et al., 2014; Barmantlo et al., 2018). In our study, the food quantity/quality was indirectly determined by information on how many potential prey were not eaten on a daily basis. Strikingly, this number decreased by 24% at the lowest test concentration compared to the control within the 'Free-feeding' treatment, indicating that these nymphs were possibly feeding more and thus indicating

compensatory feeding (Alexander *et al.*, 2013; Barmantlo *et al.*, 2018). Alternatively, a plethora of indirect effects can occur within the communities of the experimental ditches explaining the difference in toxicity between the treatments. The most obvious would be a secondary exposure to thiacloprid through contamination of the natural food supply. However, this is not that likely as neonicotinoids are highly soluble (USEPA, 2003) and thus generally considered to have a low bioaccumulation potential. More likely are thiacloprid-induced alterations in the communities residing in the experimental ditches, which altered the amount of natural food supply that entered the cages. Thus it is possible that we observed less prey in the cages at the lowest test concentration (0.1 µg/L) due to less supply from the ditch community rather than due to compensatory feeding. This is supported by the observation that the delay in emergence of caged individuals was greatest at this lowest test concentration. In any case, the outperformance of the 'Fed' nymphs importantly showcases that optimal food conditions can alter neonicotinoid-induced toxicity. As the common laboratory-based ecotoxicological approach is to feed animals *ad libitum* (according all OECD regulations, for example OECD, 2012), the already scarce toxicity data for Odonates as collected in the laboratory setting may currently be underestimating the actual risks in the field.

The total emergence of the natural populations of *I. elegans* declined strongly (39-65%) with increasing thiacloprid concentrations, whereas within our cage setup we mostly observed a delay in emergence time. The most likely explanation for this discrepancy is that in our cage setup many biotic interactions are excluded, e.g., there was no predation pressure. These biotic interactions are obviously present within the natural damselfly populations. It is recognized that the effects of contaminants depend on ecological context (Trekels *et al.*, 2011; Clements *et al.*, 2012). Thus, it is probable that the temporarily neonicotinoid-induced weakening of the nymphs increased their vulnerability to biotic pressures such as predation or by rendering them competitively inferior to other less sensitive species (as shown several times before by, for example, Mills and Semlitsch, 2004; Trekels *et al.*, 2011). The additional biotic pressure would thus reduce the actual population size and finally the total

emergence of the damselflies. We stress these results as emergence is necessary for the reproduction of *I. elegans*, and all other Odonate species, and population sizes cannot be maintained without it. A delay in emergence as observed in the caged experiment could lead to a mismatch in emergence time between different sites (polluted-unpolluted) and thus a mismatch in reproduction for Odonates (although note that our test species, *I. elegans*, has a very long imago phase). The observed total decline in emergence of the natural populations suggests less reproduction and both findings can thus potentially lower the population sizes. Damselflies (and dragonflies) play an important role in controlling mosquitos and agricultural pests (Painter et al., 1996; Sánchez-Bayo and Wyckhuys, 2019) because they are excellent predators on both nymphs and imagos of flying insects. Due to their function in the ecosystem as well as the fact that they can be inhabitants of highly anthropogenic areas, their role can be of great importance in natural pest control. Our results thus show that current environmental levels of neonicotinoids, insecticides that are meant to combat pest insects, might actually harm the natural pest control system.

Our experimental concentrations ranged from relatively low and commonly observed concentrations (0.1 µg/L) to less common higher end concentrations (10 µg/L) that are observed in surface waters all over the world. It should be noted that thiacloprid is not the only and also not the most common neonicotinoid polluting surface waters. Neonicotinoids are often found in mixtures and share a common mode of action (Morrissey et al., 2015) that can add up to toxicity (Maloney et al., 2018). We compare our findings of strong decline in natural emergence at environmentally relevant concentrations to data on Odonate abundance and distribution trends collected by citizen scientist from the ‘Dutch Dragonfly Monitoring Scheme’ of the Dutch Butterfly Conservation (Van Swaay et al., 2018). This dataset consists of standardised transect counts since 1999 and of distribution data since 1991, based on opportunistic data analysed with occupancy models (Van Swaay et al., 2018). The surveillance data for our test species, *I. elegans*, shows a decline in both abundance and distribution since 2008 (Figure 3.6). This timeline matches that of sharp increases in the amount of neonicotinoids sold and used in 2008 as reported for other

European countries (Sweden and the United Kingdom see Figure 2A/B in Simon-Delso et al., 2015). Building evidence, on the one hand our results show toxicity to *I. elegans* at environmental relevant concentrations, and on the other hand widespread decline of this species is observed. This indicates that neonicotinoids can be at least partially responsible (possibly together with other habitat altering parameters) for the current decline of the species *I. elegans*. This is in line with the study of van Dijk et al. (2013) who showed a decrease in natural *I. elegans* nymphs with increasing concentrations of the neonicotinoid imidacloprid. Such major declines in natural Odonate populations due to other systemic insecticides (imidacloprid and fipronil) have been shown before by Nakanishi et al. (2018) for the species *Sympetrum frequens*. The transect surveillance data for all Odonates shows an initial increase in Odonates between 1990 and 2008. This is likely because of improved habitat and water quality in that period (Termaat et al., 2015). However the data also shows that total Odonate distribution in the Netherlands is steadily declining, like for *I. elegans*, since the late 2000s (see Supplement Figure 3.S3). Given that our test species is one of the most widespread and eurytopic Odonates in Europe (Boudot and Kalkman, 2015), the observed neonicotinoid-induced toxicity may thus be indicative for this overall decline of Odonates.

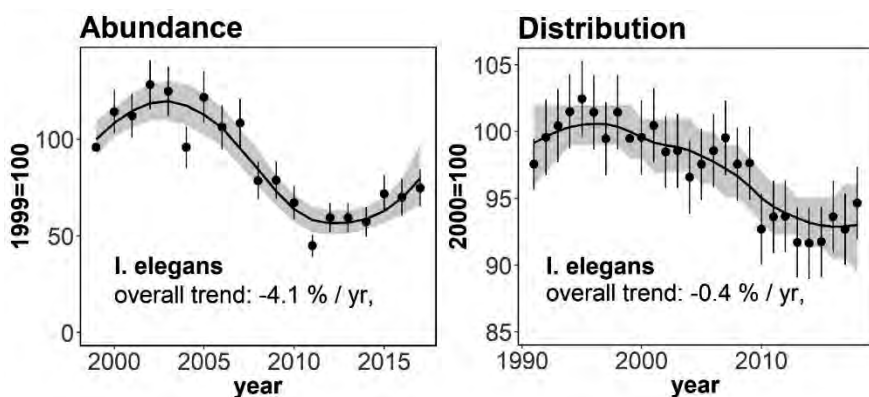


Figure 3.6. *Ischnura elegans* is declining in abundance and distribution in the Netherlands (index per year $\pm$ SE and a smoothed trend with 95% CI).

3.5 Conclusions

Clear effects of environmentally relevant concentrations of the neonicotinoid thiacloprid on the life cycle of the Odonate *I. elegans* were shown. While no direct effects on mortality were observed at environmental relevant concentrations, all sublethal endpoints tested were affected. Our results strongly depended on the food offered, which indicates that current laboratory assessments performed at *ad libitum* food underestimate neonicotinoid toxicity in the actual environment. In addition, it appears that even our realistic exposure scenario using caged individuals in experimental ditches also underestimates toxicity as the emergence of natural populations was more strongly affected. This is likely because biotic pressures such as predation add to toxicity and these pressures are not included within the caged experiment nor the common laboratory approaches. Finally, our observed reduced fitness during the nymph stage and the strong decline in natural emergence can be indicative for neonicotinoids adding to the ongoing *I. elegans* decline.

Acknowledgements

We would like to thank Justin Knetsch, Jo-Anne Bartels and Janneke van der Horst for their assistance with the experimental work. Furthermore, we thank all volunteers of the Dutch Butterfly Conservation for their valuable monitoring efforts. SB and MV were funded through a NWO-VIDI864.13.010 grant, awarded to MV. RG was supported by the ACTION project which has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement number 824603.

Authors' contributions

SB and LV conceptualized and executed the experiment; SB analyzed the data and led the writing of the manuscript; all authors contributed to the interpretation of the data, writing of the MS and gave final approval for publication.

Data accessibility

Data is available via Dryad Digital Repository

<https://doi.org/doi:10.5061/dryad.0vh187j> (Barmentlo, et al., 2019).

Supplement

Table 3.S1. Time weighted averages ($\pm$ SE) of the different water chemistry parameters over the course of the experiment.

Nominal conc. ($\mu\text{g/L}$)	Temperature ($^{\circ}\text{C}$)	pH	Conductivity ($\mu\text{S/cm}$)	Oxygen conc. (mg/L)
0.0	23.4 (± 0.11)	9.18 (± 0.05)	481 (± 4.5)	14.6 (± 0.32)
0.1	23.4 (± 0.04)	9.19 (± 0.06)	479 (± 4.4)	15.1 (± 0.34)
1.0	23.5 (± 0.04)	9.24 (± 0.07)	472 (± 5.3)	14.9 (± 0.20)
10.0	23.5 (± 0.06)	9.62 (± 0.06)	460 (± 0.37)	15.8 (± 0.18)

'conc.' = concentration.



Figure 3.S1. Photographic overview of the experimental site. From left to right: adjoining lake, wooden wave barrier, helophytes, experimental ditches. The white pyramids are the emergence traps used in the present study.

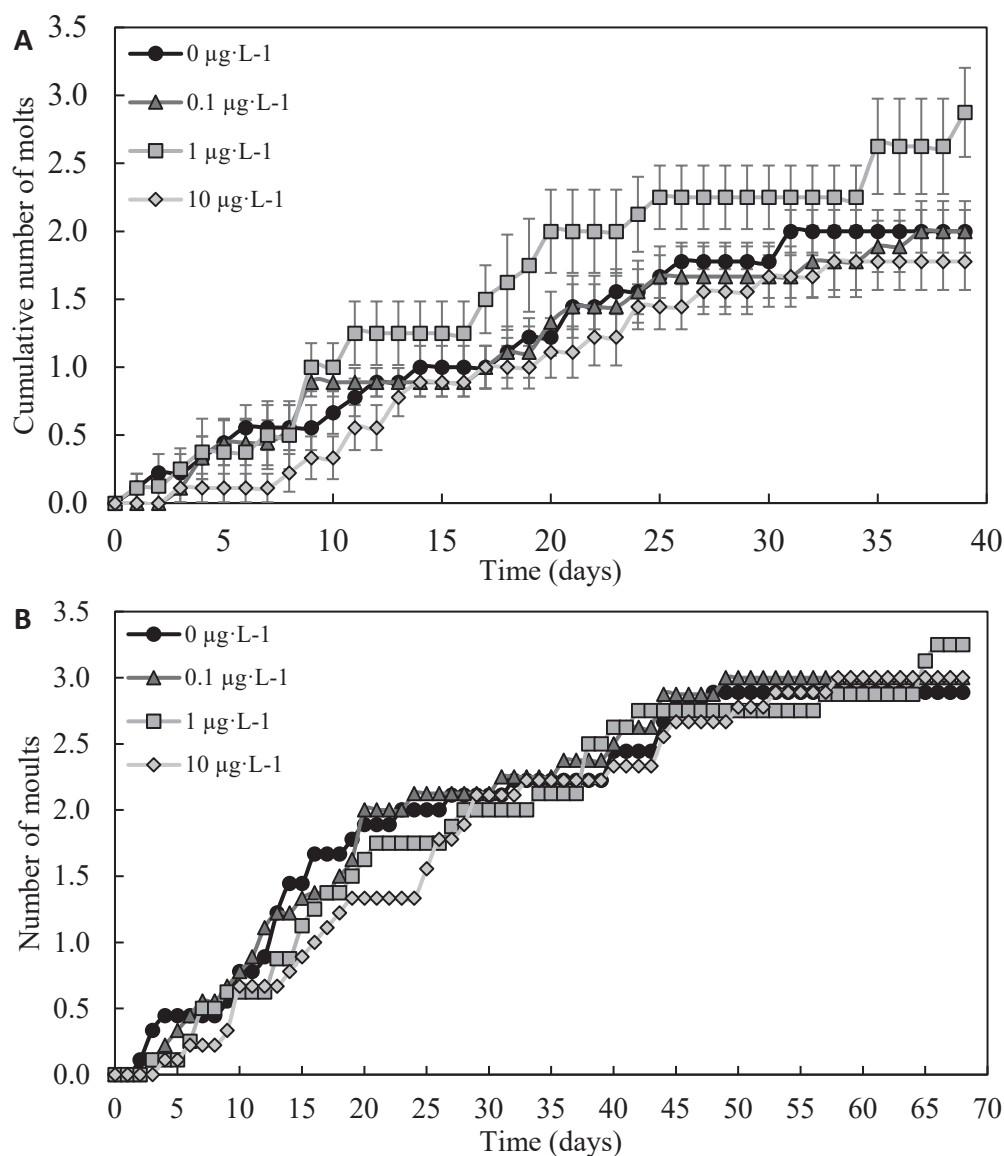


Figure 3.S2. Average cumulative number of molts ($n = 8-9$) A) 'Fed' and B) 'Free-Feeding' *I. elegans*. Spikes were applied on $t=0$ and $t=14$. Note that for B) error bars are not shown for clarification purposes.

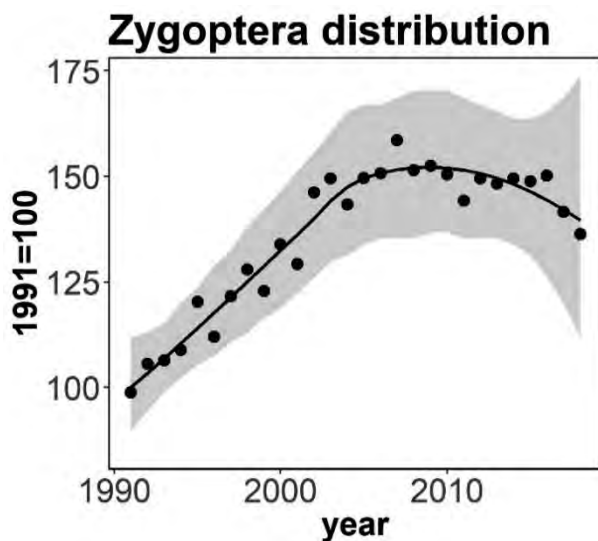
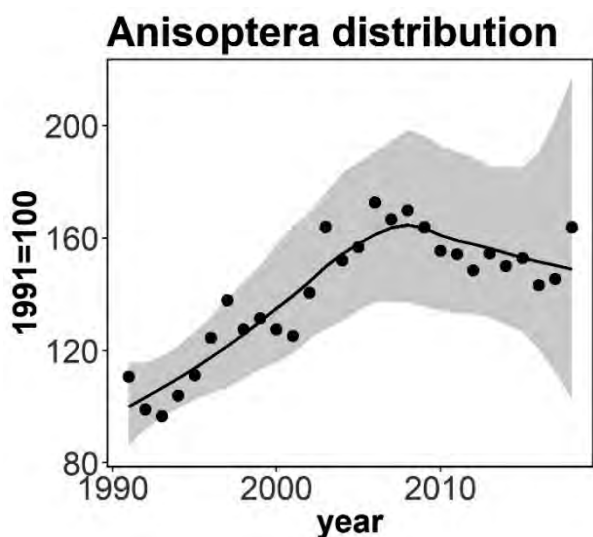


Figure 3.S3. Multispecies indicator of all Dutch Anisoptera (left) and Zygoptera (right). Initial increases in the period 1990-2008 are likely to overall improvement in water and habitat quality (see Termaat et al., 2015). Database accessed: 18-02-2019. Source: <https://www.clo.nl/indicatoren/nl138712-trend-van-libellen>

Chapter 4

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

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Highlights of this study:

1. Combined effects of mixtures of agrochemicals compared to their single effects were assessed under realistic fluctuations of abiotic conditions in an outdoor mesocosm experiment with an assemblage of aquatic invertebrates.
2. Environmentally realistic concentrations of binary mixtures of pesticides and nutrients showed additive species' responses for abundancies and species growth.
3. Treatments with tertiary mixtures of pesticides and nutrients affected species' abundances and body length in ways that could not be described by the cumulative responses of the single exposures treatments.
4. This indicates that in agricultural ditches, containing a multitude of agrochemicals residues, non-additive induced shifts in natural aquatic invertebrate assemblages occur.

Graphical abstract



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.

Keywords: agrochemicals, mixtures, outdoor testing, aquatic invertebrates, mesocosm

4.1 Introduction

Agricultural ditches collect a wide variety of pesticides from adjacent fields due to run off, direct drift and leaching (Nollet and Rathore, 2010). From the monitoring data that takes place in these water bodies (e.g. de 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 (e.g. Relyea et al., 2005) and decomposition (e.g. 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; Barmantlo 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; Barmantlo 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., 2002; 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 for an assemblage of macroinvertebrate species. We investigated the effects of single exposure as well as binary and tertiary mixtures of a commonly used insecticide, herbicide and nutrients on different endpoints for 9 functionally distinct aquatic macroinvertebrates species. To allow for more (a)biotic context, we tested these species in assemblages for 35 days in an outdoor mesocosm experiment.

4.2 Material and methods

Species selection

The species assembly chosen (Table 4.1) 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 Supplement Table 4.S1 for additional information on the species). The different test species reflected broadly the feeding mode trait distribution (retrieved from www.freshwaterecology.info; Schmidt-Kloiber and Hering, 2012) as found in dune ditch systems in order to create a coherent and stable food web (Ieromina et al., 2015).

Daphnia magna was obtained from laboratory cultures of Leiden University (Leiden, The Netherlands). *Lymnaea stagnalis* were obtained 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* and *Tubifex* sp. were purchased. 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.

Table 4.1. 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
<i>Asellus aquaticus</i>	25	28d LC ₁₀ = 1.35 ¹	NA
<i>Chironomus riparius</i>	50	4d NOEC (growth) = 0.74	2d NOEC (swimming) = 200 ²
<i>Cloeon dipterum</i>	15	28d LC ₁₀ = 0.041 ¹	NA
<i>Culex pipiens</i>	12	NA	NA
<i>Daphnia magna</i>	78	21d NOEC = 2000 ³	2d EC ₅₀ (immobility) = 21200 ⁴
<i>Lymnaea stagnalis</i>	5	NA	NA
<i>Notonecta glauca</i>	2	96h EC ₅₀ (mobility) = 18.2 ¹	NA
<i>Sigara striata</i>	12	NA	NA
<i>Tubifex sp.</i>	220	1d LC ₅₀ = 320 ⁵	NA

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

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/cm² 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. 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 4.1) 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 *N. glauca* 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. 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. 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 (Pesticide Atlas, 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 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; de 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.

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 hours after the second treatment application, as described in 2.2) and t=21. Samples were

collected by sampling the water in the middle of a mesocosm 5 cm below the surface. Terbutylazine 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 ($\text{NO}_3^-/\text{NO}_2^-$)) and PO_4^{2-} were determined colorimetrically using an auto-analyzer (Seal AA3; SEAL Analytical, UK).

Biotic endpoints

Biomass and numbers determination. In order to determine algae densities, water samples (15 mL) were collected from each mesocosm one hour 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 hours 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 abundance per species.

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 for body length determination. Only species that had not been fully grown at the start of the experiment were analyzed on body length, being *A. aquaticus*, *C. dipterum*, *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. Only egg-carrying *D. magna* were selected to ensure maturity and measured 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).

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), terbutylazine (2 levels; addition / no addition), nutrients (2 levels; addition / no addition) and all possible combinations 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, terbutylazine, nutrients, imidacloprid + nutrients, terbutylazine + nutrients, imidacloprid + terbutylazine, imidacloprid + terbutylazine + 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 the treatments. Data did not deviate from a homogeneity of variance for any data with the exception for body length of *D. magna* in relation to imidacloprid usage. Data transformation did not solve this deviation from homogeneity of variance.

Statistical analyses were performed with R-studio, version 0.99.902 (R version 3.3.0; R Core Team, 2016).

4.3 Results

Control performance

At the end of the experiment, all species except for *C. pipiens* were found in the replicated controls. *C. pipiens* had disappeared due to intense predation by *N. glauca* (personal observation) and was therefore excluded from further analysis. 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 *S. striata* 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 $\mu\text{g}/\text{cm}^2$ (SE 0.017) and free-floating algae to be 20 cells/ cm^2 (SE 18).

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 $\mu\text{g}/\text{L}$ (t=0) and 4.2 ± 0.1 $\mu\text{g}/\text{L}$ (t=18) after first and second application, respectively. Treatments that had received solely imidacloprid (TWA concentration: 2.8 $\mu\text{g}/\text{L}$) and mesocosms that had received imidacloprid application as part of the mixture showed experimental concentrations diverging no more than 0.2 $\mu\text{g}/\text{L}$ (TWA concentration: 3.0 $\mu\text{g}/\text{L}$). 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 significantly increased by terbuthylazine addition ($F_{1,40} = 4.47$, $p = 0.041$) and marginally significant by nutrient addition ($F_{1,40} = 3.666$, $p = 0.063$). 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; $F_{1,40} = 4.55$, $p = 0.039$).

There was no deviation in water chemistry parameters between the 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 ($F_{1,40} = 89.5$, $p < 0.001$) (Supplement Figure 4.S1A). Both pH and oxygen concentration were positively affected by an interaction between time and nutrients ($F_{1,16} = 22.0$, $p < 0.001$; $F_{1,16} = 9.36$, $p = 0.002$) (Supplement Figure 4.S1B and 4.A1C respectively). Water conductivity increased significantly by time, nutrient addition and herbicide addition ($F_{1,16} = 19.6$, $p < 0.001$; $F_{1,16} = 5.36$, $p = 0.022$; $F_{1,16} = 6.92$, $p = 0.009$) (Supplement Figure 4.S1D).

Effects of the agrochemicals on species' abundance

Imidacloprid negatively affected the number of surviving *C. dipterum* ($F_{1,40} = 32.7$, $p = 0.004$). Imidacloprid showed a marginally significant interaction with nutrient additions and terbuthylazine on the number of surviving *C. dipterum* ($F_{1,40} = 1.82$, $p = 0.096$), as well as the binary mixture

of terbutylazine and nutrients ($F_{1,40} = 2.25$, $p = 0.065$). 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 * terbutylazine, $p < 0.05$) except for the tertiary mixture, where no significant difference from the control was observed (Figure 4.1C). Imidacloprid also significantly decreased the abundance of *S. striata* ($F_{1,40} = 4.23$, $p = 0.046$) (Table 4.2) with a marginally significant interaction between nutrients addition and imidacloprid ($F_{1,40} = 8.33$, $p = 0.068$).

Nutrient additions increased the abundance of *D. magna* ($F_{1,40} = 19.3$, $p < 0.001$) with a positive interaction between nutrients and terbutylazine ($F_{1,40} = 8.59$, $p = 0.003$). The number of *D. magna* was lowest in the single terbutylazine treatment and the mixture of terbutylazine and imidacloprid, although this number did not significantly deviate from the control ($p > 0.05$) (Figure 4.1A). However, the *D. magna* abundance in these two treatments was significantly lower than the terbutylazine and nutrient mixture treatment ($p < 0.05$). The tertiary mixture treatment (nutrient addition, imidacloprid, terbutylazine) showed significantly more *D. magna* individuals than all other treatments ($p < 0.05$) (Figure 4.1A).

Comparable to *D. magna*, nutrient additions also significantly increased the abundance of *Cyclops* sp. ($F_{1,40} = 8.95$, $p = 0.003$; Table 4.2) with a positive marginally significant interaction between all three agrochemicals ($F_{1,40} = 2.74$, $p = 0.097$). Equal to *D. magna*, the number of *Cyclops* sp. was lowest in the single terbutylazine treatment and in the mixture of terbutylazine and imidacloprid (Figure 4.1B). 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 tertiary mixture treatments also showed marginally more *Cyclops* sp. when compared to the control ($p = 0.07$).

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

Table 4.2. The single and combined effects of the different agrochemicals on abundance and body length of the test species as assessed via factorial ANOVA.

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

Var: variable, Abund: abundance, N: nutrients, I: insecticide = imidacloprid, H: herbicide = terbuthylazine, ‘-’: indicates mixtures. Significant effects ($p \leq 0.05$) of the treatments are shown in bold while marginal significant effects are shown by underscores.

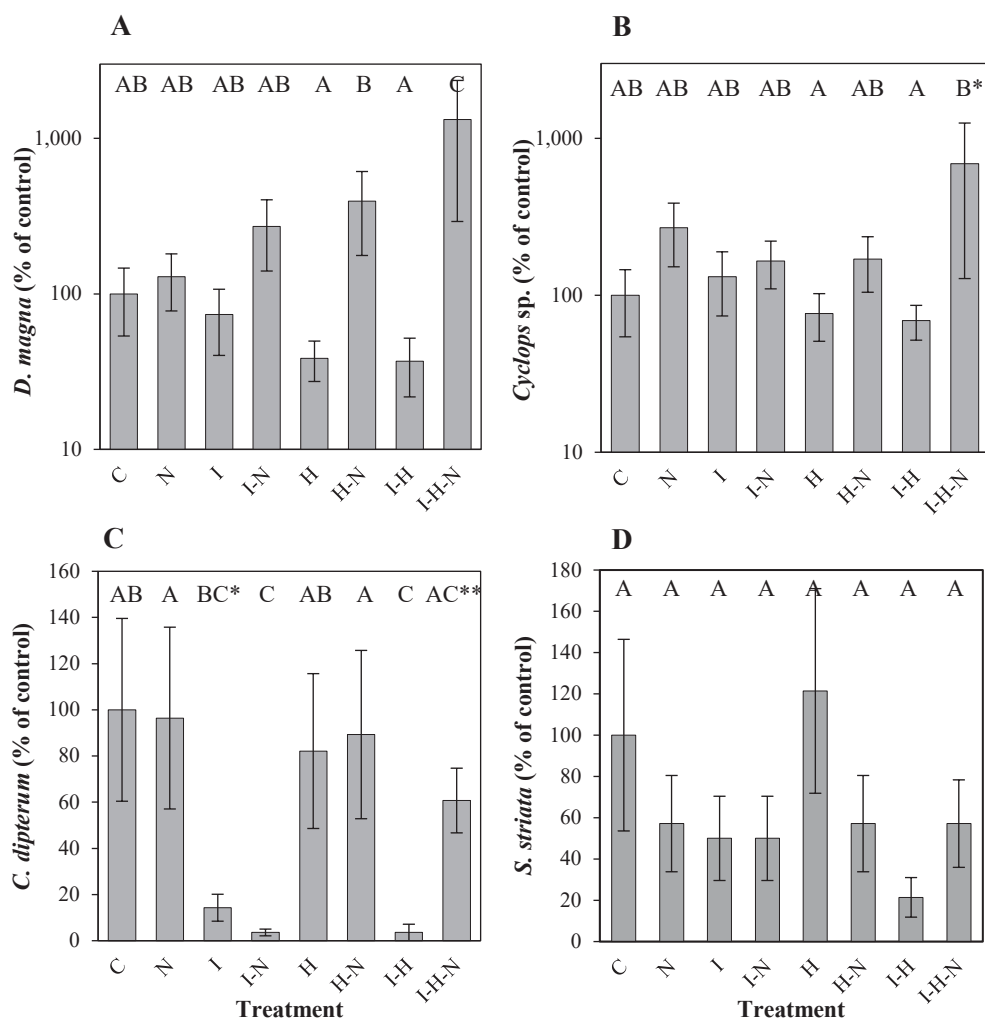


Figure 4.1. Abundance of A) *D. magna* B) *Cyclops* sp. C) *C. dipterum* and D) *S. striata*. Average number of animals are shown as the percentage of the control ($\pm$ SE). Different letters (A, B, C) indicate significant differences (One-way ANOVA + Tukey's post hoc) between treatments at significance level $p = 0.05$. *: Marginally different from the control (II: $p = 0.07$; III: $p = 0.08$). **: Marginally different from treatment I-H ($p = 0.08$). C: control, N: nutrients, I: insecticide = imidacloprid, H: herbicide = terbuthylazine, '-': indicates mixtures.

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 (Figure 4.2). 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 ($p < 0.05$ 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*.

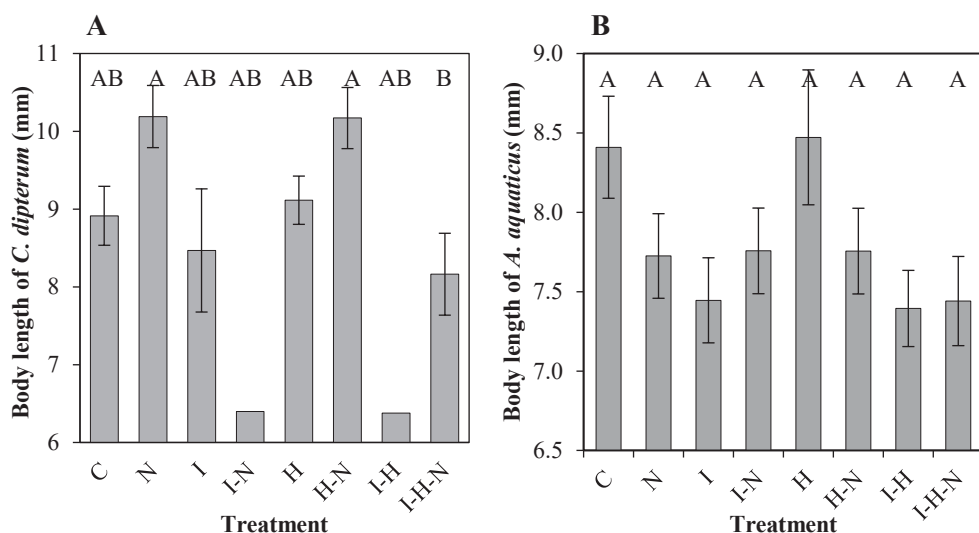


Figure 4.2. Average body length ($\pm$ SE) of A) *C. dipterum* and B) *A. aquaticus*. Different letters (A, B, C) indicate significant differences (One-way ANOVA + Tukey's post hoc) between treatments at significance level $p = 0.05$. Absent standard error bars are due to the limited surviving test animals within that treatment. C: control, N: nutrients, I: insecticide = imidacloprid, H: herbicide = terbuthylazine, '-': indicates mixtures.

4.4 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.

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* likely as a result of increased food source availability (here: periphyton). However, this could not be confirmed statistically due to high variation in the biomass of the periphyton samples. 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. It can be speculated that the observed 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.

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 EC₁₀ (10% effect concentration) for mobility of *C. dipterum* was 0.041 µg/L (Table 4.1), 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 LC₁₀ = 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 observed effects of agrochemicals thus coincided with proposed mechanisms when applied as a single substance.

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 Figure 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 4.1) 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 terbutylazine, 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.

4.5 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

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Supplement

Table 4.S1. Characteristics of the test species. GC = gatherer / collector, NA = not available.

Species	Feeding mode	Main food source	Initial body length (mm)
<i>Asellus aquaticus</i>	Grazer, shredder	Detritus	M: 9.01 ($\pm$ 0.21) F: 6.38 ($\pm$ 0.26)
<i>Chironomus riparius</i>	Filter feeder, GC	Detritus, algae	10.73 ($\pm$ 0.40)
<i>Cloeon dipterum</i>	Grazer, GC	Detritus, periphyton	5.31 ($\pm$ 0.22)
<i>Culex pipiens</i>	Grazer, Filter	Bacteria	NA
<i>Daphnia magna</i>	Filter feeder	Algae	0.98 ($\pm$ 0.02) – 1.42 ($\pm$ 0.05)
<i>Lymnaea stagnalis</i>	Grazer, Shredder	Detritus, periphyton	17.44 ($\pm$ 0.70)
<i>Notonecta glauca</i>	Predator	Macrofauna	15.72 ($\pm$ 0.30)
<i>Sigara striata</i>	GC, Predator	Algae, detritus	7.03 ($\pm$ 0.16)
<i>Tubifex sp.</i>	GC	Detritus	NA

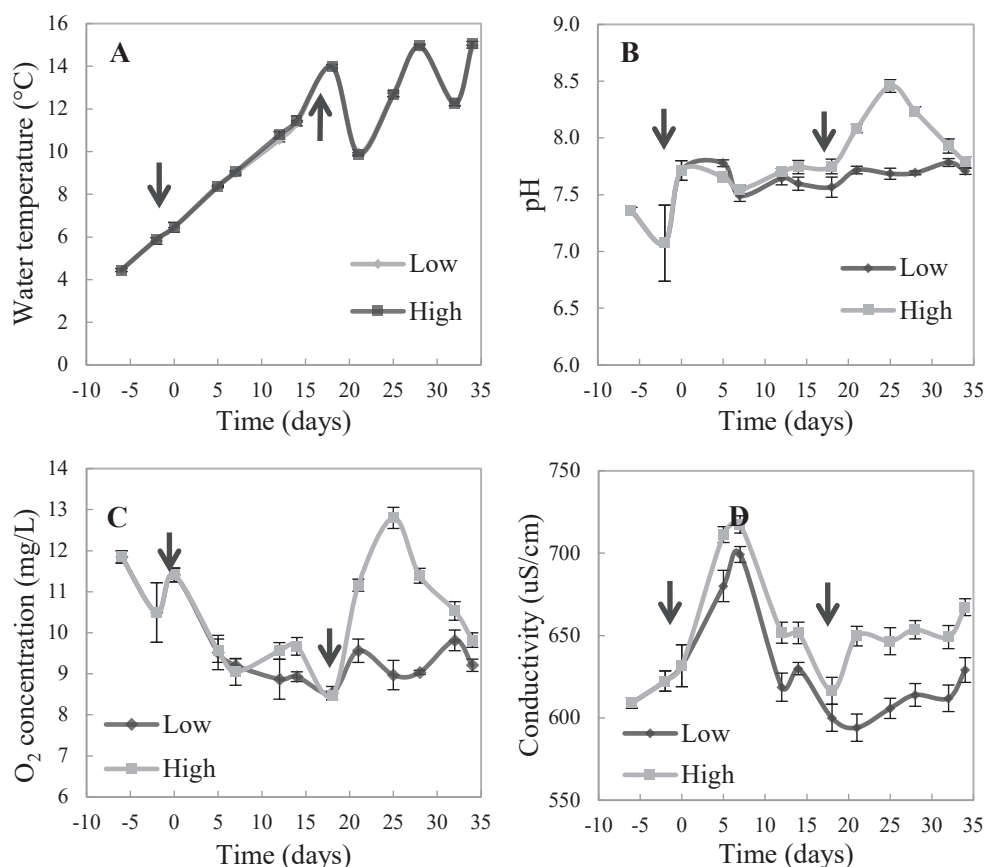


Figure 4.S1. Average ($\pm$ SE, $n = 12$) A) temperature, B) pH, C) oxygen concentration and D) conductivity for low nutrient (all treatments without nutrient addition) and high nutrient conditions (all treatment with nutrient addition). In order to improve clarity of the figure, we summarized the treatments on nutrient content (low, high). Arrows indicate the time points on which treatments (nutrients, imidacloprid and terbuthylazine) were applied. Note that error bars for A) are often smaller than the data points.

Chapter 5

Experimental evidence for neonicotinoid driven decline in aquatic emerging insects

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Abstract

We are witnessing an unprecedented loss in insects, both in terms of species richness and sheer biomass. Anthropogenic stressors, like insecticides, have been widely proposed to be responsible for the decline. However, to date, experimental evidence for this claim is lacking. Here we used an outdoor experimental approach to explore the effects of environmentally realistic concentrations of the neonicotinoid insecticide thiacloprid on the emergence of naturally assembled insect populations. Following thiacloprid application, all major insect orders declined strongly in either numbers and/or biomass. Emergence of most orders was even near absent at the highest concentration. Chironomidae diversity, the most species rich family, decreased with 50% at a monthly average concentration 0.4 µg/L and was mostly reduced to a single species in the highest concentration. This experimental approach provides causal evidence that neonicotinoid contribute to the ongoing decline of insects.

Keywords: Diversity, environmentally relevant concentrations, insect decline, insecticides, thiacloprid.

5.1 Introduction

Insects represent the most diverse class of animals on our planet and outnumber all other animal species (May, 1988; Mora et al., 2011). They play key roles in crucial ecosystem services including nutrient cycling, pollination, decomposition and pest control (Bianchi et al., 2006; Losey and Vaughan, 2006; Vanbergen et al., 2013; Yang and Gratton, 2014). In addition, many vertebrate animals, such as the majority of fish, birds and bats rely on insects as their main or only source of diet, illustrating their pivotal role in ecosystems. However, these roles may soon become vacant: several studies suggest that insects are declining at alarming rate. An growing number of reports show that we are witnessing an unprecedented loss in insects both in terms of species richness and biomass, even of common species (Hallmann et al., 2017, 2019; Homburg et al., 2019; Lister and Garcia, 2018; Powney et al., 2019; Sánchez-Bayo and Wyckhuys, 2019), likely with negative repercussions for natural and human ecosystems (Dirzo et al., 2014).

Different anthropogenic stressors have been suggested to underlie this decline, most importantly: 1) climate change and related alterations in temperature and drought/wet periods, 2) habitat loss and fragmentation and 3) insecticide usage (Vanbergen et al., 2013; Collier et al., 2016; Sánchez-Bayo and Wyckhuys, 2019; Wagner, 2020). However, inferring causality on the contribution of each of these, and other, stressors is challenging and requires large datasets that generally contain confounding factors (for examples see Hallman et al 2017, Musters et al. 2019 and Wagner 2020). As a result, studies addressing the drivers of the decline have been criticized for their correlative nature, resulting in some coining the decline “slightly exaggerated” (Thomas et al., 2019) while others state that we already know enough to act now (Leather, 2018; Forister et al., 2019;). To reconcile this debate, more well-controlled experiments are required to determine the causality of each of the proposed drivers (Wagner, 2020).

The impact of insecticides, most notably neonicotinoids, on natural insect populations is perhaps the most extensively debated. Neonicotinoids were first introduced in 1991 and are currently the most widely used class

of insecticides globally (Jeschke et al., 2011; Sparks, 2013). Their potential hazard to insects has been assessed in a large body of literature based on laboratory experiments (see <https://cfpub.epa.gov/ecotox/>) as well as field observations (Woodcock et al., 2017). While these studies show a range of insects to be at risk, including severe effects on populations of pollinators (EASAC, 2015; Woodcock et al., 2017) and other selected non-target organisms (Pisa et al., 2015), experimental studies on complete natural species assemblages are mostly lacking. Moreover, most studies focus on short term effects on insect larvae (Simon-Delso et al., 2015), while mid- to long-term effects on emerging adults are rarely addressed even though these are crucial for species propagation. As such, there exists a major knowledge gap on the causality of neonicotinoid insecticides and the ongoing decline of insects (Gilburn et al., 2015; Brain and Anderson, 2019;).

Here, we tested the hypothesis that current surface water concentrations of neonicotinoids cause a decline in the abundance, species richness and biomass of emerging insects. We explored the effect of a range of surface water concentrations (0, 0.1, 1.0 and 10 µg/L) of a model neonicotinoid, thiacloprid, on insect emergence from experimental freshwater ecosystems (Figure 5.1). These concentrations showed severe effects on the emergence, in terms of abundance and biomass, of five orders of insects: Coleoptera, Diptera, Ephemeroptera, Odonata and Trichoptera together comprising >99% of emerging insects, as well as the diversity of the most species-rich freshwater insect group, the Chironomidae.



Figure 5.1. Overview of the experimental site (top). Shown are 36 individual experimental ditches adjoining to a small lake. Each ditch is fitted with one emergence trap from which insects are directly caught and collected (bottom).

4.2 Methods

Test location

The experiment was carried out in 36 experimental freshwater ditch-ecosystems at the outdoor “Living Lab” facility of Leiden University (Leiden, the Netherlands). Each ditch-ecosystem (length-width-depth: 10-0.8-0.3 m; volume: 1,750 L) was placed side-by-side and adjoined to a small lake (Figure 5.1). This lake provides a natural source of plants, microbes and invertebrates that (re)colonize the ditches, leading to similar communities (Barmentlo et al., 2019a). The ditches are colonized by large numbers of invertebrates ranging across thirty-one different taxonomic orders, among which nine insect orders. For more details on the experimental site, see reference (Barmentlo et al., 2019a) and www.mesocosm.eu. After an initial colonization period (November 2017 – April 2018), each ditch was hydrologically isolated from the adjoining lake with 1,000:500:2 mm acrylic plates that were placed at the end of each ditch. We specifically mimic ditches as these systems are generally the first aquatic waterbodies to receive agricultural contaminants (due to processes such as run-off from the agricultural field) and consequently the highest concentrations of pesticides are found here.

Sampling of emergent insects

Each experimental ecosystem was equipped with a single emergence trap at the center of the ditch, to continuously trap and sample emerging insects between mid-April to mid-July. Each trap consisted of a pyramid of stainless steel rods (length:width:height – 60:60:74 cm) which was fitted with No-See-Um netting (300 holes / cm²). All traps were placed 3 cm below the water surface level (connected with tie-raps to bamboo sticks that were tightly stuck in the ditch banks) to prevent the escape of emerging insects. Furthermore, each trap was equipped with the insect collection system according to reference (Cadmus et al., 2016); consisting of two 500 mL polyethylene bottles connected to each other via cutout caps (Ø 3.5 cm) that are glued together (Figure 5.1). The upper (capture) bottle is connected to the trap via an 6.5 by 4.5 cm opening in both the netting and bottle. The lower (collection) bottle is filled with 100 mL of 80% ethanol (20% tap water) as a capture and preservative medium for the insects. These lower bottles were replaced twice a week (every

Monday and Thursday) for different bottles with fresh preservative. The collected insects were then transferred to 25 mL glass snap cap falcons with newly prepared 80% ethanol.

Neonicotinoid application

To confirm that experimental communities were similar prior to the experimental treatments, we assessed adult emergence in the month before the experimental treatments were applied. After this one-month period, we started application of thiacloprid in mid-May, when actual thiacloprid concentrations in surface waters are rising (Barmantlo et al., 2018; Leiden University and Rijkswaterstaat-WVL, 2018), and continued to follow emergence for two more months. We applied two biweekly spikes of thiacloprid (CAS no. 111988-49-9, purchased from Sigma-Aldrich, Zwijndrecht, the Netherlands) in four different concentrations: 0, 0.1, 1 and 10 $\mu\text{g/L}$ thiacloprid with nine replicate ditches per concentration. This range represents the range of neonicotinoid concentrations observed in surface waters across the globe (Casado et al., 2019; Sánchez-Bayo et al., 2016; Simon-Delso et al., 2015; Stehle et al., 2018; Leiden University and Rijkswaterstaat-WVL, 2018). Stock solutions of thiacloprid were prepared in 1L of demineralized water in glass bottles. These stock solutions were diluted in a 10L glass bottle with ditch water of the ditch the treatment was offered to. This solution was then evenly spread over the entire length of the ditch. We monitored the thiacloprid concentrations using LC-MS/MS analysis (see Barmantlo et al., 2018 for the sampling procedure and Roessink et al., 2013) for chemical analysis). Direct measurements after application of the two neonicotinoid spikes showed that our actual average spike concentrations were 0 (control), 0.08 (nominal 0.1), 0.85 (nominal 1) and 9.86 $\mu\text{g/L}$ (nominal 10 $\mu\text{g/L}$). Thiacloprid degraded rapidly with DT50 (half-life) of 3.6 days and a DT90 of 12.0 days (Barmantlo et al., 2019b).

Insect identification

All trapped insects were first identified to the taxonomic order level. For the order of Diptera, we identified all individuals to family level using a stereomicroscope and the European Diptera key (Oosterbroek, 2016). All identifications were performed by a single observer. In addition, we identified all individual Chironomidae to the species level for a subset of the experimental ecosystems. As this is a labor-intensive process, we selected five replicates per concentration (the middle 20 ditches within the block design) one month after the initial application, being the middle of the experimental period. Only male specimens were selected, as these are much easier to identify than females. After separating the males from the female Chironomidae under a stereomicroscope, all specimens were dissected following the protocol by reference (Langton and Pinder, 2007), embedded in Euparal and finally identified using a microscope and the literature (Hirvenoja and Hirvenoja, 1988; Langton and Pinder, 2007; Sæther, 1990) as guidelines for determination.

Insect biomass

We determined the relative contribution of the different insect groups to the total biomass by randomly weighing selected specimens per taxonomic order and per Diptera family from different treatments and dates collected. As differences in body size can skew biomass estimates, biomass from samples of the orders of Odonata, Coleoptera and Hymenoptera were determined based on different size classes. For larger taxonomic orders (such as Odonata), one to a few insects were dried for three days at 30 °C per sample to determine the biomass per individual. For smaller taxonomic orders (such as Dipterans, often <1 mg dry weight per insect), about 20 insects were dried and weighed per sample using a microbalance. All weights were divided by the number of dried specimens in order to obtain the biomass per individual. This was repeated, if specimens were sufficient, twenty times to determine an average biomass per taxon.

Statistical analyses

All collected insect numbers and biomass data were first transformed to cumulative data. We then assessed the effects of neonicotinoid concentration on total emergence and on taxon emergence both in abundance and biomass. For this, we used generalized additive models (function `gam`, package ‘`mgcv`’) with a Poisson distribution and nominal spike concentrations, time (in days) and their interaction as explanatory variables. As neonicotinoid concentrations declined rapidly (see ‘Neonicotinoid application’), we used the nominal spike concentration rather than the actual concentration per sampling date. We used the nominal rather than actual concentrations to account for data that do not follow a dose-response relationship. We smoothed the terms for nominal spike concentration and its interaction with time using factor smooths (‘`fs`’) with 24 knots, being a knot for every point in time that insects were collected. Finally, we accounted for the repeated measures design by including ditch ID as a random factor. Monthly average neonicotinoid concentration (as time-weighted averages) effects on Chironomidae diversity were investigated by dose-response modelling using Shannon diversity (‘ H' ’) and Shannon evenness (‘ J' ’) metrics as response variables (function `drm`, package ‘`drc`’). All statistical analyses were performed using R (version 3.5.1 (R Core Team, 2019)).

4.3 Results

Insects collected and exposure conditions

During a three-month timespan, we collected 55,253 emerged aquatic insects of which 51,137 flies and midges (Diptera), 1,842 mayflies (Ephemeroptera), 1,088 beetles (Coleoptera), 919 caddisflies (Trichoptera), 232 dragon- and damselflies (Odonata), 30 hymenopterans (Hymenoptera), 3 true bugs (Hemiptera) and 2 alderflies (Megaloptera). Fourteen percent of the insects was trapped in the first month before the first neonicotinoid spike (i.e. before the 18th of May 2018), meaning that 86% was caught during the experimental period (i.e. 18th of May – 12th of July 2018). This coincides with a steady increase of 7 °C in water temperature during the first month of trapping see Supplement, Figure 5.S1).

Neonicotinoid driven decline in insect abundance

Total insect emergence was significantly affected by neonicotinoid concentrations in interaction with time (adj. $R^2 = 0.67$, χ^2 (34, N = 864) = 6107, $p < 0.001$), but not following a dose-response relationship (Figure 5.2). We observed temporal increase in emergence at the lowest test concentration (52% at $t=24$ and 0.08 $\mu\text{g/L}$) and an absolute decrease at the highest test concentration (25% at $t=55$ and 9.86 $\mu\text{g/L}$ thiacloprid) compared to the control. This pattern is mostly reflected by Diptera as they made up 93% (on average, SD = 5.4%) of the total abundance and their emergence mirrored the patterns for total abundances (adj. $R^2 = 0.64$, χ^2 (34, N = 864) = 6050, $p < 0.001$, Figure 5.2). Effects of the neonicotinoid on the other four insect orders, Coleoptera, Odonata, Trichoptera and Ephemeroptera, were more severe; shortly after application of thiacloprid, we found large reductions in the number of emerging insects for all four orders. For Coleoptera, we observed no observed effects at the lowest test concentration (0.08 $\mu\text{g/L}$). However, strong declines over time, resulting in 61% and 91% reduced emergence at the final day of insect collection were found at the 0.85 and 9.86 $\mu\text{g/L}$ thiacloprid relative to the control (adj. $R^2 = 0.58$, χ^2 (34, N = 864) = 368, $p < 0.001$). Similar patterns were found for Odonata (which were strongly dominated by *Ischnura elegans*, see Barmantlo et al., 2019) and

Trichoptera as they also consistently decreased over time relative to the control (adj. $R^2 = 0.61$, χ^2 (34, N = 864) = 28, $p < 0.001$ and adj. $R^2 = 0.42$, χ^2 (34, N = 864) = 47, $p < 0.001$ respectively). At the final day of collection, we observed decreases of 37, 53 and 61% in Odonata with increasing neonicotinoid concentrations, respectively (Figure 5.1). The number of emerged Trichoptera, like Coleoptera, came to an abrupt near full stop within 10 days after application (Figure 5.1), leading to a decline in abundance of 74% at the final day of collection at the highest test concentration (9.86 $\mu\text{g/L}$). Ephemeroptera emergence only started one month after (mid-June) the initial spike, but was immediately affected by the neonicotinoid application albeit not in a dose-response manner (adj. $R^2 = 0.43$, χ^2 (34, N = 864) = 1109, $p < 0.001$). We found a sharp average decline of 98% relative to the control in the cumulative number of emerged Ephemeroptera at a concentration of 9.86 $\mu\text{g/L}$ neonicotinoid (Figure 5.2). At a concentration of 0.85 $\mu\text{g/L}$, however, the cumulative number of Ephemeroptera increased 138% relative to the control at the final day of insect collection.

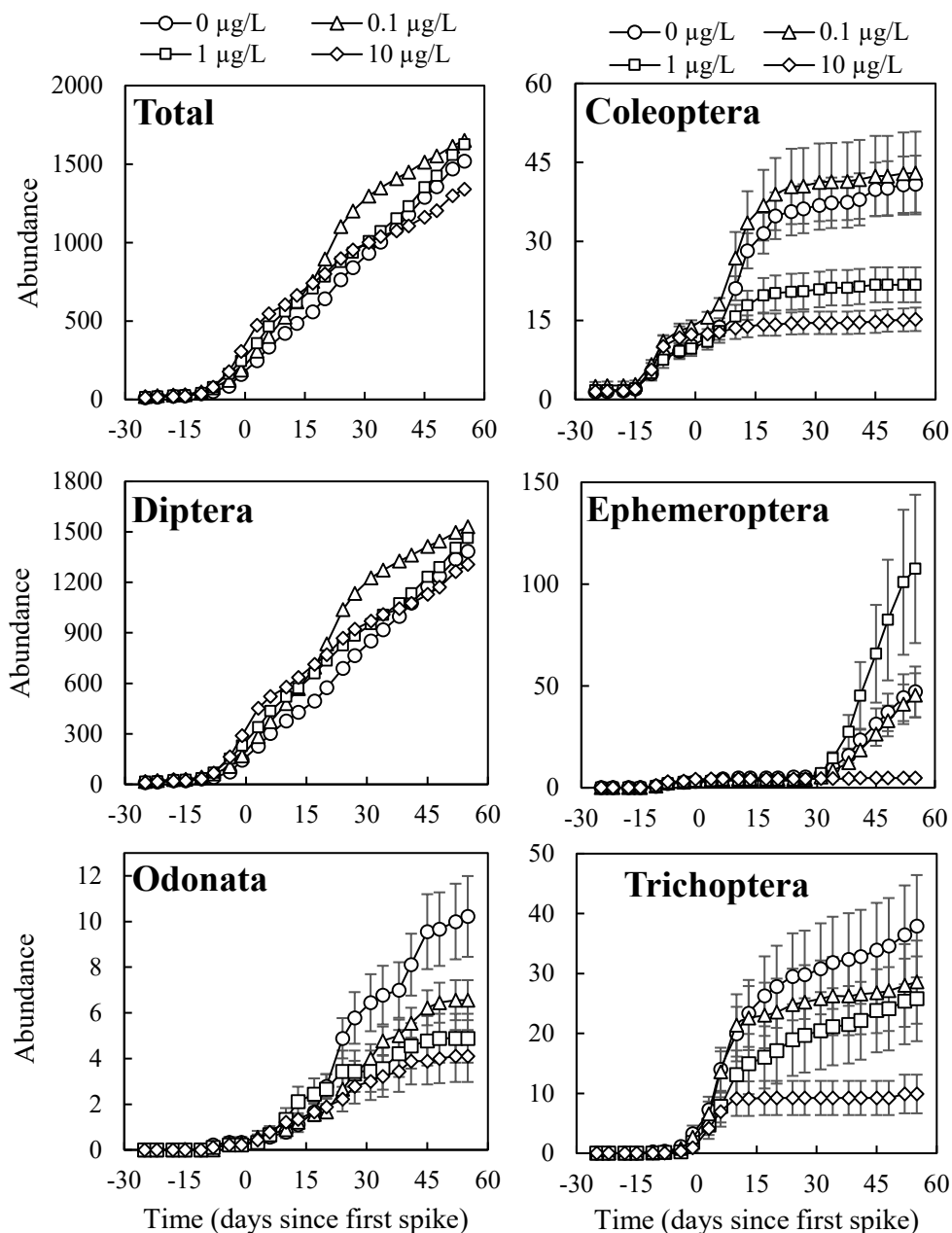


Figure 5.2. Average cumulative number of emergent insects (per 60 cm² water surface level, $\pm$ SE, n = 9) of the total emergence and that of the five major taxonomic orders over time per nominal spike concentration of the neonicotinoid thiacloprid. Errors bars for total and Diptera emergence are not shown for clarification purposes.

Effects on biomass of emerging insects

Despite the lack of severe effects on total cumulative emergence, the total collected insect biomass significantly declined with increasing neonicotinoid concentration (adj. $R^2 = 0.92$, $F_{1,34} = 0.84$, $p < 0.001$; Figure 5.3). At the final day of collection, average cumulative biomass had declined during the exposure period by 11, 4 and 50% with increasing neonicotinoid concentrations, respectively (Table 5.1). The decline in *median* cumulative biomass was 18, 27 and 48% with increasing neonicotinoid concentration, respectively (Supplement Figure 5.S2). This deviance between average and median biomass is explained by some high Ephemeroptera counts at a $TWA_{full\exp}$ of $0.16 \mu\text{g/L}$ (spike concentration $0.85 \mu\text{g/L}$), skewing the average biomass (Figure 5.2 and 5.3). The order of Diptera made up the bulk of the biomass collected, constituting to, on average, 52% of the control biomass on the final day of collection. Cumulative Diptera biomass decreased with increasing thiacloprid concentrations (adj. $R^2 = 0.91$, $F_{1,34} = 0.59$, $p < 0.001$), by 7, 17 and 25% at the final day of collection, respectively (Table 5.1). Despite these losses, the relative contribution of Diptera to total biomass actually increased to 82% at $TWA_{full\exp}$ $1.89 \mu\text{g/L}$ (spike concentration $9.86 \mu\text{g/L}$; $F_{1,34} = 18.9$, $p < 0.001$) as the contribution of the other taxonomic orders decreased more strongly (Figure 5.3). Coleopterans accounted for 10.5% of the total controls biomass which decreased to 9.5, 3.3 and 1.9% with increasing neonicotinoid concentrations, respectively ($F_{1,34} = 15.1$, $p < 0.001$). Both Trichoptera and Odonata appeared to also experience a similar decrease in percentual contribution (Figure 5.3), but these were not confirmed statistically ($p > 0.05$ for both comparisons). Nevertheless, as reflected by their abundances, the overall biomass of all orders decreased with increasing thiacloprid concentrations.

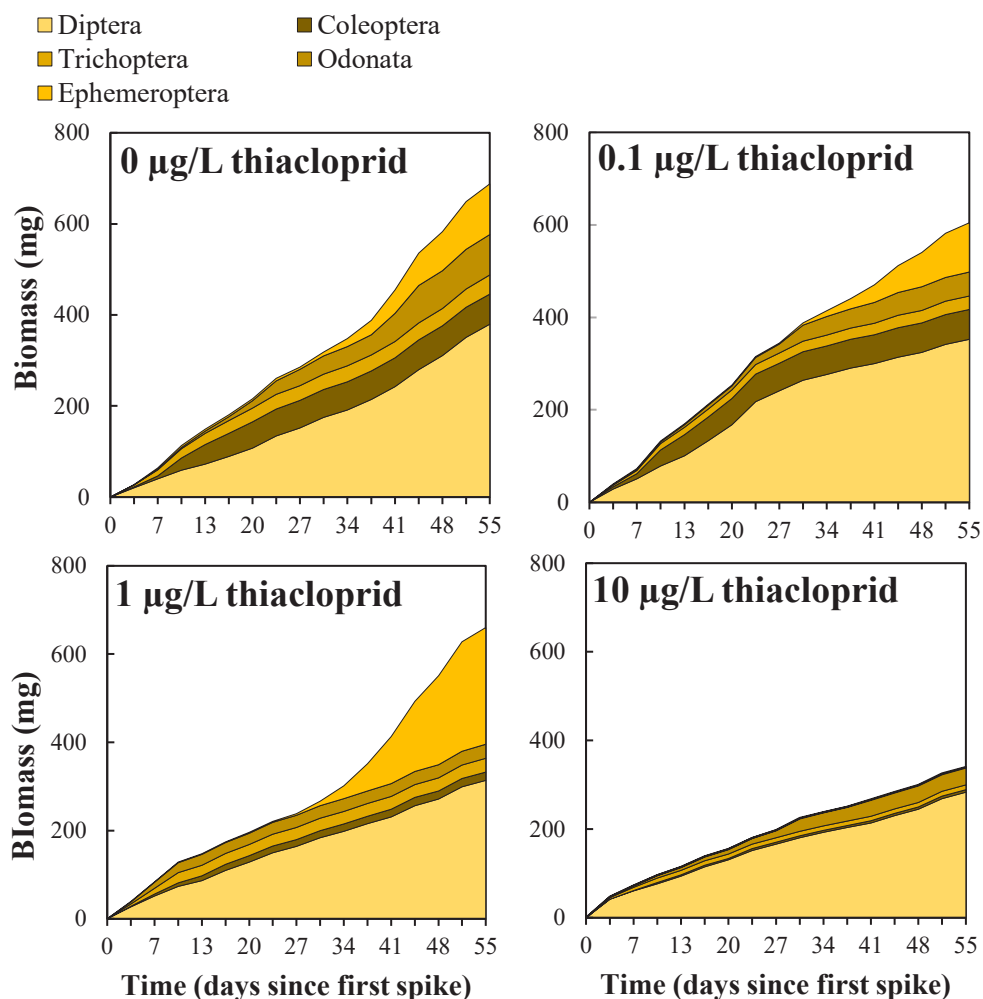


Figure 5.3. Thiachloprid-induced changes in average insect biomass of the different taxonomic orders over time per nominal spike concentration of neonicotinoid. Data before the addition of the neonicotinoid is not show for clarification purposes. The neonicotinoid thiachloprid was applied at $t=0$ and $t=15$ days.

Table 5.1. Cumulative biomass (in mg/60 cm² surface water ± SE) per insect taxonomic order on the final day of collection (t=55) since the first neonicotinoid application (t=0).

Biomass per taxonomic order	Nominal spike / TWA_{full exp} concentration thiacloprid (µg/L)			
	0 / 0 (control)	0.1 / 0.02	1 / 0.16	10 / 1.89
Total	690.1 (±96.0)	612.7 (±73.2)	662.4 (±112.2)	344.6 (±27.2)
Coleoptera	65.8 (±15.1)	64.7 (±26.5)	18.6 (±3.3)	5.4 (±1.6)
Diptera	379.8 (±86.0)	352.3 (±38.3)	313.8 (±28.6)	283.3 (±29.5)
Ephemeroptera	111.1 (±31.1)	106.6 (±27.5)	264.7 (±92.1)	1.7 (±0.6)
Trichoptera	42.2 (±9.0)	29.2 (±7.7)	31.5 (±7.8)	11.0 (±3.3)
Odonata	88.9 (±17.9)	51.6 (±8.7)	33.1 (±9.0)	38.7 (±16.0)
Others	2.3 (±1.3)	8.2 (±3.9)	0.7 (±0.6)	4.5 (±2.3)

TWA_{full exp}: Time weighted average concentration from the first neonicotinoid application until the end of insect collection (t=55). Others: sum of Hemiptera and Hymenoptera.

Effects of neonicotinoid exposure on diversity

Effects of neonicotinoid exposure on diversity were investigated for the most species-rich family of insects, the Chironomidae (order: Diptera). One month after the first application of the neonicotinoid (i.e. after >95% neonicotinoid removal from the aqueous phase), we identified 477 individuals belonging to 29 different species of three different sub-families of Chironomidae (Chironominae, Orthocladiinae and Tanypodinae, see Supplement Table 5.S1 for the full list of species). Neonicotinoid exposure induced a strong dose-response driven decline in species diversity. We calculated a 50% reduction (or 'EC₅₀') of Shannon *H*-diversity at a time-weighted average (TWA) concentration of 0.40 µg/L thiacloprid (SE 0.15; spike concentration: 1.11 µg/L, SE 0.41). Mean Shannon diversity (*H'*) decreased from 1.60 to 1.67, 0.95, and 0.16 at the TWA concentrations of 0, 0.03, 0.31 and 3.33 µg/L respectively (Figure 5.4). A similar decline was observed in Shannon evenness (*J'*): 0.88 to 0.87, 0.60 and 0.33 with increasing concentration respectively (time-weighted average EC₅₀ = 1.16 µg/L, SE 0.65). These effects on evenness likely represent an underestimation as species richness was reduced to only one species in 60% of samples of the highest concentration, *Procladius choreus*, not allowing calculating evenness (Supplement Figure 5.S3). In contrast to all other Chironomidae, this species actually strongly increased in abundance by 301-310% on average at the two highest test concentrations relative to the control (Dunnett's post-hoc test: *T* = 2.5, *p* = 0.024 and *T* = 2.4, *p* = 0.030 respectively). This strong increase also explains part of the lack of large effects on total emergence as *P. choreus* (assuming equal effects on females) made up 77% of this total emergence at this point in time at the highest test concentration versus 17% for the control (Supplement Figure 5.S4).

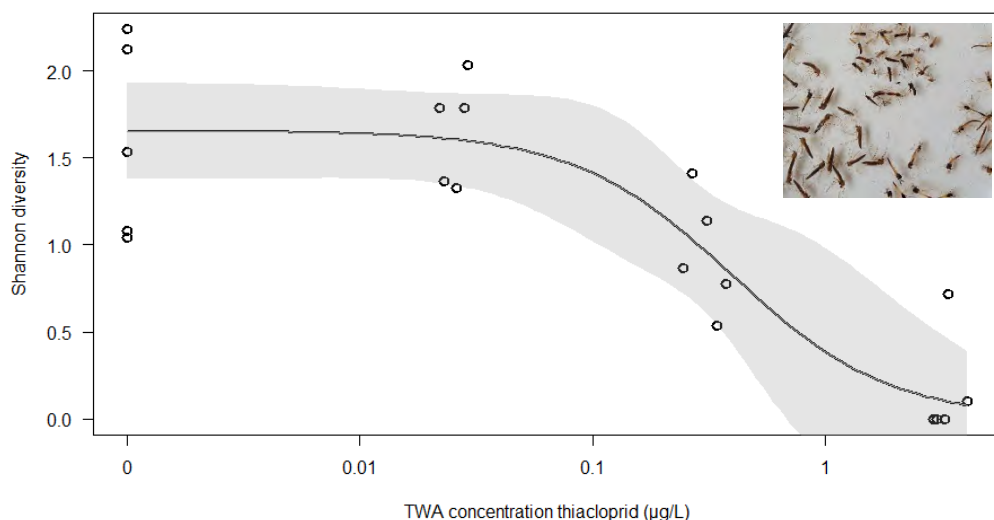


Figure 5.4. Dose-response model ($\pm 95\%$ confidence intervals in grey shading, all data points) of male chironomid Shannon diversity (H' , $n=5$) per actual time weighed average concentration of the neonicotinoid thiacloprid one month after the first spike. The x-axis is log transformed for presentation purposes.

5.4 Discussion

Both scientific (Goulson, 2013; Hallmann et al., 2014; Powney et al., 2019), policy (EASAC, 2015), as well as public debates (amongst all in The Guardian [Carrington, 2019] and the New York Times [Jarvis, 2018]) on insecticides center around the hazard of neonicotinoids to natural insect populations. Despite this awareness, the actual risks to natural insect populations and ecosystems have remained largely unknown due to a lack of well controlled experiments (Brain and Anderson, 2019; Gilburn et al., 2015). Here, we show by using experimental ditch-ecosystems that current neonicotinoid surface water levels decrease abundance and biomass of five major insect orders. In addition, we show that a monthly average concentration of $0.4 \mu\text{g/L}$ halves the diversity of the most species-rich freshwater insect family, the chironomids. At our highest spike concentration ($9.86 \mu\text{g/L}$), chironomid diversity was even close to zero and emergence of three out of five major insect orders (Coleoptera, Ephemeroptera and Trichoptera) was close to non-existent.

These strong insecticide-induced impacts were not as apparent in the total cumulative abundance, in a large part due to the contrary response of a single Chironomidae species. This thereby shows that identification to the species level is sometimes needed to unravel specific patterns. The responses of Coleoptera, Ephemeroptera, Trichoptera and Odonata were more straight forward; current neonicotinoid surface water concentrations reduce their abundance and their relative contribution to total biomass. These results for Trichoptera and Ephemeroptera are somewhat in line with results of single species bioassays within these orders (Roessink et al., 2013; Morrissey et al., 2015; van den Brink et al., 2016). Indeed, close to none Trichoptera and Ephemeroptera emerged at our highest test concentration. Nevertheless, we observed that Ephemeroptera were thriving at spike concentrations that are deemed toxic (0.85 µg/L) (Roessink et al., 2013). As our ditches are continuously (re)colonized, we hypothesize that Ephemeroptera egg deposit had occurred after removal of most of the neonicotinoid from the aqueous phase. This is supported by the observed very rapid removal of the neonicotinoid (DT50 = 3.6 days) and the fast development of several Ephemeroptera species (generally a few weeks at our measured water temperature (Sweeney et al., 2018)). In contrast, we observed a temporary increase in abundance at the lowest test concentration shortly after neonicotinoid application for the order of Diptera. Nevertheless, biomass of Diptera was reduced at this same concentration, indicating that the diversity shifted towards species with smaller and/or lighter imagos (such as *P. choreus*). Smaller species often have shorter life cycles (Danks, 2006) and therefore, this finding, like for Ephemeroptera, is another indication that species with a short aquatic lifespan might avoid neonicotinoid exposure or even benefit from opened niche space.

Within the order of Diptera, Chironomidae made up the vast majority. Chironomidae are considered useful indicators for water quality (Lencioni et al., 2012) due to the wide range of species with specific habitat preferences. In our study, we observed that the total abundance of Chironomidae is not a sensitive indicator for neonicotinoid pollution as the impacts of different concentrations were relatively small compared to the impacts on other insects. Chironomidae diversity, however, proved to

be a highly sensitive indicator for neonicotinoid pollution: diversity was halved at a monthly average concentration of 0.4 µg/L, which is below the current Dutch admission norm (0.51 µg/L; Leiden University & Rijkswaterstaat-WVL, 2018). One species of Chironomidae, *P. choreus*, actually increased strongly in abundance with increasing neonicotinoid concentration which explains the only small decline in total emergence. We hypothesize that *P. choreus* larvae, being mostly predaceous, occupied opened up niche space as earlier experiments in the same experimental ditches show losses in several other predator taxa (Barmantlo et al., 2019a) and predator activity (Barmantlo et al., 2019b).

Neonicotinoid surface water concentrations have earlier been associated to a decrease in several insectivorous bird species (Hallmann et al., 2014). It has been hypothesized and discussed that such decreases in insectivorous birds could be due to a food source (insects) depletion because of neonicotinoid toxicity during the aquatic larval stage (Hallmann et al., 2014; Gibbons et al., 2015; Lennon et al., 2019). Our results experimentally show that such a depletion in food source can indeed be caused by neonicotinoids as biomass decreased for all five major insect orders. We tested this in the same timespan when neonicotinoid surface water concentrations are increasing in the Netherlands: May-July, which matches the timeline when insectivorous birds in the Netherlands are scavenging for insects to feed their young. In this sense, our results might even underestimate the magnitude of the effects considering that we only applied two spikes and neonicotinoid concentrations reduced rapidly. In the actual environment, however, neonicotinoid concentrations are typically found to continuously exceed environmental standards in surface waters (due to processes such as leaching (Vijver et al., 2017) meaning that exposure time would be higher than within our test setup.

Our experiment with well-replicated and naturally assembled insect populations which approximate the conditions of real freshwater ecosystems allowed us to identify causal effects of neonicotinoids to insects. The ecological communities were exposed to a realistic range of neonicotinoid concentrations as observed in surface waters around the

globe. From this, we conclude that current neonicotinoid surface water levels have pronounced, negative effects on abundance, biomass and diversity of emerging aquatic insects. While our data experimentally proved that neonicotinoids cause insect decline, a quantitative estimate on the magnitude of neonicotinoid contribution to the ongoing insect decline is still open for research. The main reason for this is that in the field situation neonicotinoids often occur in mixtures with other agrochemicals in surface waters (Vijver and van den Brink, 2014) and interactions with other drivers might enhance or decrease their toxicity (Clements et al., 2012; Ieromina et al., 2014; Gessner and Tlili, 2016; Barmantlo et al., 2018). Nevertheless, given the urgency of the insect decline, we consider that the usage of neonicotinoids should be halted or at least strongly reevaluated in order to preserve freshwater insects, as well as the life and services depending on them.

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Author contributions

SHB performed the experiment. SHB and AvN carried out insect identifications. SHB and PMvB performed the data analysis. All authors contributed to the theory and the writing of the manuscript.

Conflict of interest

The authors report no conflict of interest.

Supplement

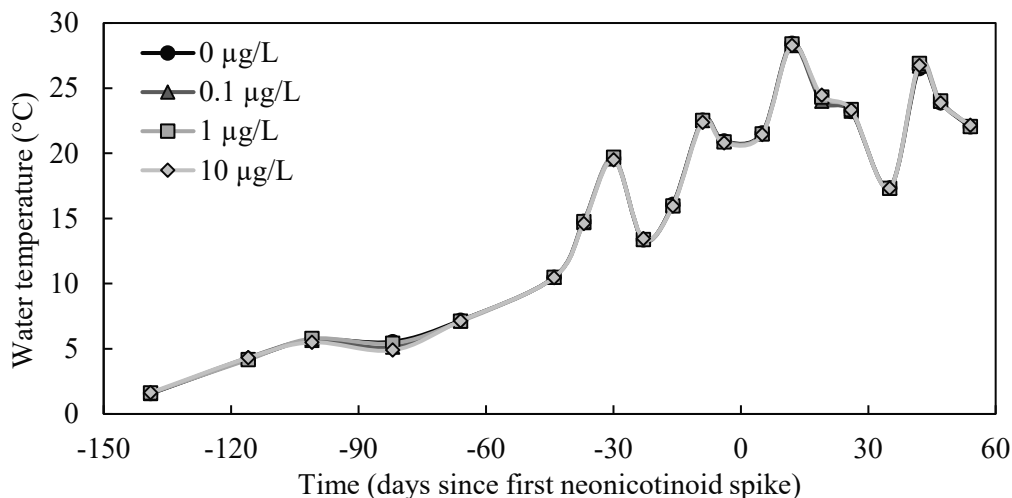


Figure 5.S1. Average water temperature per concentration thiacloprid ($n = 9$). There were no significant differences in water temperature between treatments (SD: 0.1-0.6 °C) at any given time ($p > 0.05$).

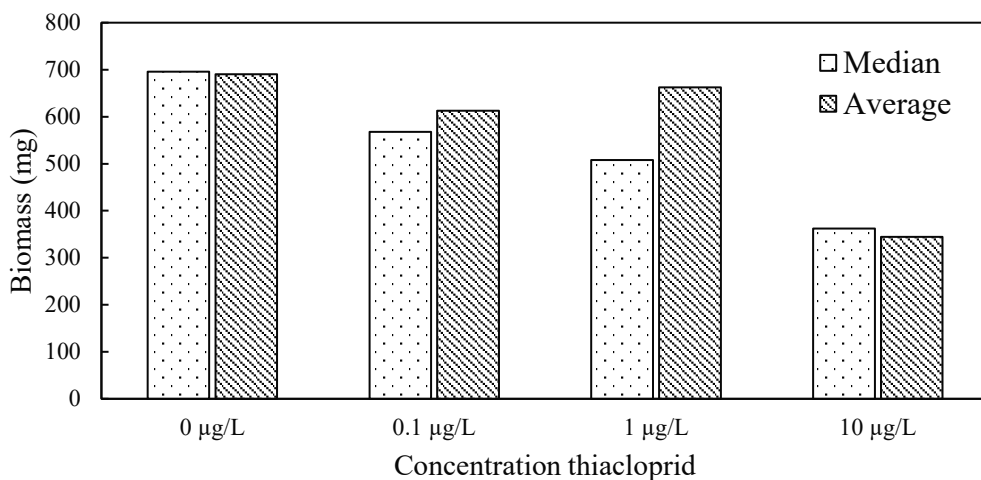


Figure 5.S2. Median and average cumulative insect biomass with increasing nominal neonicotinoid spike concentration since the first spike ($t=0$).

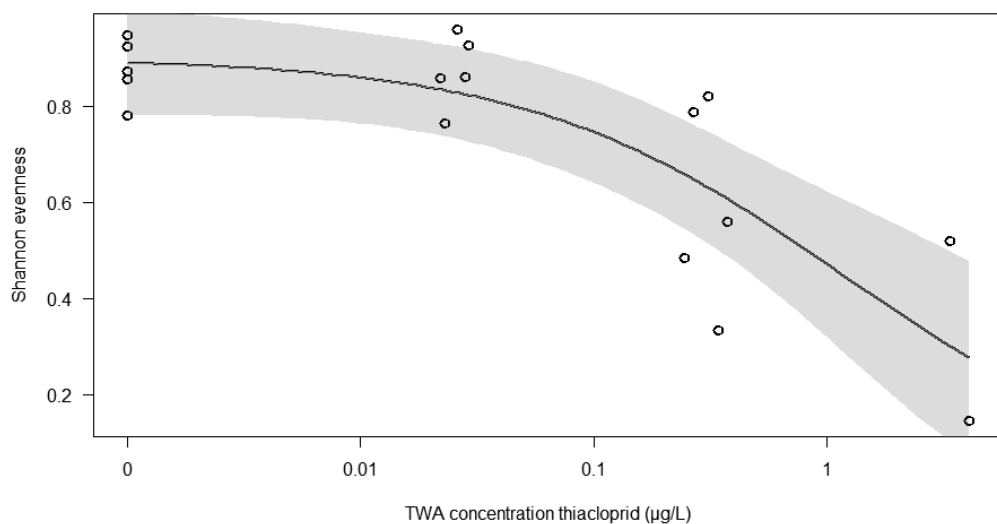


Figure 5.S3. Dose-response model ($\pm$ 95% CI in grey shading, all data points) of male chironomid Shannon evenness (J' , $n=5$) per actual time weighed average concentration of the neonicotinoid thiacloprid one month after the first spike. The x-axis is log transformed for presentation purposes. Note that at the highest test concentration, evenness could not be calculated in 3/5 samples as species richness was equal to 1.

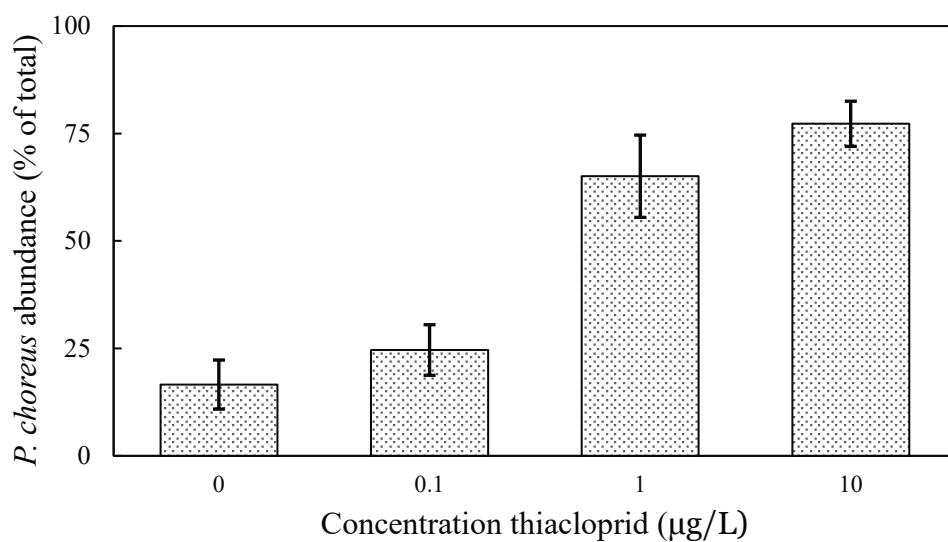


Figure 5.S4. Abundance of the chironomid *Procladius choreus* as percentage of total insect emergence one month after the first application of thiacloprid.

Table 5.S1. List of identified Chironomidae species (male specimens only) in alphabetical order.

Family	Sub-family	Genus	Species
Chironomidae	Chironominae	Cladotanytarsus	mancus
Chironomidae	Chironominae	Cryptochironomus	supplicans
Chironomidae	Chironominae	Endochironomus	tendens
Chironomidae	Chironominae	Microtendipes	pedellus
Chironomidae	Chironominae	Parachironomus	vitiosus
Chironomidae	Chironominae	Polypedilum	nubeculosum
Chironomidae	Chironominae	Tanytarsini	Sp.
Chironomidae	Chironominae	Tanytarsus	cf. striatulus
Chironomidae	Chironominae	Tanytarsus	Mendax
Chironomidae	Chironominae	Tanytarsus	pallidicornis
Chironomidae	Chironominae	Tanytarsus	Sp.
Chironomidae	Chironominae	Tanytarsus	usmaensis
Chironomidae	Orthoclaadiinae	Acricotopus	lucens
Chironomidae	Orthoclaadiinae	Corynoneura	carriana
Chironomidae	Orthoclaadiinae	Cricotopus	cf. cylindraceus
Chironomidae	Orthoclaadiinae	Cricotopus	cf. flavocinctus
Chironomidae	Orthoclaadiinae	Cricotopus	sp.
Chironomidae	Orthoclaadiinae	Cricotopus	sylvestris
Chironomidae	Orthoclaadiinae	Metriocnemus	sp.
Chironomidae	Orthoclaadiinae	Nanocladius	dichromus
Chironomidae	Orthoclaadiinae	Psectrocladius	cf. sordidellus
Chironomidae	Orthoclaadiinae	Psectrocladius	limbatellus
Chironomidae	Orthoclaadiinae	Psectrocladius	obvius
Chironomidae	Orthoclaadiinae	Psectrocladius	sordidellus
Chironomidae	Tanypodinae	Ablabesmyia	monilis
Chironomidae	Tanypodinae	Ablabesmyia	sp.
Chironomidae	Tanypodinae	Paramerina	cingulata
Chironomidae	Tanypodinae	Procladius	choreus
Chironomidae	Tanypodinae	Xenopelopia	falcigera

Chapter 6

Neonicotinoids and fertilizers jointly structure naturally assembled freshwater macroinvertebrate communities

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Abstract

Although it is widely acknowledged that a decline of freshwater biodiversity jeopardizes the functioning of freshwater ecosystems, the large number of (human-induced) pressures jointly acting on these systems hampers managing its biodiversity. To disentangle the magnitude and the temporal effects of these single and interacting pressures, experiments are required that study how these pressures affect the structuring of natural communities.

We performed experiments with naturally assembled invertebrate communities in 36 experimental ditches to assess the single and joint effects of environmentally relevant concentrations of two commonly co-occurring stressors: fertilizer inputs and neonicotinoid insecticides, in this case thiacloprid. Specifically, we explored whether these agrochemicals result in sustained changes in community structure by inspecting divergence, convergence and short- /long-lived dissimilarity of communities, when compared to a control treatment.

Our results indicate strong impacts on the abundance of different taxa by exposure to the agrochemicals. However, we found no effect of any treatment on total abundance, taxon richness or convergence/divergence (measured as beta dispersion) of the communities. Moreover, we found contrasting responses when both joint stressors were present: when considering abundance of different taxa, we observed that fertilizer additions reduced some of the thiacloprid toxicity. But when assessing the community structure, we found that exposure to both stressors consistently resulted in a more dissimilar community compared to the control. This dissimilarity was persistent up to four months after applying the agrochemicals, even though there was a turnover in taxa explaining this dissimilarity. This turnover indicates that the persistent dissimilarity can potentially be attributed to a rippling effect in the community rather than continued toxicity. Such shifts in natural freshwater invertebrate communities, months after the actual exposure, suggests that stressors may have important long-term repercussions for which may subsequently lead to changes in ecosystem functioning.

Keywords: freshwater ecosystem, thiacloprid, nutrients, dissimilarity, divergence, convergence

6.1 Introduction

Globally, we are witnessing a strong decline in freshwater aquatic biodiversity, which negatively affects the functioning of freshwater ecosystems (Dudgeon et al., 2006; Schwarzenbach et al., 2006). These declines have been widely attributed to anthropogenic pressures (Schwarzenbach et al., 2006; Rockström et al., 2009), but the large number of different pressures that are found jointly in freshwater ecosystems hampers the identification and prediction of their adverse effects to freshwater ecosystems. As biota drives ecosystem functioning, an understanding of how different anthropogenic pressures jointly affect and structure freshwater communities is thus essential to effectively manage and conserve these ecosystems.

Studying how anthropogenic stressors impact the structure of aquatic communities has proven notoriously difficult as natural communities are affected by a variety of pressures that generally operate at the same time (Côté et al., 2016). Two of the most prominent anthropological pressures on freshwater ecosystems result from the emission of agricultural fields: pesticides and fertilizers (Matson et al., 1997; Davis et al., 2010; Malaj et al., 2014), agrochemicals that are often applied and found together (Schreiner et al., 2016). Especially the effects of neonicotinoid insecticides have gained increasing attention (see e.g. Hallmann et al., 2014; Pisa et al., 2015) as they are among the most commonly used pesticides on the global market (Jeschke et al., 2011) and consequently show elevated concentrations in surface waters (Morrissey et al., 2015). Maximum observed concentrations are typically found around 0.1 – 1 µg/L (see Figure 2b in Morrissey et al., 2015), but were also found to reach up to 320 µg/L for the neonicotinoid imidacloprid in Dutch surface waters (van Dijk et al., 2013).

Over the past decades, it has become apparent that, in addition to agricultural pest insects, non-target invertebrates including freshwater macrofauna experience high toxicity from neonicotinoids at

concentrations that are found in surface waters across the world (Morrissey et al., 2015; Miles et al., 2017; Vijver et al., 2017). Several authors have provided evidence that such concentrations also affect invertebrate populations and communities (Beketov et al., 2008; Miles et al., 2017; Barmantlo et al., 2018a; Rico et al., 2018). However, recent data suggests that such effects can be reduced by increased nutrient levels or food quality as this may allow for compensatory feeding (Ieromina et al., 2014; Barmantlo et al., 2018b). Fertilizers (or nutrients) themselves also present an important stressor for aquatic communities (e.g. Davis et al., 2010). This entanglement of different types of processes thus explains why the study of the combined effects of different stressors, such as neonicotinoids and fertilizers, to natural communities has been proven to be so challenging (Alexander et al., 2013; Côté et al., 2016).

Obtaining understanding on how the single and joint effect of pesticides and fertilizers affect communities requires experiments that disentangle the single and interacting pressures that operate on natural freshwater communities. When comparing to a control situation, such data allow for the examining of changes in the structuring of communities. A powerful tool to analyze such changes is by identifying whether the stressors alter the state of the communities, distinguishing two important changes in community state: 1) convergence: a perturbation causes all communities to become reduced to one state and 2) divergence: a perturbation results in communities to evolve to multiple states or no clear state (Houseman et al., 2008). A lower degree of variation between communities (lower beta dispersion) after a disturbance indicates convergence and can possibly be a result of ecological filtering, for example, due to neonicotinoids that harm specific species (Figure 6.1A, scenario i). On the other hand, a higher degree of variation after a disturbance (higher beta dispersion) would indicate divergence, which would for example be due to fertilizer inputs that broaden or add ecological niches by increasing productivity; Figure 6.1A, scenario iii). In addition, communities can become more or less dissimilar compared to a control state (Figure 6.1B) irrespective of the degree of variation between communities.

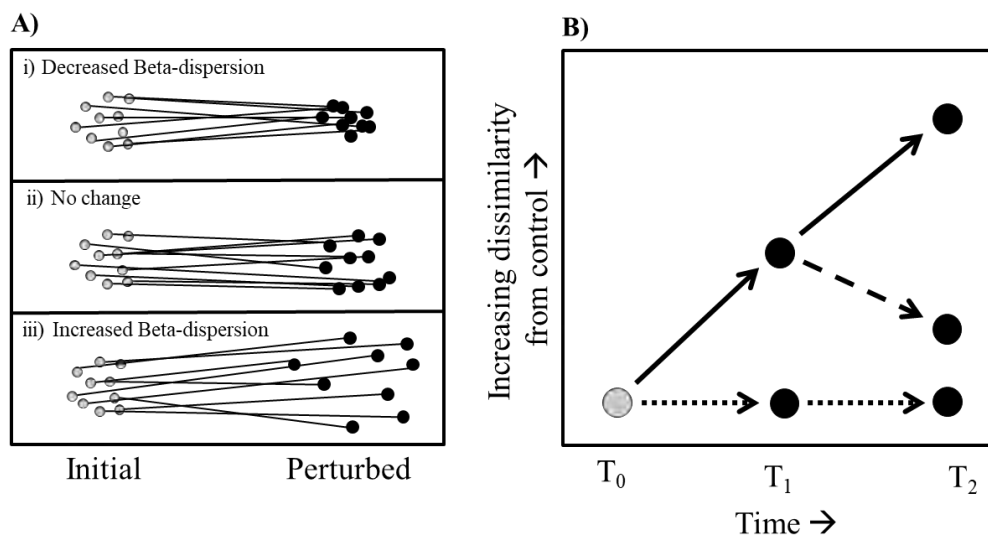


Figure 6.1. (A) Effects on beta dispersion; communities can either gain niche width between the initial and perturbed state (top panel; divergence), do not change (middle panel), or lose niche width (lower panel; convergence). (B) Effects on community similarity; pressures can cause the communities to become more dissimilar (straight arrows), veer back to the control (dashed arrow) or stay similar (finely dashed arrow) to the control. Circles denote the individual communities.

To move towards an integral understanding of the joint effects of fertilizers and neonicotinoids under natural conditions, we distinguish three possible outcomes of these agrochemicals on naturally assembled freshwater invertebrate communities. Assuming that environmentally relevant concentrations of both agrochemicals indeed affect the communities, the joint effect can result in i) a qualitatively different shift in community composition, but one of the pressures masks or reduces the effect of the other ii) an additive effect of both agrochemicals, or iii) a combined effect of both agrochemicals that is stronger than the single effects. This study aims to evaluate the single and joint effect of agrochemical stressors on taxa abundance and structuring of communities, both in terms of beta dispersion (Figure 6.1A) and dissimilarity (figure 6.1B). Moreover, we test whether shifts in community composition are short-lived or relatively long-lived, i.e. whether the

changes persist up to a four-month period (Figure 6.1B). To this end, we assessed the impact of environmentally relevant concentrations of a model neonicotinoid, thiacloprid, and fertilizer in experimental ditches on naturally assembled freshwater communities at three different time points: one month before treatments (assuming no effects and similar communities), one month after and four months after application of the agrochemicals.

6.2 Methods

Experimental site

The experiment took place in the outdoor facility ‘Living Lab’ of Leiden University (the Netherlands; Figure 6.2). In November 2016, 36 adjacent ditches with a length of 10 m, a width of 0.8 m at the surface level and 0.4 m at the sediment level on a depth of 0.3 m were dug adjoining to an existing water level compensation reservoir in Oegstgeest, The Netherlands (Figure 6.2). The reservoir has an average depth of 1.5 m and is connected to the Old-Rhine watershed through ditches and canals. The reservoir is located next to uncultivated grassland (for the past ~30 years, owned by Leiden University) and characterized by low levels of soluble nutrients and absence of any pesticide residues as there are no agricultural (or agrochemical maintenance) practices in close proximity. A longstanding undisturbed ecological community of invertebrates, fish, birds and plants is present in the reservoir, yielding full ecosystem complexity. The ditches are protected from waves from the reservoir by a wooden barrier, thus more naturally mimicking actual drainage ditches and protecting the testing facility from erosion. All ditch banks are covered by organically grown grass. Furthermore, between the wooden barrier and the ditch entrance, a row of *Typha latifolia* was planted in order to attract invertebrates that would colonize the ditches. Fish were retained from accessing the ditches by placing 5 mm netting (dug into the sediment and reaching 10 cm above the water surface level) between the wooden barrier and the ditch entrance. Birds were effectively repelled from the ditches by a brightly colored orange wire hanging 5 cm above the water surface level over the length of each ditch.



Figure 6.2. Photographic overview of the experimental site ‘the Living Lab’. Top to bottom: adjoining reservoir, wave barrier, macrophytes, netting, experimental ditches.

Experimental setup

The 36 experimental ditches had been dug by removing 1.8 m of topsoil and were left connected to the reservoir for six months (November – April) to form naturally colonized communities of invertebrates and plants. As we dug nearly two meters down, the ditches were initially characterized by clay with low levels of organic matter. Therefore, we transplanted as little as 10 L of organically rich sediment from the adjoining reservoir into each experimental ditch in February 2017. In April 2017, we sampled the biota residing in the ditches (see 2.3 for the sampling procedure) in order to establish if natural communities have formed comparably between the ditches. Then, in order to avoid cross-contamination of agrochemicals between treatments, we ensured that all ditches became hydrologically isolated from the reservoir using 1000:500:2 mm acrylic plates that were hammered firmly into the ditch banks and 15 cm deep into the sediment. After this, the total ditch length was 9 m. Hereafter, we started application of the different agrochemicals. The experiment entailed a full factorial design of four different treatments with nine replicates per treatment: i) control (no added substances, ‘C’),

ii) thiacloprid addition ('T'), iii) fertilizer addition ('F') and iv) thiacloprid and fertilizer addition ('TF') (See Supplement Figure 6.S1 for a schematic overview of the block design). Substances were added to the ditches following a block design in order to account for the effects of possible naturally occurring gradients. We use thiacloprid as a model neonicotinoid as it is among the most toxic of neonicotinoids to non-target organisms and like most neonicotinoids abundantly present in surface waters (Morrissey et al., 2015; Leiden University and Rijkswaterstaat-WVL, 2016).

Starting in April 2017 (after hydrologically isolating the ditches), we enriched half of the ditches continuously with fertilizer by hanging three sachets filled with 75 g of slow-releasing granulates (Osmocote; N:P:K = 15:9:11 combined with microelements, Dordrecht, The Netherlands) in each ditch. These sachets were replaced with fresh granulates every six weeks. Fertilizer addition was aimed to approach nutrient levels that have been experimentally showed to stimulate freshwater algal growth (see Ieromina et al., 2014). In May 2017, we started two biweekly applications of thiacloprid (99.9% purity; Sigma-Aldrich, Zwijndrecht, The Netherlands; see Supplement for details on the application procedure). We aimed to maintain a nominal time weighed average concentration (TWA) of 0.4 µg/L for the duration of one month. This environmentally relevant concentration of thiacloprid was determined from data retrieved from the Leiden University and Rijkswaterstaat-WVL dataset (2017) that shows grab samples of surface water concentrations of thiacloprid in the Netherlands in the period 2011-2015 (discussed and shown in Barmentlo et al., 2018). The experimental concentration (0.4 µg/L) falls in the top 10% of detect concentrations measured in the month May. However, note that thiacloprid is highly likely to quickly adsorb to sediment (given its log K_{ow} of 1.26; USEPA, 2003) and consequently the chance to detect the maximal surface water concentration via grab sampling is low. In addition, the smaller waters like we simulate here are often not monitored and are generally the first recipient of agrochemical loadings, meaning that they likely receive higher neonicotinoid concentrations. Our experimental concentration is not exclusive to the Netherlands but also fits the range for other countries and neonicotinoids (see Figure 1B in Morrissey et al.,

2015) To investigate the effectiveness of our nutrient enrichments and thiacloprid addition, we monitored dissolved nitrate (NO_3^-) and phosphate (PO_4^{3-}) concentrations using a NOVA 60 Spectroquant® photometer (Merck). These analyses were performed with water samples that were retrieved from the middle of each ditch, 5 cm below the surface. From these water samples, we also determined the thiacloprid concentration one hour after application of a spike. Subsequently, we measured randomly selected blocks within the block design daily for a week after the first thiacloprid application, then biweekly and finally monthly over the summer period. Thiacloprid concentrations were determined using liquid chromatography tandem mass spectrometry (Agilent Technologies; see Roessink et al., 2013 for the detailed procedure). We also monitored the possible effects of the agrochemicals on several physicochemical parameters including, water temperature, pH, dissolved oxygen (DO) and conductivity. See the Supplement for a detailed description on the methods and results for thiacloprid and nutrient concentration determination and for the physicochemical responses.

Community sampling

One month before (April), one month after (June) and four months after (September) the first application of thiacloprid (May), we sampled the macroinvertebrate communities residing in each experimental ditch. Sampling was conducted by quickly and simultaneously placing two acrylic plates with a width of 1 m into a ditch, thus isolating 1/9 of its length. By sampling all macroinvertebrates in this meter of ditch, we were able to standardize the number of taxa and number of animals caught to a fixed volume of water and sediment rather than the number of sweeps with a dipping net over a fixed length (as is the common practice, for example Ieromina et al., 2015). Within this 1 m of ditch, macroinvertebrates were sampled by sweeping with a 25*25 cm dipping net (150 μm). All plants within the compartment were carefully washed by hand to retrieve all macroinvertebrates. In order to catch the benthic macroinvertebrates, we sampled the top 3-5 cm of the sediment and ditch banks and sieved out the invertebrates using a 500 μm sieve. We stopped sampling when subsequent nets remained empty. All invertebrates were carefully collected in large white trays, sorted on species groups with the

naked eye (e.g. leeches, beetles) and then immediately identified to the lowest possible level using a stereomicroscope (magnification: 20-40x) at the on-site laboratory. In order to determine the species composition in all 36 communities with minimum disturbance to the community within the shortest possible time span (i.e. within one week to minimize time-dependent effects), we estimated the abundances of highly dominant taxa (such as Branchiopoda and Maxillopoda) by sieving these animals over a fine sieve (106 μm) and subsequently placing them in 500 mL of water. A subsample of this fixed volume was analyzed until at least 50 individuals were counted or 25% of the total sample volume was counted to ensure a representative sample of the community. The number of animals within the subsample was then multiplied to fit the total filtrate volume. Directly after identification, macroinvertebrates were released back into the ditch compartment they originated from in order to avoid a decline in communities in the ditches. The whole procedure from sampling to release took 1 – 2 h per ditch.

Statistical analyses

In order to detect the formation of multiple community states as a result of either thiacloprid addition ('added' or 'not added') or fertilizer addition ('added' or 'not added') and their possible interaction, we tested for dissimilarity of the communities using Permutational multivariate analysis of variance (PERMANOVA, function 'adonis', R package 'Vegan') with 999 permutations. We $\log_{10}(x+1)$ transformed all count data and used Bray-Curtis as our measure of dissimilarity as recommend by Tebby et al. (2017) for data 1) with large differences in abundances between species and 2) where the less abundant species may be affected. This was evaluated by examining the histograms per species. Our full model investigated the effects of fertilizer, thiacloprid, time (April, June, September, included numerically) and all possible interactions while including the respective ditch measured as a random variable to account for potential effects of the ditch. As freshwater communities are highly variable between seasons (because of the high turnover in abundances and species), we investigated the relative effects of both fertilizer and thiacloprid per month as well to gain more insight on the specific

community responses. In these monthly models, we included ditch number as a fixed effect (ranging from 1 to 36, aligned from left to right at the experimental site) and all possible interactions with any of the treatments in order to evaluate the effects of possible natural gradients within the experimental setup. To test whether the dispersion of the data was homogeneous across the different treatments, we used permuted (999) distance-based dispersion tests (function 'betadisper', R package 'Vegan'). Deviations from such homogeneity can point to convergence (decreased distances) or divergence (increased distances) within a treatment compared to the control. Finally, when treatments indicated a significant effect on the community centroids, we calculated the average contribution of individual taxa to the overall Bray-Curtis dissimilarity using SIMPER (similarity percentage, R package 'Vegan').

We also investigated for possible effects of thiacloprid ('added' or 'not added'), nutrients ('added' or 'not added'), time and all possible interactions on total taxon richness, total abundance and average abundance per taxon using linear mixed effect modelling (function 'lme') while accounting for the repeated measure design by including the ditch as a random effect. In order to more closely examine the effects of both agrochemicals and their possible interaction on the community structure, we investigated their potential effect on the taxonomic class abundance per month using factorial ANOVA (function 'lm') We tested for these differences to identify whether group-specific effects of the agrochemicals occurred: for example lower insect abundance because of thiacloprid (an insecticide) addition. We tested for normal distribution of the model residuals using Quantile Quantile-plots and homogeneity of variances using Levene's. If either assumption was not met (i.e., for the models on Insecta and Maxillopoda in June and for Malacostraca and Gastropoda in September), we log transformed the data accordingly. Prior to the analyses, we omitted one value in the thiacloprid treatment in September due to the extremely high number of Daphniidae observed (18,400 individuals), which heavily skewed the data as the treatment average was 571 individuals in total. This was due to an unexplained and unusual high concentration of phosphate (>1 mg/L) in this ditch in September. Statistical significance was considered at $p < 0.05$ and marginal

significance is reported at $0.05 < p < 0.10$. All statistical analyses were performed with R (version 3.5.0; R Core Team, 2018).

6.3 Results

Effectiveness of the treatments.

The actual TWA concentration of thiacloprid during the month after the first spike was 0.46 $\mu\text{g/L}$ (see Supplement, Table 6.S1). Thiacloprid concentrations in the water declined rapidly with an average DT50 of 3.3 days (SD 0.1) and DT90 of 11.1 days (SD 0.4) (Supplement Figure 6.S2). This was expected due to the high log octanol-water partition coefficient of thiacloprid ($\log K_{ow} = 1.26$; USEPA, 2003), thus thiacloprid was likely to adsorb to the sediment. There was no effect of fertilizer addition on the thiacloprid concentration at any given time ($p > 0.05$), meaning that thiacloprid did not degrade more rapidly because of e.g. increased bacterial degradation. Fertilizer addition significantly increased the TWA concentrations of both nitrate and phosphate ($F_{1,32} = 4.7$, $p = 0.037$; $F_{1,31} = 12.0$, $p = 0.002$) with a factor of 1.3 and 1.4 respectively (see Supplement, Table S1 for the actual concentrations).

Colonization, taxon richness and total abundances.

The broad range of observed taxa from different classes of macroinvertebrates illustrate that the experimental ditches were effectively colonized (Figure 6.3, Figure 6.4A,B,C) with a total of 59, 83 and 97 identified taxa in the months April, June and September, respectively (see the Supplement for the full lists of taxa per month). Average taxon richness per meter ditch in the controls increased significantly over time from 18.0 taxa in April to 20.9 taxa in June and 27.2 in September ($F_{1,68} = 127.9$, $p < 0.001$; Figure 6.3A). However, note that the abundance per taxon significantly decreased in September (5.8) compared to July (25.8, $F_{1,8} = 39.6$, $p < 0.001$). There were no effects of the treatments on the total taxon richness at any given month (thiacloprid: $F_{1,65} = 1.5$, $p > 0.05$ and fertilizer: $F_{1,65} = 1.2$, $p > 0.05$). There were also no observed significant effects of the treatments on the total number of animals in either April or

June (thiacloprid: $F_{1,32} = 0.3$, $p > 0.05$ and fertilizer: $F_{1,32} = 1.4$, $p > 0.05$). However, fertilizer addition increased the total number of animals four months after the first application ($F_{1,32} = 18.5$, $p < 0.001$; Figure 6.3B) by 100% compared to the control.

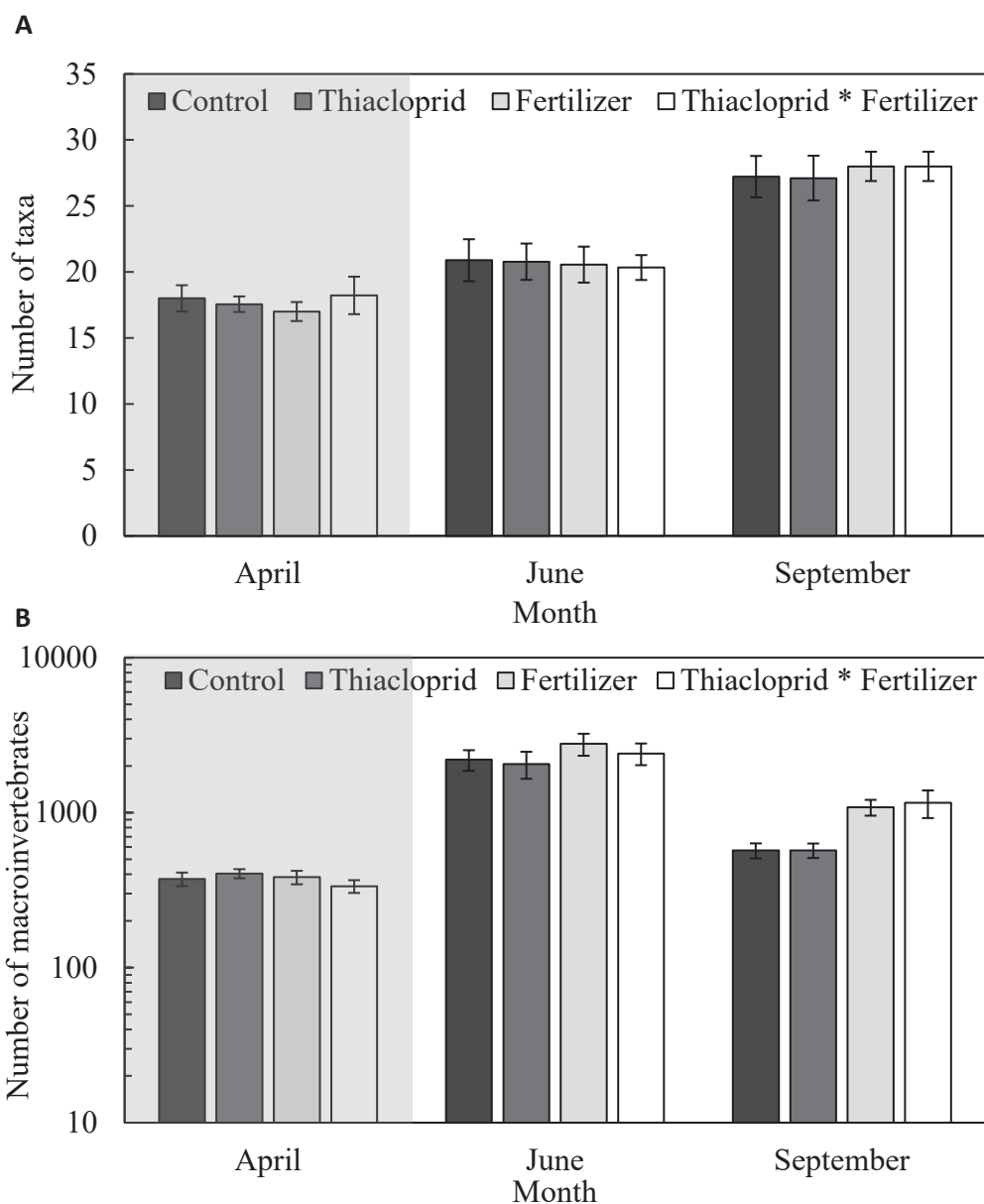


Figure 6.3. Average ($n=9$) A) macroinvertebrate taxon richness ($\pm$ SE, total number of identified taxa) and B) total macroinvertebrate abundance ($\pm$ SE) per treatment in 1 m of ditch per month (spiking was conducted in May 2017). Grey shading shows data before the addition of the agrochemicals.

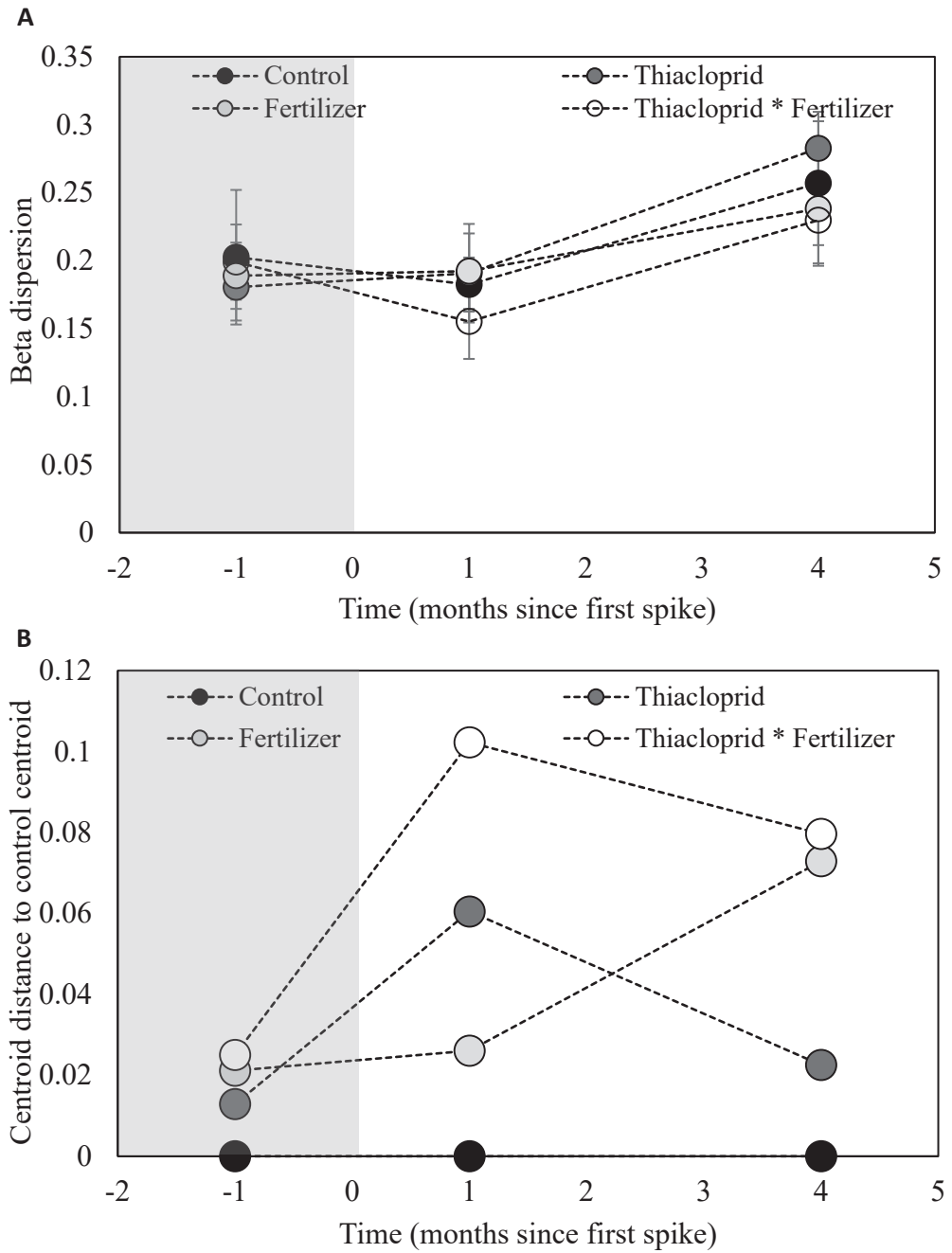


Figure 6.4. A) Average beta dispersion (distance to centroid, $\pm$ SD) per treatment and B) Centroid distance (Principal Coordinates Axes 1 and 2) to the control centroid per month. Treatments were applied at $t = 0$ (May). Grey shading shows data before the addition of the agrochemicals.

Temporal dynamics in dissimilarity and dispersion.

Both thiacloprid and fertilizer induced significant dissimilarity interacting with time relative to the control ($F_{1,100} = 12.5$, $R^2 = 0.06$, $p < 0.001$ and $F_{1,100} = 6.2$, $R^2 = 0.03$, $p < 0.001$ respectively); the thiacloprid treatment more strongly affected the community centroid in June, while the effect of fertilizer application was stronger in September (Figure 6.4B). Time, fertilizer and thiacloprid also showed a significant three-way interaction resulting in communities that were most dissimilar from the control in both June and September compared to the single treatments ($F_{1,100} = 3.4$, $R^2 = 0.02$, $p < 0.001$; Figure 6.4B). The effect of time on the community dissimilarity was strong ($F_{1,100} = 59.7$, $R^2 = 0.27$, $p < 0.001$), showing that the communities were highly dynamic in time. When investigating the communities more closely per month, we found no difference between community centroids in the prospective treatments before the addition of the agrochemicals (April; PERMANOVA: $p > 0.05$, Figure 6.4A). Community centroid shifted significantly with ditch number, which is a (fixed effect) spatial variable in these analyses ($F_{1,28} = 5.8$, $R^2 = 0.15$, $p < 0.001$), indicating a possible underlying spatial gradient affecting macroinvertebrate communities. This spatial impact on the communities was also present, but weaker over time, in both June and September ($F_{1,28} = 5.7$, $R^2 = 0.13$, $p < 0.001$ and $F_{1,28} = 2.6$, $R^2 = 0.07$, $p < 0.001$ respectively), but never interacted with any treatment effect ($p > 0.05$ for all possible interactions).

We found no significant effect on the distances to the centroids (beta dispersion) in the prospective treatments before the addition of the agrochemicals (April; beta dispersion tests, thiacloprid: $F_{1,34} = 0.4$, $p > 0.05$ and fertilizer: $F_{1,34} = 0.1$, $p > 0.05$, Figure 6.4A). Moreover, there was no observed difference in the distances to the centroid (beta dispersion) for both thiacloprid and fertilizer addition in June ($F_{1,34} = 1.6$, $p > 0.05$ and $F_{1,34} = 1.7$, $p > 0.05$ respectively; Figure 6.4A). Fertilizer addition did appear to reduce the average distance to the centroid in September ($F_{1,34} = 4.7$, $p = 0.037$; Figure 6.4A), but only in the thiacloprid – thiacloprid*fertilizer comparison. We also evaluated this effect of

fertilizer using raw data (as recommended for data with large differences in abundancies, see Tebby et al., 2017) and observed no deviation from homogeneity ($F_{1,34} = 0.2$, $p > 0.05$). Because of the large differences in total abundancies (Figure 6.3C) and the incoherency with the raw data, we consider that the slight deviation from homogeneity of the $\log(x+1)$ transformed model, if at all present, is negligible.

Monthly taxonomic class-specific responses to the agrochemicals.

While there was no effect of the treatments on total abundance in June, the abundances of the different taxonomic classes were affected in this month (Figure 6.5B). We observed that the addition of thiacloprid resulted 51% lower abundances of individuals belonging to the class Insecta compared to the control treatment in June, one month after application ($F_{1,32} = 5.0$, $p = 0.032$; Figure 6.5B). In June, the Insecta class was dominated by the family Chironomidae which contributed, on average, 83% to the total abundance within the control treatment. This explained most of the observed lower insect abundance, but there were clear effects in other insect taxa as well (see Table 6.1), most notably a 97-100% lower abundance of the alderfly *Sialis lutaria*. As a result of fertilizer addition, we observed marginally significant higher abundances for Insecta (35%) and Maxillopoda (29%) in June relative to the control ($F_{1,32} = 3.8$, $p = 0.060$ and $F_{1,32} = 4.0$, $p = 0.055$ respectively; Figure 6.5B). The class Malacostraca showed significantly higher abundances in the fertilization treatment (93%) in June ($F_{1,32} = 4.2$, $p = 0.048$; Figure 6.5B) relative to the control. In September, the number of Malacostraca again significantly higher by fertilizer addition ($F_{1,32} = 10.2$, $p = 0.003$; Figure 6.5C) compared to the control. The number of Gastropoda in September was also significantly higher due to fertilizer addition compared to the control (104%, $F_{1,32} = 16.9$, $p < 0.001$, Figure 6.5C). For Insecta, we observed a marginal higher number by fertilizer addition, similar to June (75%, $F_{1,32} = 3.9$, $p = 0.058$).

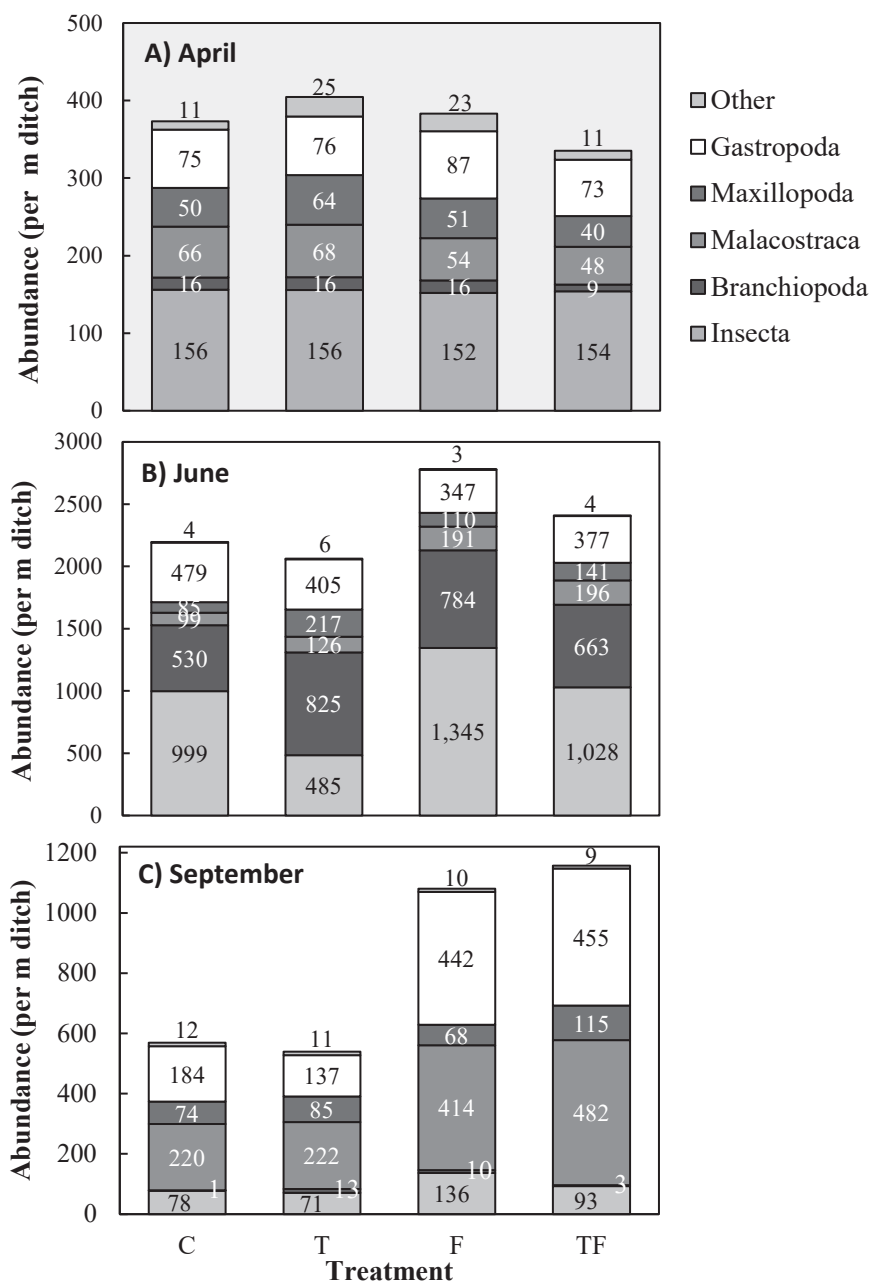


Figure 6.5. Average macroinvertebrate class abundance (n=9) of the most abundant classes one month before (A), one month after (B) C) and four months after the first thiocloprid application. C = control, T = thiocloprid, F = fertilizer and TF = thiocloprid and fertilizer. 'Other' is the sum of all other identified classes. Grey shading shows data before the addition of the agrochemicals.

Table 6.1. Average contribution ('cont.', %, highest to lowest, SIMPER analysis) to the observed dissimilarity per taxon (log10[x+1] transformed). Shown are taxa (species groups and *species*) that contribute for >2.5% to the observed dissimilarity. 'NS': no sign. difference (PERMANOVA). Yellow colors indicate average lower and blue colors higher abundances compared to the control.

Month	Comparison					
	Control - Thiachloprid		Control - Fertilizer		Control - Thia*Fert	
	Taxon	Cont. (%)	Taxon	Cont. (%)	Taxon	Cont. (%)
June	<i>S. lutaria</i>	6.93	NS	NS	<i>S. lutaria</i>	7.27
	Daphniidae	6.50			Daphniidae	5.52
	<i>Chaoborus</i> sp.	4.62			<i>H. minutus</i>	5.05
	<i>N. viridus</i>	4.30			<i>H. aequalis</i>	4.69
	Cyclopidae	3.88			Chironomidae	4.17
	<i>G. thoracicus</i>	3.81			<i>V. piscinalis</i>	4.15
	<i>C. coleoptrata</i>	3.78			<i>N. viridis</i>	4.11
	<i>H. aequalis</i>	3.76			<i>G. thoracicus</i>	3.70
	<i>V. piscinalis</i>	3.63			<i>H. pusillus</i>	3.33
	Chironomidae	3.62			Cyclopidae	3.06
	<i>P. antipodarum</i>	3.09			<i>P. coxalis</i>	2.94
	<i>H. minutus</i>	2.93			<i>H. complanatus</i>	2.74
	<i>G. tigrinus</i>	2.59			<i>G. tigrinus</i>	2.66
Sept.	<i>P. coxalis</i>	5.29	<i>P. coxalis</i>	5.74	<i>P. coxalis</i>	4.48
	Daphniidae	3.71	<i>C. coleoptrata</i>	4.58	<i>H. complanatus</i>	4.00
	<i>O. cancellatum</i>	3.38	<i>H. complanatus</i>	3.68	<i>O. cancellatum</i>	3.61
	<i>C. coleoptrata</i>	3.15	Coenagrionidae	3.28	<i>S. lateralis</i>	3.60
	<i>A. aquaticus</i>	2.81	Daphniidae	3.00	<i>C. coleoptrata</i>	3.12
	Cyclopidae	2.74	<i>A. aquaticus</i>	2.72	<i>A. aquaticus</i>	2.91
	<i>Sigara</i> sp.	2.73	<i>Sigara</i> sp.	2.69	<i>E. octoculata</i>	2.84
	<i>L. hyalinus</i>	2.64			Coenagrionidae	2.83
	Coenagrionidae	2.60			Cyclopidae	2.80
	Chironomidae	2.60			<i>G. tigrinus</i>	2.61
					<i>Tubifex</i> sp.	2.52

Taxon specific responses to the agrochemicals.

Both in June and September, thiacloprid significantly shifted the community centroids ($F_{1,28} = 3.7$, $R^2 = 0.09$, $p < 0.001$ and $F_{1,28} = 2.1$, $R^2 = 0.05$, $p = 0.002$ respectively; Figure 6.4B). The taxa that contributed most on average to the observed dissimilarity between the 'C' and the 'T' treatments in June are *S. lutaria*, Daphniidae, *Chaoborus* sp. and *Notonecta viridis* (average contribution to the dissimilarity: 6.93, 6.50, 4.62 and 4.30%, respectively; Table 6.1). This was partially comparable to those taxa contributing most to the dissimilarity between the 'C' and 'TF' treatments: *S. lutaria*, Daphniidae, *Helophorus minutus* and *Helophorus aequalis* (average contribution to the dissimilarity: 7.27, 5.52, 5.05 and 4.69%, respectively; Table 6.1). For *S. lutaria*, we observed 0.8 and 0.1 individuals on average in the 'T' and 'TF' treatments versus 25.0 in the control. Four months after application, in September, the taxa contributing most to dissimilarities between the 'C' and 'T' treatments shifted to *Proasellus coxalis*, Daphniidae and *Orthetrum cancellatum* and *Cymatia coleoptrata* (average contribution to the dissimilarity: 5.29, 3.71, 3.38 and 3.15%, respectively). This was partially similar to the C-TF comparison where *P. coxalis*, *Hippeutis complanatus*, *O. cancellatum* and *Sigara lateralis* contributed most (average contribution to the dissimilarity: 4.48, 4.00, 3.61 and 3.60%, respectively). Solely fertilizer addition also significantly shifted the community centroid in September ($F_{1,28} = 2.6$, $R^2 = 0.07$, $p < 0.001$; Figure 6.4). Contributing most to this observed dissimilarity between the 'C' and the 'F' treatment were *P. coxalis*, *C. coleoptrata*, *H. complanatus* and Coenagrionidae (average contribution to the dissimilarity: 5.74, 4.58, 3.68 and 3.28% respectively, Table 6.1).

6.4 Discussion

Using a well-replicated experimental design with a semi-natural setup, our results show that at environmentally relevant concentrations fertilizers and neonicotinoid insecticides jointly structure aquatic macroinvertebrate communities. One of our expected outcomes was that fertilizer input could ‘mask’ or reduce the effects of neonicotinoids. While we found such effects at the species or family level, our results suggest the opposite effect for communities: the magnitude of effects on community structure was greater for the joint application when compared to the effects of the single stressors. Moreover, our results indicate long-term legacy effects of the stressors; community structure was still altered four months after the neonicotinoid was applied. Our observations with natural aquatic community assemblages add to previous laboratory and mesocosm studies which show isolated effects on individual sensitive species (e.g. Beketov et al., 2008; Roessink et al., 2013), as we show that effects can feed through to higher levels of organization in aquatic ecosystems and that species turnover strongly affect community composition in response to toxic stress.

This is, to our knowledge, the first study to investigate the joint effect of agricultural pressures to fully naturally (re-)assembled aquatic invertebrate communities of macroinvertebrates. Previous studies have addressed effects of neonicotinoids on communities by focusing on either recovery time of sensitive species (e.g. univoltine species, see Beketov et al., 2008), species with sensitive traits to chemical pollution (SPEAR, see Liess and Beketov, 2011), predator-prey interactions (Alexander et al., 2016), or food-web structure (Schrama et al., 2017). Most of these studies report that application of neonicotinoids result in a decline in total abundance and/or richness (for example: Beketov et al., 2008; Alexander et al., 2013; Rico et al., 2018). Using our study system, experimental ditches that are open to natural (re)colonization, we find that none of these relatively simple metrics were affected by the treatments: total abundance, taxon richness and beta dispersion (variation) between communities all remained remarkably similar between treatments.

However, our results do show pronounced treatment-induced changes in community composition, where single and combined stressors result in different communities compared to a situation not exposed to stressors. For example, thiacloprid application resulted in 51% lower insect abundance, especially pronounced in Chironomidae (50% lower) and larger predator species (*S. lutaria*, *Notonecta viridis* and *Gerris thoracicus*; combined lower abundance of 96%). In contrast, we observed a strong increase in the abundance of multivoltine taxa such as Daphniidae and Cyclopidae, thus explaining the lack of effects on total abundance. While chironomids have previously been identified as a family of insects that is particularly sensitive to neonicotinoids (Langer-Jaesrich et al., 2010), the lower abundances in large predators was not expected as toxicity values for these species generally are several-fold higher than our concentrations (see Roessink et al., 2013 and Morrissey et al., 2015). Several of these species that showed lower abundances fulfill important ecosystem process within as well as outside of the aquatic ecosystem as they are, for example, common food sources for terrestrial predators such as birds and bats (Hallmann et al., 2014). Not only do these results suggest a potential impact on the (aquatic) predator trophic level by neonicotinoids, they also suggest that studying metrics such as diversity and abundance under strongly controlled conditions may paint a too simple story. As such, the inclusion of natural (re)colonizing communities in the experimental setup (to allow for species turnover) opens up unique research possibilities to evaluate effects of single and multiple co-occurring stressors on natural communities and its concomitant impacts on ecosystem functioning. The current results already point towards changes in ecosystem functioning as the several species that showed relative higher or lower abundances are important players for several ecosystem processes (such as emergent taxa as a food source for birds and bats, chironomids that might be important for phytoplankton abundance and OM degradation or planktonic species that might be important for phytoplankton abundance).

Results from our study show that joint stressors have a qualitatively different effect from the effect of the single stressors, both at the individual species and at the community level. The sole addition of thiacloprid resulted in a community structure that was dissimilar from the control. These results are in line with Beketov et al. (2008) who already showed that inoculated macroinvertebrate communities in artificial stream mesocosms were affected by thiacloprid at a nominal concentration of 3.2 µg/L. We found different communities at over six times lower TWA concentration (0.5 µg/L), that is representative for concentrations observed in surface waters (Morrissey et al., 2015). Like thiacloprid, ditches with fertilizer were also statistically significantly dissimilar to the control, but this was found only four months after the introduction of the treatment. These effects were most clear when considering total abundance, which increased by 100%. Such increases in abundance by additional nutrients are well-known (for example Davis et al., 2010) and were anticipated. When studying both stressors in concert we found that fertilizer caused a marked decrease in toxicity of the neonicotinoid to some taxa; Chironomidae abundance was 50% lower after thiacloprid application, but the addition of fertilizer nearly nullified this response. This is in accordance with previous studies that found reduced effects on nutrients on species-specific neonicotinoid toxicity (Alexander et al., 2013; Ieromina et al., 2014; Barmantlo et al., 2018). However, fertilizer addition did not reduce the effect of thiacloprid on community structure as a whole. In fact, the joint application of both agrochemicals resulted in a community composition that was most dissimilar from the control. This is likely caused by a combination of two effects. On the one hand, taxa that disappeared after thiacloprid application were also absent from the mixture treatment as exemplified by the alderfly *S. lutaria* (97 and 100% lower abundances, respectively). On the other hand, some taxa that reacted strongly and positively to fertilizer increased even stronger in the mixture treatment. For example, abundance of beetles of the genus *Helophorus* showed 49% higher abundances in the ditches with solely fertilizer addition compared to the control, whereas they had 265% higher abundances in the mixture. This could potentially be explained by an initial filling of a (wider) opened ecological niche (a double indirect effect, see Gessner and Tlili, 2016) and, subsequently, dissimilarity remained as the community state did not return to the control state. Overall, such observed shifts in community

structure suggest that neonicotinoids can cause a rippling effect in the community that can even be amplified by the presence of nutrients.

Our results indicate that stressor-induced ecological differentiation can persist onto the next season. Particularly for the treatments containing neonicotinoids this is a remarkable effect because thiacloprid was removed rapidly from the water column (>95% concentration decrease within two weeks). Dispersal limitation cannot explain this phenomenon as 'polluted' ditches were directly next to 'unpolluted' ditches and the adjacent lake. We speculate that there are three important explanations for the lack of, or slow, recovery. First, the slow recovery of uni- and semivoltine species (as suggested and found earlier by Beketov et al., 2008 and Rico and van den Brink, 2015) may be underlying the lower abundances in both June and September, as shown by for example *S. lutaria* (explaining 2.48% of the dissimilarity in September, not shown in Table 6.1) and the water boatmen *Cymatia coleoptrata* (uni/bivoltine species, Table 6.1). Second, neonicotinoids form a nearly irreversible bond to the post-synaptic nicotinic acetylcholine receptors (nAChR) in insect central nervous systems (Tennekes, 2010), likely continuing to hamper the performance of the toxicologically sensitive species that were developing over these months. Third, effects in spring could lead to cascading effects later in the season. This idea is supported by the observation that taxa that explained most of the dissimilarity between the control and thiacloprid treatment in September were not necessarily the same taxa as in June (see Table 6.1). The most likely ecological explanation for this is that thiacloprid induced not only a short-term toxicity effect (as discussed in the previous paragraph) but also a long-term rippling effect in the aquatic communities. As we found no differences in beta dispersion (for all months and treatments), we found no indications for divergence, convergence or unidentified community states. This means that the observed agrochemical-induced (rippling) effects on the community structure had a similar pattern within treatments and were thus consistent. Such consistent patterns of structuring of aquatic communities may help future studies and managers to help predict the effect of fertilizers and neonicotinoids in the natural aquatic environment.

6.5 Conclusions

We identify fertilizer and neonicotinoids as important single and joint drivers of freshwater macroinvertebrate communities. The application of both agrochemicals resulted in consistently altered macroinvertebrate communities. The neonicotinoid thiacloprid induced persistent changes in the communities without affecting the magnitude of the variation between the communities (i.e., no changes in beta dispersion). Given the key role of biota in maintaining ecosystem processes and ultimately the functioning of the ecosystem (Hooper et al., 2005), our results strongly suggest that a variety of ecosystem processes may be affected. These impacts go beyond the period that these substances can be retrieved from the water column and even beyond aquatic ecosystems per se. Our findings illustrate how agricultural stressors can propagate through aquatic ecosystems with inherent risks for their functioning and the services they provide.

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Supplement

Methods

Thiacloprid application. A stock solution of 25 mg/L of thiacloprid was prepared by dissolving the compound in demineralized water in a glass bottle. We diluted this stock solution in a 10 L glass bottle with filtered (mesh width: 106 μ m) ditch water of the respective ditch that received the application. Filtering was conducted to prevent that any macro-invertebrates would be present in the 10 L glass bottle and would thus receive an unplanned short-term exposure to high concentrations of thiacloprid. The 10 L glass bottle was then mounted with a watering can head and the mixture was spread evenly over the ditch, simulating spray drift. In order to maintain experimental concentrations for the duration of one month, we provided a second spike two weeks after the first spike, following the same method.

Physicochemical characteristics. We characterized each ditch by monitoring several variables. Temperature, pH, dissolved oxygen (DO) and conductivity were measured using a Hach hq 40d electronic multiparameter on a weekly basis from March to July and on a monthly basis between July and September.

Results

Physicochemical characteristics. Oxygen concentrations were consistently higher (on average 2.4 mg/L, Table 6.1) after fertilizer addition ($F_{1,32} = 7.6$, $p = 0.010$), probably due to increased photosynthesis of algae or macrophytes which likely also explains the increased pH (0.46 on average, $F_{1,32} = 5.2$, $p = 0.029$; see Supplement, Table 6.S1). Unexpectedly, fertilizer, thiacloprid and time interacted significantly on both oxygen concentration and pH ($F_{1,32} = 6.1$, $p = 0.013$ and $F_{1,32} = 4.53$, $p = 0.033$), leading to even higher levels for both variables at some time points within the mixture treatment (for oxygen: 0 – 3.31 mg/L and for pH: 0 - 0.45 compared to the control respectively) compared to the already raised

levels for the fertilizer treatment. Fertilizer addition also significantly decreased the conductivity although the effect was small (22 $\mu\text{S}/\text{cm}$ on average, $F_{1,32} = 7.6$, $p = 0.010$; see Supplement, Table 6.S1).

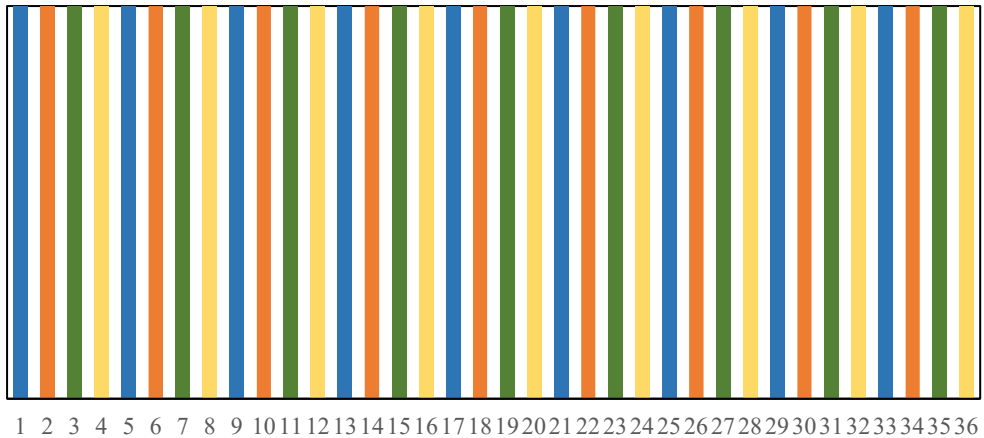


Figure 6.S1. Schematic overview of the treatment order. Treatments followed a block design ($n = 9$) with increasing experimental ditch number (1:36, left to right at the experimental site). Blue = control, orange = thiacloprid, green = fertilizer and yellow = thiacloprid + fertilizer.

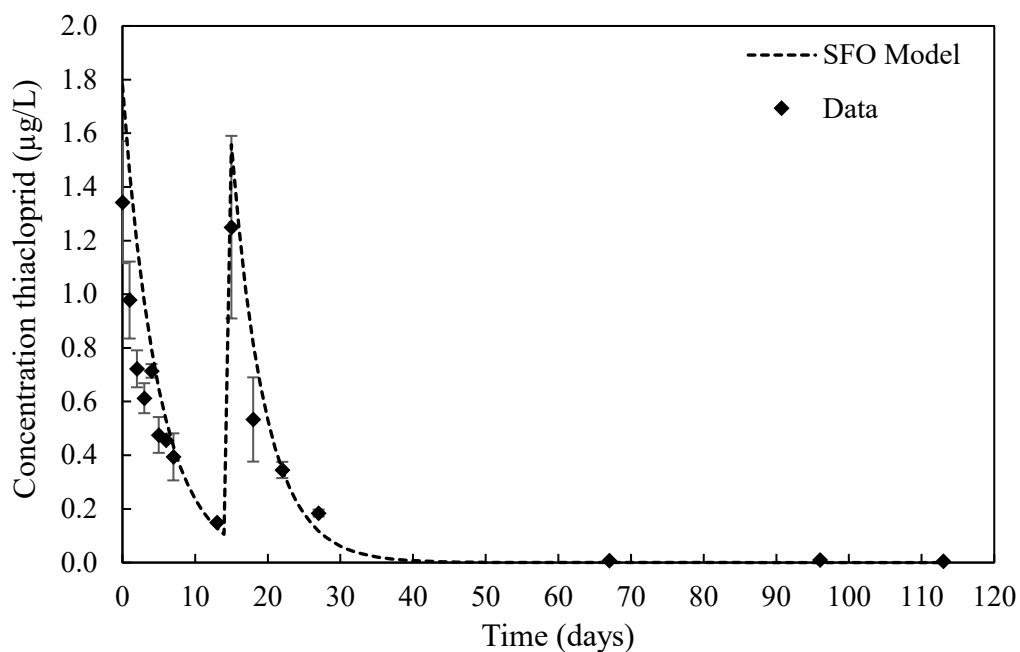


Figure 6.S2. Average thiacloprid concentration ($\pm$ standard deviation; $n = 18$ at $t=0$ and $t=14$, $n=2$ for all other time points) of the ‘T’ and ‘TF’ treatment over the course of the experiment. Dotted line represent the first-order kinetics equation (Single First order, SFO). Thiacloprid was spiked at $t=0$ and $t=14$. Detection limit = $0.1 \mu\text{g/L}$.

Table 6.S1. Time-weighted averages ($\pm$ SE, n = 9) of physicochemical parameters since the start of nutrient enrichment.

Variable	Treatment			
	Control	Thiacloprid	Fertilizer	Thia*Fert
Conduc. (μ S/cm)	637 ($\pm$ 7.88)	622 ($\pm$ 7.78)	611 ($\pm$ 8.65)	591 ($\pm$ 8.34)
Oxygen (mg/L)	11.9 ($\pm$ 0.21)	12.0 ($\pm$ 0.42)	13.8 ($\pm$ 0.18)	13.9 ($\pm$ 0.30)
Nitrate (mg/L)	0.97 ($\pm$ 0.08)	0.89 ($\pm$ 0.12)	1.25 ($\pm$ 0.13)	1.13 ($\pm$ 0.11)
pH	8.62 ($\pm$ 0.03)	8.53 ($\pm$ 0.03)	8.99 ($\pm$ 0.04)	9.07 ($\pm$ 0.06)
Phosphate (mg/L)	0.29 ($\pm$ 0.03)	0.30 ($\pm$ 0.04)	0.41 ($\pm$ 0.04)	0.37 ($\pm$ 0.03)
Temperature ($^{\circ}$ C)	21.4 ($\pm$ 0.04)	21.3 ($\pm$ 0.05)	21.4 ($\pm$ 0.04)	21.4 ($\pm$ 0.03)
Thiacloprid (μ g/L)	<DL	0.43 ($\pm$ 0.05)	<DL	0.49 ($\pm$ 0.06)

Conduc: conductivity. Time-weighted averages for thiacloprid are since the first spike until degradation below the detection limit.

Identified macroinvertebrate taxa lists per sampling month (alphabetical order).

April

Phylum	Class	Order	Family	Genus	Species
Annelida	Clitellata	Arhynchobdellida	Erpobdellidae	Erpobdella	octoculata
Annelida	Clitellata	Haplotaxida	Tubificidae	Stylaria	lacustris
Annelida	Clitellata	Oligochaeta	Tubificidae	Tubifex	
Annelida	Clitellata	Rhynchobdellida	Glossiphoniidae	Alboglossiphonia	striata
Annelida	Hirudinea	Rhynchobdellida	Piscicolidae	Piscicola	geometra
Annelida	Hirunidea	Rhynchobdellida	Glossiphoniidae	Helobdella	stagnalis
Annelida	Hirunidea	Rhynchobdellida	Glossiphoniidae	Hemiclepsis	marginata
Annelida	Hirunidea	Rhynchobdellida	Glossiphoniidae	Theromyzon	tessulatum
Arthropoda	Arachnida	Prostigmata	Hydryphantidae	Diplodontus	despiciens
Arthropoda	Arachnida	Prostigmata	Lebertiidae	Lebertia	
Arthropoda	Arachnida	Trombidiformes	Arrenuridae	Arrenurus	
Arthropoda	Arachnida	Trombidiformes	Eylaidae	Eylais	
Arthropoda	Arachnida	Trombidiformes	Hydrachnidae	Hydrachna	
Arthropoda	Arachnida	Trombidiformes	Limnesiidae	Limnesia	
Arthropoda	Arachnida	Trombidiformes	Utonicolidae	Neumannia	
Arthropoda	Branchiopoda	Diplostraca	Daphniidae	Daphnia	longispina
Arthropoda	Branchiopoda	Diplostraca	Daphniidae	Daphnia	magna
Arthropoda	Entognatha	Collembola			
Arthropoda	Insecta	Coleoptera	Dytiscidae	Hydroporus	planus
Arthropoda	Insecta	Coleoptera	Dytiscidae	Hygrotus	impressopunctatus

April (continued)

Phylum	Class	Order	Family	Genus	Species
Arthropoda	Insecta	Coleoptera	Dytiscidae	Hygrotus	versicolor
Arthropoda	Insecta	Coleoptera	Dytiscidae	Laccophylus	hyalinus
Arthropoda	Insecta	Coleoptera	Dytiscidae	Laccophylus	minutus
Arthropoda	Insecta	Coleoptera	Dytiscidae	Graptodytes	pictus
Arthropoda	Insecta	Coleoptera	Dytiscidae	Hydroglyphus	pusillus
Arthropoda	Insecta	Coleoptera	Halplidae	Halplus	
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Helochares	obscurus
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Helophorus	aequalis/grandis
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Helophorus	brevipalpis/minutus
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Hydrobius	fuscipes
Arthropoda	Insecta	Diptera	Ceratopogoninae		
Arthropoda	Insecta	Diptera	Chironomidae		
Arthropoda	Insecta	Diptera	Culicidae	Culicinae	
Arthropoda	Insecta	Ephemeroptera	Baetidae	Cloeon	dipterum
Arthropoda	Insecta	Ephemeroptera	Caenidae	Caenis	horaria
Arthropoda	Insecta	Hemiptera	Corixidae	Arctocoris	germari
Arthropoda	Insecta	Hemiptera	Corixidae	Cymatia	Coleopttrata
Arthropoda	Insecta	Hemiptera	Corixidae	Paracorixa	concinna
Arthropoda	Insecta	Hemiptera	Corixidae	Sigara	lateralis
Arthropoda	Insecta	Hemiptera	Corixidae	Sigara	
Arthropoda	Insecta	Hemiptera	Gerridae	Gerris	thoracicus
Arthropoda	Insecta	Hemiptera	Notonectidae	Notonecta	

April (continued)

Phylum	Class	Order	Family	Genus	Species
Arthropoda	Insecta	Hemiptera	Pleidae	Plea	minutissisima
Arthropoda	Insecta	Odonata	Coenagrionidae		
Arthropoda	Insecta	Odonata	Libellulidae	Ortheterum	cancellatum
Arthropoda	Malacostraca	Amphipoda	Gammaridae	Gammarus	sp.
Arthropoda	Malacostraca	Isopoda	Asellidae	Proasellus	sp
Arthropoda	Malacostraca	Mysida	Mysidae	Limnomysis	benedeni
Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae		
Mollusca	Bivalvia	Veneroidea	Sphaeriidae	Musculium	lacustre
Mollusca	Bivalvia				
Mollusca	Gastropoda	Ectobranchia	Valvatidae	Valvata	piscinalis
Mollusca	Gastropoda	Neotaenioglossa	Bithyniidae	Bithynia	leachii
Mollusca	Gastropoda	Neotaenioglossa	Bithyniidae	Bithynia	tentaculata
Mollusca	Gastropoda	Neotaenioglossa	Hydrobiidae	Potamopyrgus	antipodarum
Mollusca	Gastropoda	Pulmonata	Lymnaeidae	Lymnaea	stagnalis
Mollusca	Gastropoda	Pulmonata	Planorbidae	Gyraulus	crista
Mollusca	Gastropoda	Pulmonata	Planorbidae	Planorbarius	corneus
Platyhelminthes	Turbellaria	Tricladida	Dugesidae	Dugesia	lugubris

June

Phylum	Class	Order	Family	Genus	Species
Annelida	Clitellata	Arhynchobdellida	Erpobdellidae	Erpobdella	octoculata
Annelida	Clitellata	Oligochaeta	Tubificidae	Tubifex	
Annelida	Hirudinea	Rhynchobdellida	Piscicolidae	Piscicola	geometra
Annelida	Hirunidea	Rhynchobdellida	Glossiphoniidae	Helobdella	stagnalis
Annelida	Hirunidea	Rhynchobdellida	Glossiphoniidae	Hemiclepsis	marginata
Annelida	Hirunidea	Rhynchobdellida	Glossiphoniidae	Theromyzon	tessulatum
Arthropoda	Arachnida	Araneae	Lycosidae	Pirata	piraticus
Arthropoda	Arachnida	Prostigmata	Lebertiidae	Lebertia	
Arthropoda	Arachnida	Trombidiformes	Hydrachnidae	Hydrachna	
Arthropoda	Arachnida	Trombidiformes	Limnesiidae	Limnesia	
Arthropoda	Arachnida	Trombidiformes	Unionicolidae	Neumannia	
Arthropoda	Branchiopoda	Diplostraca	Daphniidae		
Arthropoda	Entognatha	Collembola			
Arthropoda	Insecta	Coleoptera	Dytiscidae	Hydroporinae	
Arthropoda	Insecta	Coleoptera	Dytiscidae	Hydroporus	planus
Arthropoda	Insecta	Coleoptera	Dytiscidae	Hygrotus	confluens
Arthropoda	Insecta	Coleoptera	Dytiscidae	Hygrotus	impressopunctatus
Arthropoda	Insecta	Coleoptera	Dytiscidae	Hygrotus	inaequalis
Arthropoda	Insecta	Coleoptera	Dytiscidae	Hygrotus	versicolor
Arthropoda	Insecta	Coleoptera	Dytiscidae	Laccophylus	hyalinus
Arthropoda	Insecta	Coleoptera	Dytiscidae	Laccophylus	minutus

June (continued)

Phylum	Class	Order	Family	Genus	Species
Arthropoda	Insecta	Coleoptera	Dytiscidae	Laccophylus	sp.
Arthropoda	Insecta	Coleoptera	Dytiscidae	Agabus	bipustulatus
Arthropoda	Insecta	Coleoptera	Dytiscidae	Colymbetinae	
Arthropoda	Insecta	Coleoptera	Dytiscidae	Graptodytes	pictus
Arthropoda	Insecta	Coleoptera	Dytiscidae	Hydroglyphus	pusillus
Arthropoda	Insecta	Coleoptera	Halplidae	Halplus	ruficollis/ lineaticollis
Arthropoda	Insecta	Coleoptera	Halplidae	Halplus	
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Cercyon	cf. granarius
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Helochares	cf. obscurus
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Helochares	
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Helophorus	aequalis/grandis
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Helophorus	minutus/ paraminutus
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Hydrobius	fuscipes
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Laccobius	cf. bipunctatus
Arthropoda	Insecta	Coleoptera	Scirtidae	Scirtes	
Arthropoda	Insecta	Diptera	Ceratopogonidae	Atrichopogon	
Arthropoda	Insecta	Diptera	Chaoboridae	Chaoborus	
Arthropoda	Insecta	Diptera	Chironomidae		
Arthropoda	Insecta	Diptera	Culicidae		
Arthropoda	Insecta	Diptera	Ephydridae	Ephydra	

June (continued)

Phylum	Class	Order	Family	Genus	Species
Arthropoda	Insecta	Diptera	Syrphidae	Eristalis	tenax
Arthropoda	Insecta	Diptera			
Arthropoda	Insecta	Ephemeroptera	Baetidae	Cloeon	dipterum
Arthropoda	Insecta	Ephemeroptera	Caenidae	Caenis	horaria
Arthropoda	Insecta	Ephemeroptera	Caenidae	Caenis	robusta
Arthropoda	Insecta	Hemiptera	Corixidae	Arctocoris	germari
Arthropoda	Insecta	Hemiptera	Corixidae	Cymatia	coleoptrata
Arthropoda	Insecta	Hemiptera	Corixidae	Paracorixa	concinna
Arthropoda	Insecta	Hemiptera	Corixidae	Sigara	iacans
Arthropoda	Insecta	Hemiptera	Corixidae	Sigara	lateralis
Arthropoda	Insecta	Hemiptera	Corixidae	Sigara	scotti/fossarum
Arthropoda	Insecta	Hemiptera	Corixidae	Sigara	striata
Arthropoda	Insecta	Hemiptera	Corixidae	Sigara	
Arthropoda	Insecta	Hemiptera	Corixidae	Subfamily: Corixinae	
Arthropoda	Insecta	Hemiptera	Gerridae	Gerris	thoracicus
Arthropoda	Insecta	Hemiptera	Naucoridae	Ilyocoris	cimicoides
Arthropoda	Insecta	Hemiptera	Nepidae	Nepa	cinerea
Arthropoda	Insecta	Hemiptera	Notonectidae	Notonecta	viridis
Arthropoda	Insecta	Hemiptera	Pleidae	Plea	minutissisima
Arthropoda	Insecta	Hemiptera	Saldidae	Saldula	saltatoria
Arthropoda	Insecta	Megaloptera	Sialidae	Sialis	lutaria
Arthropoda	Insecta	Odonata	Coenagrionidae	Coenagrion	puella

June (continued)

Phylum	Class	Order	Family	Genus	Species
Arthropoda	Insecta	Odonata	Coenagrionidae	Enallagma	cyathigerum
Arthropoda	Insecta	Odonata	Coenagrionidae	Ischnura	elegans
Arthropoda	Insecta	Odonata	Coenagrionidae		
Arthropoda	Insecta	Odonata	Libellulidae	Orthetrum	cancellatum
Arthropoda	Insecta	Trichoptera	Leptoceridae	Oecetis	furva
Arthropoda	Insecta	Trichoptera	Leptoceridae	Oecetis	lacustris
Arthropoda	Malacostraca	Amphipoda	Gammaridae	Gammarus	tigrinus
Arthropoda	Malacostraca	Amphipoda	Mysidae	Neomysis	integer
Arthropoda	Malacostraca	Isopoda	Asellidae	Proasellus	
Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae		
Arthropoda	Ostracoda				
Mollusca	Bivalvia	Veneroida	Sphaeriidae	Musculium	lacustre
Mollusca	Gastropoda	Ectobranchia	Valvatidae	Valvata	piscinalis
Mollusca	Gastropoda	Neotaenioglossa	Bithyniidae	Bithynia	tentaculata
Mollusca	Gastropoda	Neotaenioglossa	Hydrobiidae	Potamopyrgus	antipodarum
Mollusca	Gastropoda	Pulmonata	Lymnaeidae	Lymnaea	stagnalis
Mollusca	Gastropoda	Pulmonata	Lymnaeidae	Radix	auricularia
Mollusca	Gastropoda	Pulmonata	Planorbidae	Hippeutis	complanatus
Mollusca	Gastropoda	Pulmonata	Planorbidae	Planorbarius	corneus
Platyhelminthes	Rhabditophora	Tricladida	Planariidae	Polycelis	nigra/tenuis

September

Phylum	Class	Order	Family	Genus	Species
Annelida	Clitellata	Arhynchobdellida	Erpobdellidae	Erpobdella	octoculata
Annelida	Clitellata	Oligochaeta	Tubificidae	Tubifex	
Annelida	Hirunidea	Rhynchobdellida	Glossiphoniidae	albughossiphonia	striata
Annelida	Hirunidea	Rhynchobdellida	Glossiphoniidae	Helobdella	stagnalis
Annelida	Hirunidea	Rhynchobdellida	Glossiphoniidae	Theromyzon	tessulatum
Annelida	Hirunidea	Rhynchobdellida	Hirudidae	Haemopsis	sanguisua
Arthropoda	Arachnida	Araneae	Lycosidae	Pirata	piraticus
Arthropoda	Arachnida	Prostigmata	Lebertiidae	Lebertia	
Arthropoda	Arachnida	Trombidiformes	Arrenuridae	Arrenurus	
Arthropoda	Arachnida	Trombidiformes	Hydrachnidae	Hydrachna	
Arthropoda	Arachnida	Trombidiformes	Limnesiidae	Limnesia	
Arthropoda	Branchiopoda	Diplostraca	Daphniidae		
Arthropoda	Entognatha	Collembola			
Arthropoda	Insecta	Coleoptera	Dytiscidae	Hygrotus	impressopunctatus
Arthropoda	Insecta	Coleoptera	Dytiscidae	Hygrotus	inaequalis
Arthropoda	Insecta	Coleoptera	Dytiscidae	Laccophylus	hyalinus
Arthropoda	Insecta	Coleoptera	Dytiscidae	Laccophylus	minutus
Arthropoda	Insecta	Coleoptera	Dytiscidae	Rhantus	suturalis
Arthropoda	Insecta	Coleoptera	Dytiscidae	Agabus	bipustulatus
Arthropoda	Insecta	Coleoptera	Dytiscidae	Agabus	nebulosus
Arthropoda	Insecta	Coleoptera	Dytiscidae	Agabus	sturnii

Sept. (continued)

Phylum	Class	Order	Family	Genus	Species
Arthropoda	Insecta	Coleoptera	Dytiscidae	Graptodytes	pictus
Arthropoda	Insecta	Coleoptera	Dytiscidae	Hydroglyphus	pusillus
Arthropoda	Insecta	Coleoptera	Halplidae	Halplus	confinis
Arthropoda	Insecta	Coleoptera	Halplidae	Halplus	flavicollis
Arthropoda	Insecta	Coleoptera	Halplidae	Halplus	immaculatus
Arthropoda	Insecta	Coleoptera	Halplidae	Halplus	lineatocollis
Arthropoda	Insecta	Coleoptera	Halplidae	Halplus	lineolatus
Arthropoda	Insecta	Coleoptera	Halplidae	Halplus	ruficollis
Arthropoda	Insecta	Coleoptera	Halplidae	Halplus	
Arthropoda	Insecta	Coleoptera	Halplidae	Peltodytes	caesus
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Anacaena	lutescens
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Enochrus	melanocephalus
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Helochares	lividus
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Helochares	obscurus
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Helophorus	aequalis
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Helophorus	brevipalpis
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Helophorus	minutus
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Hydrobius	fuscipes
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Hydrophilus	piceus
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Laccobius	cf. bipunctatus
Arthropoda	Insecta	Coleoptera	Hydrophilidae	Limnoxenus	niger
Arthropoda	Insecta	Coleoptera	Noteridae	Noterus	clavicornis

Sept. (continued)

Phylum	Class	Order	Family	Genus	Species
Arthropoda	Insecta	Coleoptera	Paelobiidae	Hygrobia	hermanni
Arthropoda	Insecta	Diptera	Chaoboridae	Chaoborus	
Arthropoda	Insecta	Diptera	Chironomidae		
Arthropoda	Insecta	Diptera	Culicidae	Aedes	
Arthropoda	Insecta	Diptera	Culicidae	Anopheles	maculipennis/ complex
Arthropoda	Insecta	Diptera	Limoniidae		
Arthropoda	Insecta	Diptera	Stratiomyidae	Nemotelus	
Arthropoda	Insecta	Diptera	Tabanidae	Chrysops	
Arthropoda	Insecta	Diptera	Tabanidae	Tabaninae	
Arthropoda	Insecta	Diptera	Tipulidae		
Arthropoda	Insecta	Diptera			
Arthropoda	Insecta	Ephemeroptera	Baetidae	Cloeon	dipterum
Arthropoda	Insecta	Ephemeroptera	Caenidae	Caenis	horaria
Arthropoda	Insecta	Hemiptera	Corixidae	Arctocorisa	germari
Arthropoda	Insecta	Hemiptera	Corixidae	Cymatia	coleoptrata
Arthropoda	Insecta	Hemiptera	Corixidae	Paracorixa	concinna
Arthropoda	Insecta	Hemiptera	Corixidae	Sigara	iactans
Arthropoda	Insecta	Hemiptera	Corixidae	Sigara	lateralis
Arthropoda	Insecta	Hemiptera	Corixidae	Sigara	striata
Arthropoda	Insecta	Hemiptera	Corixidae	Sigara	
Arthropoda	Insecta	Hemiptera	Gerridae	Gerris	thoracicus

Sept. (continued)

Phylum	Class	Order	Family	Genus	Species
Arthropoda	Insecta	Hemiptera	Naucoridae	Ilyocoris	cimicoides
Arthropoda	Insecta	Hemiptera	Nepidae	Ranatra	linearis
Arthropoda	Insecta	Hemiptera	Notonectidae	Notonecta	glauca
Arthropoda	Insecta	Hemiptera	Notonectidae	Notonecta	viridis
Arthropoda	Insecta	Hemiptera	Pleidae	Plea	minutissima
Arthropoda	Insecta	Hemiptera	Veliidae	Microvelia	reticulata
Arthropoda	Insecta	Lepidoptera	Crambidae	Cataglyphis	lemnata
Arthropoda	Insecta	Megaloptera	Sialidae	Sialis	lutaria
Arthropoda	Insecta	Odonata	Coenagrionidae	Coenagrion	pulchellum
Arthropoda	Insecta	Odonata	Coenagrionidae	Enallagma	cyathigerum
Arthropoda	Insecta	Odonata	Coenagrionidae	Ischnura	elegans
Arthropoda	Insecta	Odonata	Coenagrionidae		
Arthropoda	Insecta	Odonata	Libellulidae	Orthetrum	cancellatum
Arthropoda	Insecta	Trichoptera	Leptoceridae	Athripsodes	aterrimus
Arthropoda	Insecta	Trichoptera	Leptoceridae	Mystacides	longicornis
Arthropoda	Malacostraca	Amphipoda	Gammaridae	Gammarus	pulex
Arthropoda	Malacostraca	Amphipoda	Gammaridae	Gammarus	tigrinus
Arthropoda	Malacostraca	Amphipoda	Mysidae	Neomysis	integer
Arthropoda	Malacostraca	Decapoda	Cambaridae	Procambarus	clarkii
Arthropoda	Malacostraca	Isopoda	Asellidae	Asellus	aquaticus
Arthropoda	Malacostraca	Isopoda	Asellidae	Proasellus	coxalis

Sept. (continued)

Phylum	Class	Order	Family	Genus	Species
Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae		
Arthropoda	Ostracoda				
Mollusca	Bivalvia	Veneroida	Sphaeriidae	Musculium	lacustre
Mollusca	Bivalvia	Veneroida	Sphaeriidae	Sphaerium	
Mollusca	Gastropoda	Ectobranchia	Valvatidae	Valvata	piscinalis
Mollusca	Gastropoda	Neotaenioglossa	Bithyniidae	Bithynia	tentaculata
Mollusca	Gastropoda	Neotaenioglossa	Physidae	Physa	fontinalis
Mollusca	Gastropoda	Pulmonata	Lymnaeidae	Lymnaea	stagnalis
Mollusca	Gastropoda	Pulmonata	Planorbidae	Hippeutis	complanatus
Mollusca	Gastropoda	unranked	Hydrobiidae	Potamopyrgus	antipodarum
Platyhelminthes	Rhabditophora	Tricladida	Planariidae	Planaria	torva
Platyhelminthes	Rhabditophora	Tricladida	Planariidae	Planaria	

Chapter 7

Networks in aquatic communities collapse upon neonicotinoid-induced stress

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Abstract

In light of the unprecedented decline in biodiversity, a major question is how and to what extent the functioning of ecosystems will be affected. In contrast to commonly used diversity metrics, interaction networks are now considered key to provide such understanding, but experimental evidence of the effectiveness of such networks are limited and particularly scarce in relation to changes in ecosystem functioning. Here, we show how the ecological integrity of naturally developed freshwater communities degrades as increasing levels of neonicotinoid insecticide lead to strong losses in the structural complexity of interaction networks of macroinvertebrates. Loss of structural complexity in these neonicotinoid-exposed communities coincided with a severe erosion of ecosystem functioning, exemplified by a near complete loss of organic matter consumption and dominance of floating algal beds, which in turn suppressed phytoplankton growth. Our research thereby experimentally illustrates how network analyses identified that neonicotinoids threaten freshwater community structure which in turn causes cascades that severely impairs the functioning of freshwater ecosystems.

Keywords: Biodiversity, ecosystem functioning, insecticides, invertebrates.

7.1 Introduction

We are currently witnessing an unprecedented decline in biodiversity as well as ecosystem functioning (Díaz et al., 2019; Rockström et al., 2009; Living Planet Report, 2018). A long-standing question in ecology is how this global decline in biodiversity affects the functioning of ecosystems. At the local level, species richness or α -diversity is often remarkably constant and does not change in absolute values, even after human disturbance (Blowes et al., 2019; Dornelas et al., 2014). However, the assemblage of species at this local scale can markedly differ after disturbance due to species replacement (Blowes et al., 2019). As organisms are responsible for the functioning of an ecosystem (Hooper et al., 2005), this begs the question: do disturbed communities with a shifted community composition function equally well?

While there are few actual laws in ecology, at least a simple and perhaps trivial consensus is that ecological systems are characterized by “species that interact with one another (...) and their environment”(Lawton, 1999). Hence, it may not be the species richness of a community per se, but rather the network of interactions between species, and the environment, that provides the link between biodiversity and ecosystem functioning. Indeed, previous studies on the effect of disturbances (or upon stress sensu Pickett et al., 1989 in general) on ecosystems have shown that individual species interactions can be altered, consequently altering ecosystem productivity or functioning (Hooper et al., 2005). However, defining all possible ecological interactions and individually analyzing how these respond to different stressors within a given ecosystem is close to impossible. This hampers the extent to which we can predict, diagnose, and mitigate effects of anthropogenic-induced stresses to communities and ecosystems. Recently, network-based analyses have been adopted by ecologists to characterize the entire interaction web of ecological communities (Baiser et al., 2012; Encinas-Viso et al., 2016; Morriën et al., 2017; Simons et al., 2019), thereby providing an approximation for the structural complexity of species interactions in a given community (Ramirez et al., 2018). Although evaluated in a theoretical way, building on concepts from the last decades (Berlow, 1999; Hooper et al., 2005; Hutchinson, 1959; Lawton, 1999; Pimm et al., 1991), experimental evidence for the effectiveness of such network approaches is currently lacking. With this, we are also missing evidence on the causal link between

the ongoing species transition that potentially alter species interactions and ecosystem functioning.

Here, we experimentally explore if network approaches can identify the potential effects of stressors to communities and, if so, whether disruption of the community structural complexity coincides with losses in ecosystem functioning. We tested this by experimentally assessing how a common freshwater stressor, a neonicotinoid insecticide, disrupts natural invertebrate communities and the concurrent ecosystem processes they fulfill. Neonicotinoids are a well-known stressor as they are used worldwide (Morrissey et al., 2015; Sánchez-Bayo et al., 2016; Leiden University and Rijkswaterstaat-WVL, 2018; Casado et al., 2019) and are toxic to a range of individual invertebrate species (Goulson, 2013; Morrissey et al., 2015; Pisa et al., 2015; Vijver and van den Brink, 2014; Woodcock et al., 2017). However, knowledge is lacking to what extent neonicotinoid surface water levels actually affect natural freshwater biodiversity and, potentially, species interactions and ecosystem functioning. Therefore we analyzed naturally assembled freshwater communities that were exposed to an environmentally realistic neonicotinoid concentration range by using a two-tiered network-based approach. Firstly, we assessed community structural complexity compared to randomly generated communities (i.e. a null model) and secondly we assessed how the neonicotinoid-induced stress moves through the community and disrupts the concomitant ecosystem processes.

7.2 Methods

Study site

The experiments were conducted at the outdoor research facility ‘Living Lab’ of Leiden University (located in Oegstgeest, the Netherlands, see Barmantlo et al., 2019a and www.mesocosm.eu for an extensive site description). The site consists of 36 side-by-side experimental ditches, each with a length of 10 m, width of 0.8 m at the surface level and depth of 0.3 m. Ditch banks are covered in an organically grown mixture of grass and clover. The ditches are connected to an adjacent water compensation level reservoir that provides a natural source of microbes, plants and invertebrates. After a 5-month colonization period, the ditches were

isolated hydrologically by placing acrylic sheets (1000:500:2 mm) at the end of each ditch to prevent in- and outflow of the treatments (i.e., the neonicotinoid insecticide, see 'Neonicotinoid application').

Neonicotinoid application

We selected the insecticide thiacloprid as our model neonicotinoid as it is one of the most toxic neonicotinoids, but also because its usage was not as restricted as other neonicotinoids (e.g. outdoor use of clothianidin, imidacloprid, thiamethoxam in the EU (European Union, 2018a,b,c). Thiacloprid stock concentrations were prepared prior to spiking in demineralized water in 1 L glass bottles. Thiacloprid was applied to the ditches in two two-week apart spikes starting in May in four nominal concentrations; 0, 0.1, 1 and 10 µg/L with nine replicate ditches per concentration. This range of neonicotinoid concentrations is considered environmentally relevant (Barmantlo et al., 2019b) as they have been measured in surface waters across the globe (Morrissey et al., 2015; Sánchez-Bayo et al., 2016; Leiden University and Rijkswaterstaat-WVL, 2018; Stehle et al., 2018; Casado et al., 2019). Stock concentrations of thiacloprid were diluted to the target concentration using 10 L of filtered water (i.e. free of macroinvertebrates) of the ditch to which the spike was applied. This mixture was then spread evenly over the entire length of the ditch. Treatments were applied following a block design and all data collection followed this same design to minimize the effects of 1) potentially naturally occurring gradients and 2) time of sampling efforts on the collected data.

Abiotic conditions.

Water samples were taken 5 cm below the water surface level daily during the first week after the first thiacloprid spike and thereafter twice per week. Subsets of these samples were analyzed on thiacloprid concentration using liquid chromatography-tandem mass spectrometry and degradation was modelled via first order kinetics (see Barmantlo et al., 2019b). To control that abiotic water conditions were (initially) similar, the standard water chemistry parameters temperature, pH, oxygen concentration, conductivity were monitored on a weekly basis using a portable Hach multimeter (HQ 40d) and turbidity using a portable

Aquafluor® fluorometer. In addition, nitrate and phosphate concentrations were measured from water samples collected 5 cm below the surface water level in the middle of each ditch. These samples were collected and measured (using a NOVA 60 Spectroquant® photometer, Merck) one month before, 2 h after and one month after the first neonicotinoid spike. All these measured parameters showed no statistical differences between treatments before or after application of the neonicotinoid, except for a small decrease in turbidity at higher neonicotinoid concentrations (19% decrease at 10 µg/L, see Supplement Table 7.S2). This was likely due to losses in phytoplankton (see ‘Loss of ecosystem functioning’).

Community assay

In April 2018, one month before application of the neonicotinoid, we explored if comparable communities had developed among the ditches. For this, we followed the same method as described by Barmantlo et al. (2019a). One meter compartments were temporally isolated from the remainder of the ditch by quickly placing acrylic sheets over the entire width of a ditch. Within this 1 m compartment, we caught all macroinvertebrates using dipping nets (150 µm) for the water column and sieves (500 µm) for the top layer (3-5 cm) of the sediment. We stopped sampling when subsequent nets and sieves remained empty, i.e. when all macroinvertebrates had been caught. All invertebrates were then transferred to large white trays and a preselection was made by sorting specific species groups (e.g. beetles, dragonflies etc.) in Petri dishes. Next, the invertebrates were identified to the lowest possible taxonomic level using stereomicroscopes before being returned alive to their respective ditch. We reduced the total time of the identification effort by counting homogenized subsets of highly abundant taxa (such as zooplankton) until at least 50 individuals or 25% of the whole sample had been counted. The whole procedure from catch to release took 1-2 hours per ditch. In June, one month after the first thiacloprid spike, we again sampled and identified all invertebrates following the same method as in April.

Statistics on community network and structure

In order to identify whether we caught and identified all macroinvertebrates within our ditch system (γ -diversity), we built randomized species accumulation curves per month using the function *specaccum* (999 permutations, package 'Vegan'). For both sampling months, less than one new species was found within each ditch after half of the samples (18/36) were processed, indicating that the (near) full range of invertebrate species was caught (data not shown).

We then tested for effects of actual neonicotinoid concentration over time on total macroinvertebrate abundance using linear mixed effect modelling (function *lme*, package 'nlme') with ditch ID as a random factor to account for the repeated measures design. In a similar fashion, we tested for effects of the neonicotinoid on species richness and Shannon diversity (H' ; being the most commonly used index). We also tested these same parameters per month using one-way ANOVAs with Dunnett's post-hoc tests to indicate differences between treatments after neonicotinoid application.

We assessed community structural complexity (i.e. the number of links and link density, see also (Neutel et al., 2002)) using Spearman-rank based correlation network analysis on the abundance data of the invertebrates. This type of analysis does not provide direct causal relationships between species simply because correlation $\neq$ causation. However, a correlation between two species does provide evidence that there are one or more factors (such as other species or abiotic variables) governing the abundance of both species. Inherently, the number and strength of all correlations is a proxy of the structural complexity of communities, without the need to identify specific species-species relationships. The underlying assumption of these approaches is that networks of co-occurring species are, to some degree, structured and non-random (Pimm, 2002). Prior to correlation analysis, we removed rare species (≤ 5 individuals per species per treatment) to avoid effects of random co-occurrences. Then Spearman-rank correlation matrices was built per treatment and all strong correlations ($Rho > 0.8$) between species in these matrices were visualized (see Figure 7.2). Next, we compared the correlation matrix of each treatment (i.e. the neonicotinoid concentration) to null models in order to identify potential effects of the

treatment on biological organization. The null models consisted of 1000 generated 'communities' with abundances randomly distributed across the actual observed taxa within the treatment (>5 individuals per species). Abundances were generated following a zero-inflated Poisson distribution using the null probability (i.e. species non-occurrences probability) and total abundances as observed in nine replicate ditches. Differences between the number of correlations of differing strengths between the observed and randomized communities were assessed using a one-sample Kolmogorov-Smirnov test (with the null hypothesis being that the distribution function which generated the number of observed correlations is the distribution of correlations found in the randomized community). More numerous strong correlations than in a randomized community are a clear indication of biological structural complexity. A fewer number of (strong) correlations points towards a loss in biological organization due to environmental filtering. These comparisons of the correlation matrix per treatment versus the control null model were then visualized in Figure 7.3. Finally network 'connectance' (see Morriën et al., 2017) was calculated as a percentage by dividing the number of strong correlations ($Rho > 0.8$) and all possible correlations of the respective treatment.

Effects of thiacloprid concentration, ditch position (1 to 36, left to right at the experimental site) and their possible interaction on community composition were assessed using Permutational multivariate analysis of variance (PERMANOVA, function *adonis*, package '*Vegan*', 999 permutations). We analyzed the data both using raw abundances as well as presence/absence in order to identify whether potential shifts in macroinvertebrate communities were due to shifts in relative abundances or because of species turnover. We used Bray-Curtis as measure for dissimilarity for both the raw abundance and presence/absence data but also tested the presence/absence dataset using Jaccard's index as this is usually the more preferred type of distance. As these results showed highly similar results to the Bray-Curtis measure (as also found by others, for example see Tebby et al. (2017)) we chose to show Bray-Curtis results for comparison with the raw abundance data purposes. Potential community converging or diverging effects of the neonicotinoid were assessed by analyzing the homogeneity of multivariate dispersion using

beta-dispersion tests (function *betadisper*, package 'Vegan', 999 permutations), but no significant effects were found (both April and June: $p > 0.05$). All statistical analyses were performed using R (version 3.5.1; R Core Team, 2019).

Primary production

Plants and filamentous algae were collected during the invertebrate community assay from the same meter of ditch. These samples were dried for three days at 40 °C before weighing to determine dry weight per taxa. Monthly growth of periphyton was determined 1 cm above the sediment level (to minimize the effect of sediment particles) from standardized surface areas on acrylic plates that had been inserted vertically in each ditch for one month. Periphyton was scraped from the plates and vacuum filtered onto a pre-weighed GF/C Whatmann filter which was subsequently dried at 40 °C for three days before weighing again to determine periphyton dry weight. Phytoplankton abundance was estimated as Chlorophyll A by weekly measurements of each ditch using a handheld fluorometer (*Aquafluor*®). These weekly measurements were averaged using a time-weighted average during the first month after the first thiachloprid spike.

Organic matter decomposition and consumption

The monthly rate of microbial decomposition and invertebrate consumption were measured by using Decomposition and Consumption Tablets (DECOTAB) (Kampfraath et al., 2012). DECOTABs were prepared by blending finely ground hay (30 g) into an agar-agar solution (10 g per 500 mL demineralized water) which was allowed to set in cubicle silicon templates of 2*2 cm. Each ditch then received a DECOTAB enclosure constructed of 2 cm high round PVC circles (Ø 11 cm, fitted with 0.5 cm mesh to hold the DECOTABs in place). Each enclosure was fitted with two identically prepared DECOTABs except that one was covered with fine mesh (100 µm) to exclude invertebrate consumption thus allowing for the measurement of microbial decomposition only. DECOTABs were retrieved after one month, carefully rinsed with water and then dried for 3 days at 40 °C before weighing. Invertebrate consumption was inferred

by subtracting microbial decomposition from the total degradation as derived from the DECOTAB without fine mesh.

Feeding guilds

The number of individuals in a feeding guild is a relatively poor predictor of their effect on ecosystem processes compared with feeding guild biomass. We therefore first converted all abundance data to biomass. As we performed live invertebrate identifications, we used the maximal body length as described in identification literature as a proxy for biomass per species instead of determining dry weight. For individuals that were too young to be identified to the species level, we used 25% of the maximal length of the genus. As invertebrate biomass generally does not increase linearly with body length, we used the mass-length relationship for aquatic invertebrates as determined by Sabo et al. (2002) to estimate the mass per specimen (k):

$$mass_k = 0.11 \times length_k^{1.79}$$

where mass is in mg and length in mm. Information on the feeding guild trait modalities per species was then obtained from www.freshwaterecology.info (accessed 6/3/2019) (Schmidt-Kloiber and Hering, 2015). The obtained feeding guild modalities were: Miners, Xylophagous, Predator, Grazer, Filter Feeder (active and passive), Gatherer-Collector (GC), Parasite and Other. We did not identify Miner or Xylophagous species and therefore these guilds were omitted from further analyses. Importantly, a species can occur in multiple feeding guilds due to shared feeding modes. For taxa that were not identified to the species level, we averaged the trait modality for all species within the respective taxonomic level. We summed the trait modalities of 1) 'Predator' and 'Parasite', 2) 'Active Filter Feeder' and 'Passive Filter Feeder' and those of 3) 'GC' and 'Shredder' as they filled similar niches within our ecosystem functioning model (for example: passive and active filter feeder both filter phytoplankton) and/or because they were strongly correlated (i.e. 'GC' and 'Shredder'; Pearson correlation: $R^2 = 0.95$, $p < 0.001$). Biomass per species was multiplied with trait modalities to obtain the biomass per feeding guild per ditch.

Statistics on ecosystem functioning

We analyzed the possible effects of the neonicotinoid on the measured ecosystem processes using Piecewise Structural Equation Modelling (pSEM, package '*piecewiseSEM*'). We made two base assumptions for the effects of the neonicotinoid; 1) the neonicotinoid can only directly affect the invertebrate feeding guilds and consequently 2) all possible effects of the neonicotinoid on ecosystem processes must be through changes in the feeding guilds. These assumptions are supported by literature that showed that neonicotinoids are roughly a factor 10-10,000 more toxic to invertebrates than to algae or plants and that at our test concentrations no plant or algae toxicity is expected (Raby et al., 2018a). Our base model included linear models of all direct effects of the neonicotinoid (as actual average spike concentration) on feeding guild biomass and all direct effects of feeding guild biomass on ecosystem processes (i.e. phytoplankton, periphyton, FLAB and plant growth rates and invertebrate OM consumption). As one species can occur in multiple feeding guilds (see 'Feeding guilds'), we did not draw relationships between the different consumer feeding guilds. Next, we evaluated additional indirect effects of ecosystem processes that could potentially alter other ecosystem processes. Model evaluation and comparison was performed using Bayesian Information Criterion (BIC), χ^2 and insignificance of the p-value of the different models. As FLAB was generally not observed in the control or low insecticide treatment (14/18 samples), this data was inflated by zero values. Therefore, we included FLAB in the pSEM as either present or absent and used a general linear model with a binomial distribution to explore the effects of GC + Shredder biomass on FLAB presence or absence.

7.3 Results

Colonization of experimental ecosystems

Our setup of 36 experimental freshwater ditch ecosystems (Figure 7.1) used aspects of 'mesocosm' setups in the fact that they were well replicated ($n = 9$) and similar in habitat structure, but it also used aspects from the field as colonization occurs naturally and we thus did not control how and what ecological communities developed. This provided a

controlled outdoor experimental design, while studying the effects of the neonicotinoid on naturally-formed interactions between species and their environment. To confirm that starting conditions were similar, we sampled communities per meter ditch in April, before adding different concentrations of neonicotinoid insecticide. Results based on invertebrate counts showed that the natural colonization of the ditches was similar as the number of individuals ($1002 \pm \text{SE } 12$ individuals/meter ditch), taxon richness ($30 \pm \text{SE } 0.13$) and β -diversity (Bray-Curtis) showed no statistical differences between the prospective treatments ($p > 0.05$ for all comparisons, Supplement Figure S7.1).



Figure 7.1. Top-view of the experimental ditch system. Top-left to bottom-right: adjoining lake, wave barrier, helophyte layer, netting, hydrological barrier, experimental ditch.

Neonicotinoid driven community composition

After the initial characterization of the invertebrate communities, we applied the neonicotinoid thiacloprid in two biweekly spikes in four different nominal concentrations: 0, 0.1, 1 and 10 µg/L to the ditches. This concentration range was determined from field observations of neonicotinoid concentrations and was thus deemed environmentally relevant (see methods, section 'Neonicotinoid application'). The increasing neonicotinoid concentration had no significant effects on total taxon richness or Shannon diversity ($p > 0.05$ for both comparisons; Figure 7.2A). However, total invertebrate abundance decreased by 18% at the lowest test concentration and by 36% at the two higher test concentrations ($F_{1,32} = 4.6$, $p = 0.037$; Figure 7.2B). This decrease in total abundance was due to lower abundances in the taxonomic orders of Hirudinea, Insecta, Gastropoda, Malacostraca and Maxillopoda (Supplement Table 7.S1). In contrast, we observed higher abundances with increasing neonicotinoid concentration for the orders of Arachnida and Branchiopoda (Supplement Table 7.S1).

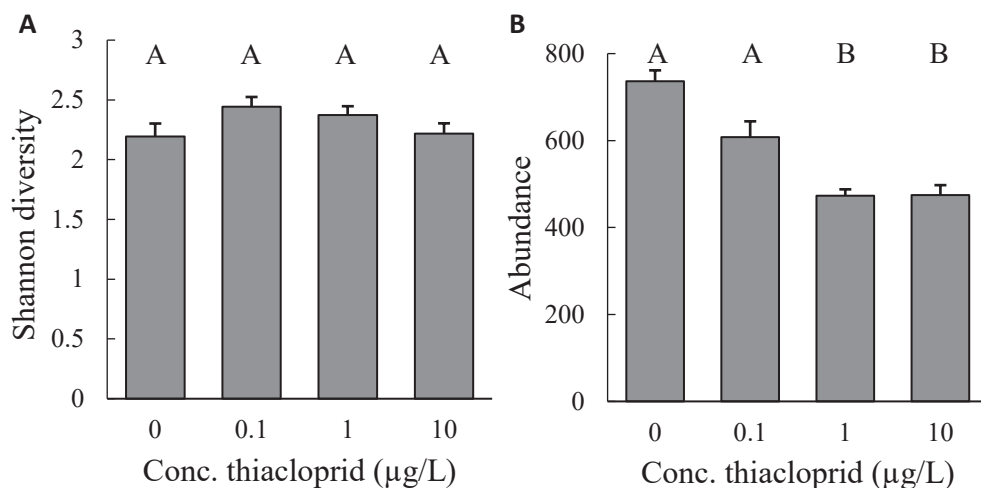


Figure 7.2. Average ($n = 9$, $\pm$ SE) A) Shannon diversity (H) and B) total invertebrate abundance (per meter ditch) with increasing concentrations ('conc.') of thiacloprid. Different letters indicate differences (One-way ANOVA with Dunnett's post hoc test) at significance level $p \leq 0.05$.

Increasing thiacloprid concentrations induced increasingly significant dissimilarities in species composition compared to the control communities ($R^2 = 0.29$, $p < 0.001$; Supplement Figure 7.S2A). A comparable result was obtained when communities were tested on the presence or absence of taxa ($R^2 = 0.24$, $p < 0.001$; Supplement Figure 7.S2B), implying that the observed dissimilarity can be largely attributed to taxon replacement, which in turn explains the lack of effects on total taxon richness and Shannon diversity (H).

Disruption of the invertebrate network

Losses in invertebrate abundance also reduced the total number of possible species-species correlations (co-occurrences) relative to the control by 8, 30 and 33% for 0.1, 1 and 10 µg/L thiacloprid, respectively (Table 7.1). Indeed, fewer significant (sign. level: $p < 0.05$) Spearman's rank correlations were observed (20 – 36%) relative to the control (Table 7.1). The relative strength of the correlations also decreased with median R^2 decreasing from 0.60 (control) to 0.55 (0.1 µg/L) to 0.53 (1 µg/L) to 0.51 (10 µg/L). These decreases were especially pronounced in the number of strong correlations (Spearman's rank > 0.8); 23 strong correlations were observed within the control and this number decreased to 14, 9 and 4 correlations, respectively, with increasing insecticide concentrations (Figure 7.3A). The remaining strong species correlations within the neonicotinoid treatments were inconsistent with those observed within the control (i.e. mostly different co-occurrences were found). The decline in correlation was both absolute and relative to the number of possible correlations as observed in the decreasing connectance (i.e., the percentage of strong correlations of the total number of correlations, Table 7.1). Control communities consistently showed more and stronger species' co-occurrences when compared to randomly generated communities (73% at $\text{Rho} \geq 0.8$, one-sample Kolmogorov-Smirnov test = 0.99, $p = 0.006$, Figure 7.3B), which is a clear indication of non-random structure of the control communities. We observed a clear insecticide dose-dependent loss in structural complexity with insecticide application as the neonicotinoid treatments 0.1, 1 and 10 µg/L were not statistically differentiated from their generated random communities (one-sample KS test = 0.72, $p = 0.56$, one-sample KS test = 0.53, $p = 0.93$ and one-samples KS-test = 0.95, $p = 0.10$, respectively). While compared to the null model generated for the control communities, the number of correlations were even fewer for the neonicotinoid treatments 1 and 10 µg/L (32% and 70% fewer at $\text{Rho} \geq 0.8$, respectively; Figure 7.3B), indicating severe impairment of the structural complexity at higher neonicotinoid concentrations.

A) Species-species co-occurrence plots

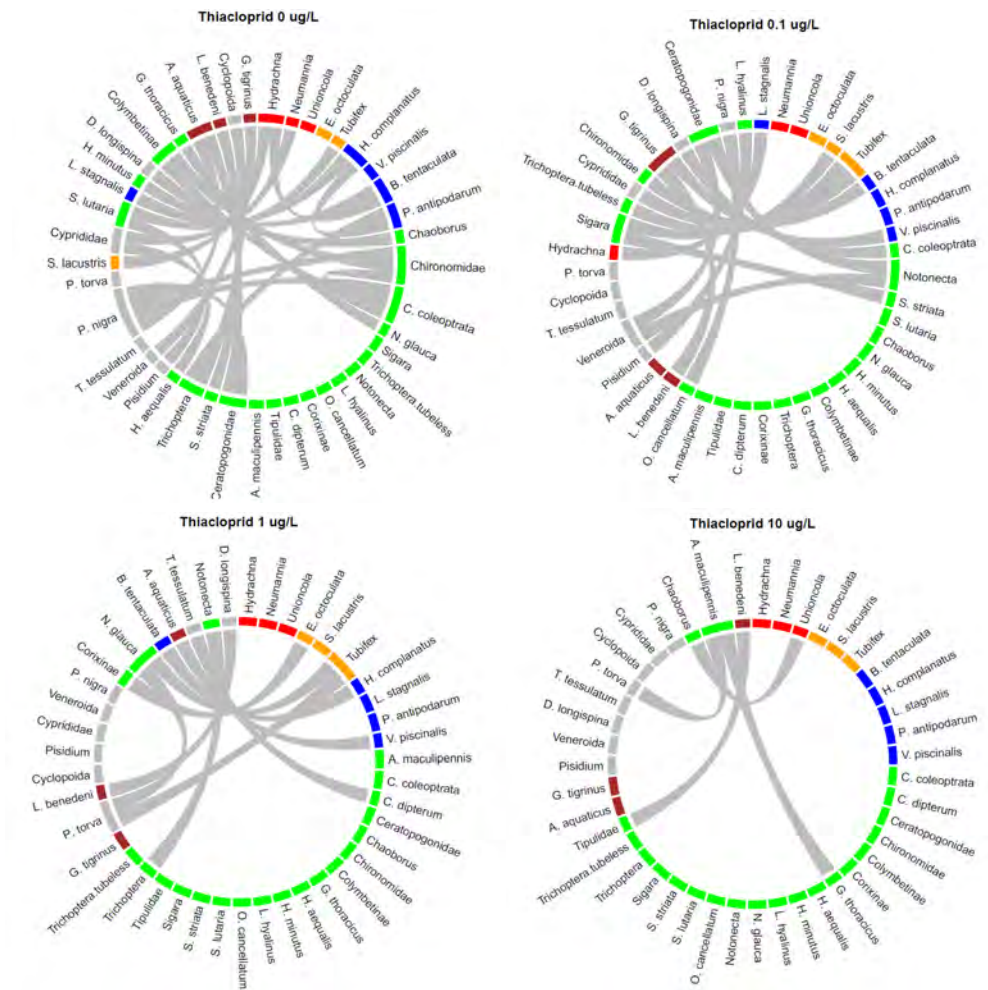


Figure 7.3. (see next page for figure description)

B) Network strengths

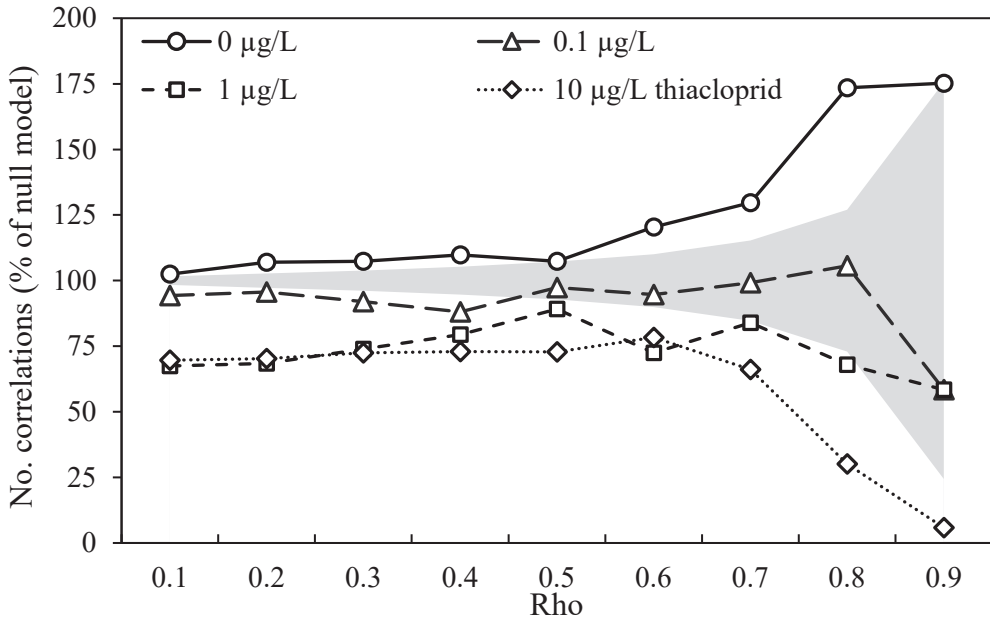


Figure 7.3. A) Invertebrate community correlation web ($Rho > 0.8$) between different taxa per spike concentration thiacloprid (0, 0.1, 1 and 10 $\mu\text{g/L}$). Green: Insecta, Orange: Clitellata, Blue: Gastropoda, Brown: Malacostraca, Red: Arachnida, Grey: Others. B) relative strength of the ecological networks (as number of species co-occurrences) per thiacloprid concentration, expressed as percentage of the community null model mean (grey shading represents the SD of the control null model).

Table 7.1. Network co-occurrence table based on species-species Spearman's Rho correlations.

Concentration thiacloprid ($\mu\text{g/L}$)	# of possible corr.	# of sign. corr. ($p < 0.05$)	Median R^2 of sign. corr.	# of strong corr. ($Rho > 0.8$)	Connectance (%)
0	946	63	0.60	23	2.43
0.1	861	50	0.55	14	1.63
1	666	40	0.53	9	1.35
10	630	40	0.51	4	0.63

'Connectance' is the percentage of strong correlations (Spearman's Rho > 0.8) per total number of possible correlations (see Morriën et al., 2017). 'corr' = correlations.

Loss of ecosystem functioning.

Insecticide-induced stress might affect ecosystem functioning through many different direct and indirect pathways. We used a piece-wise structural equation model to disentangle direct effects of the neonicotinoid on invertebrate feeding guilds and indirect effects on coherent ecosystem processes (OM consumption and primary productivity). The observed changes in community structural complexity also led to shifts in functional groups, as indicated by the piece-wise structural equation model. The biomass of the 'gatherer/collectors + shredders' guild decreased significantly by 15, 46 and 85% for the insecticide concentrations 0.1, 1 and 10 $\mu\text{g/L}$ relative to the control (std. estimate: -0.76, $p < 0.001$; Figure 7.4 and Supplement Figure 7.S3). This coincided with changes in the ecosystem processes fulfilled by these feeding guilds; invertebrate organic matter consumption strongly declined (std. estimate: 0.53, $p < 0.001$) and ditches became dominated by floating algal beds ('FLABs'; std. estimate: -0.70, $p = 0.012$; Figure 7.4), probably due to a lack of herbivory by the shredder guild. Phytoplankton levels (as Chl. A concentration) also declined (9 and 23% at concentrations 1 and 10 $\mu\text{g/L}$, respectively) due to the increasing presence of FLABs (std. estimate: -0.44, $p = 0.008$) but not due to changes within the filter feeders ($p > 0.05$, Figure 7.4). This was probably due to competition for light as the FLABs covered most of the water surface level (see Supplement Figure 7.S4). There were no pronounced differences in the biomass of the other herbivorous feeding guilds and as such we also

found no significant effects on the different ecosystem processes in which these guilds play a role. However, there were other noteworthy effects on predators as median biomass ratio of insect to non-insect predator shifted from 4.7:1 (control) to 0.9:1 (10 µg/L; stats) without statistical differences in total predator biomass ($p > 0.05$; Supplement Figure 7.S3), strongly indicating that there are predator-prey relationships affected that are not incorporated in the pSEM.

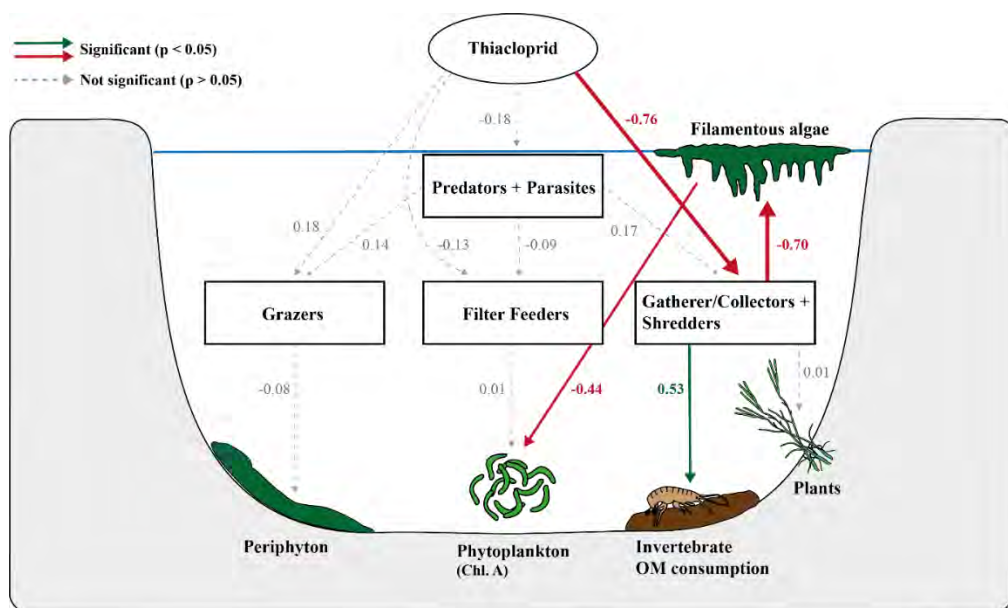


Figure 7.4. Piecewise Structural Equation Model (pSEM) of top-down effects of increasing neonicotinoid insecticide (thiacloprid) concentrations on freshwater ecosystem functioning. Rectangular boxes indicate the different invertebrate feeding guilds. Green and red arrows depict increasing and decreasing effects respectively and line width depicts the shown interaction strength (positive and negative standardized estimates for green and red arrows, respectively). Values for invertebrate OM consumption, periphyton, plant and FLAB biomass were derived from monthly growth rates and phytoplankton from a monthly time-weighted average.

7.4 Discussion

Our study illustrates how neonicotinoid insecticide impaired communities can be identified by a correlation-based network approach. Our control community showed significant structural complexity compared to a random state. After introduction of a common freshwater stressor, the neonicotinoid, community structure was compromised to such an extent that it no longer deviated from generated random assemblages of species. This loss of structure coincided with severely impaired ecosystem functioning due to strong losses within invertebrate feeding guilds. Importantly, these impacts on community structure and ecosystem functioning could not be estimated from relatively simple metrics such as species richness or α -diversity as these metrics remained stable while a clear turnover of taxa occurred. Thus our observations plead for the hypothesis that interactions between species and environment, rather than species richness, is the key link between biodiversity and ecosystem functioning.

Here we show that a relatively simple method, interaction networks, is able to identify disturbances within a community, despite the intrinsic differences in community composition and ecosystem processes. This method uses the ‘observation’, as described by Hutchinson (1959) and Lawton (1999), that ‘organisms interact with one another’, without analyzing each interaction individually as this has proven to be notoriously difficult. Importantly, the observation that we can already identify disturbed communities before ecosystem functioning becomes impaired offers the ability that we can potentially screen for (anthropogenically) disturbed communities early on. Experts on different types of ecosystems have also assessed this network approach to assess community vitality using field observations (Barberán et al., 2012; Baiser et al., 2016; Encinas-Viso et al., 2016; Morriën et al., 2017; Simons et al., 2019). While showing great promise and effectiveness, the general applicability of this method still needs to be evaluated. In addition, our system references to a natural control state, while observational field studies are limited in possibilities in this sense. In theory, null models can be built site-specifically and the degree of deviation from the null can yield as a measure for the structural complexity of each interaction network.

Using these network approaches, our results show that current environmentally relevant levels of neonicotinoid insecticides induce major compositional shifts and degradation of the aquatic food web. These effects are well-beyond any effects that could be estimated from species-specific effects (as obtained from bioassays) described by standard risk assessment methodology (e.g. OECD, 2011, 2012). In addition, we already found significantly impaired structural complexity of invertebrate communities at spike concentrations as low as 0.08 µg/L, which lies well below nearly all recognized toxic concentrations as obtained within standardized risk assessment of aquatic fauna (Morrissey et al., 2015; Pisa et al., 2015; Raby et al., 2018a). This clearly shows that pesticide risk assessment has its short comings as it is not able to predict effects on community structural complexity. The loss of the Shredder and GC feeding guilds had pronounced consequences for ecosystem functionality both in terms of invertebrate consumption as well as cascading effects on primary production. These signs of stress are typically ascribed to effects of eutrophication, which is the result fertilizer runoff from the agricultural field. As these agricultural chemicals are generally applied together and found to jointly impair freshwater communities (Barmantlo et al., 2019a), their effects on ecosystem functioning might even exacerbate each other.

Here we showed for the first time, using ecological network theory, that current surface water levels of neonicotinoid insecticides are major stressors of freshwater communities. The neonicotinoid thiacloprid showed strong impairment of community structure to such an extent that it no longer deviated from random assemblages of species. This consequently induced strong cascading ecosystem deterioration in freshwater ecosystems. Such ecosystem deteriorating effects are not assessed within current standardized pesticide risk assessment. Recently, the usage of thiacloprid has been discontinued within the European Union for different reasons (such as suspected human carcinogenic properties (European Parliament, 2019)). Our results strongly warrant this

discontinuation of further usage of these neonicotinoid insecticides in order to also preserve freshwater life and functioning.

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Author contributions

The manuscript was conceptualized by SHB, MS, GRdS, MV. SHB designed the experiment. SHB and MS collected the data. Statistical analyses were performed by SHB and EC. All authors wrote and discussed the manuscript.

Competing interests

The authors declare no competing interests.

Supplement

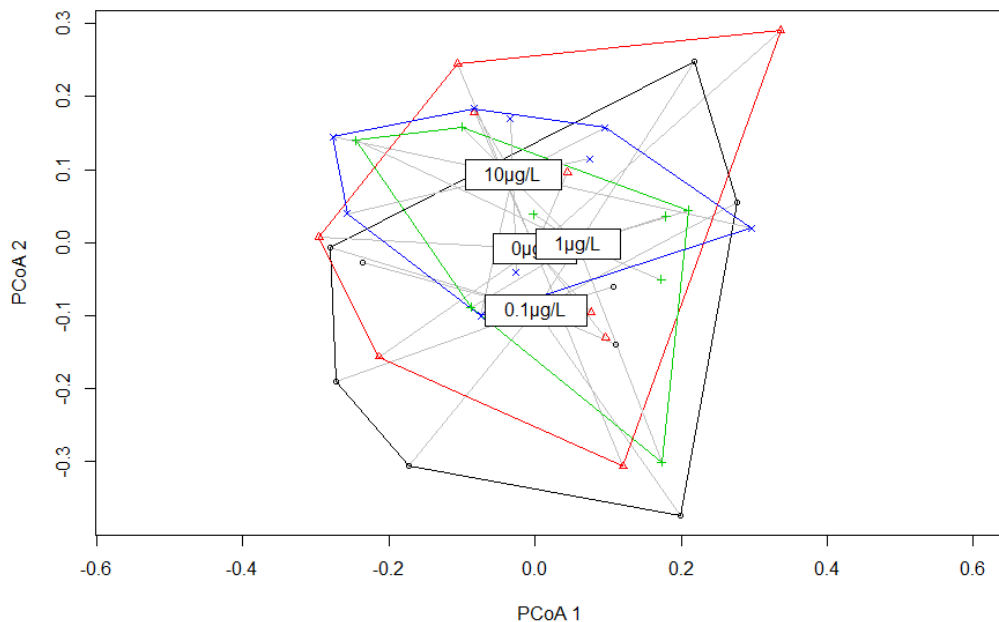


Figure 7.S1. Principal coordinate analysis (PCoA; Bray-Curtis dissimilarity) of the prospective treatments ($n = 9$, black: 0, red: 0.1, green: 1 and blue: 10 $\mu\text{g/L}$ thiachloprid). Communities were sampled in April, before addition of the treatments. Multivariate analyses (permanova and beta dispersion tests) showed no significant differences ($p > 0.05$) between the prospective treatments.

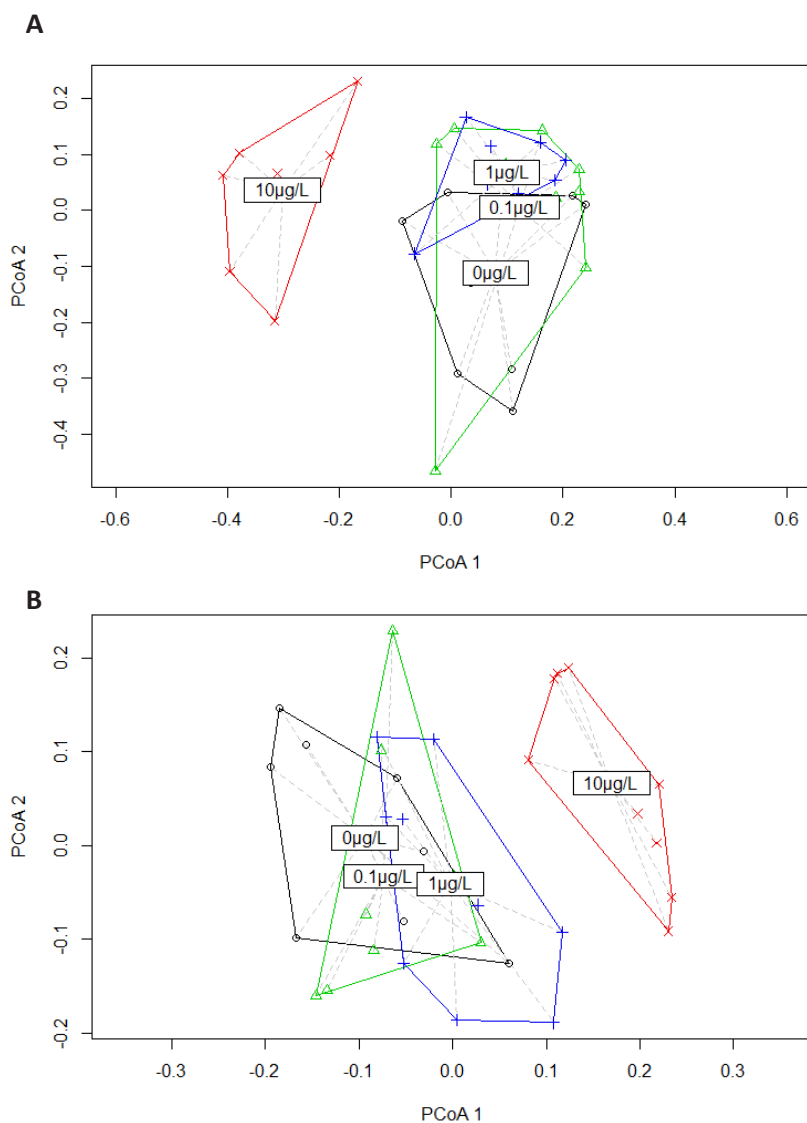


Figure 7.S2. Principal coordinate analysis (PCoA; Bray-Curtis dissimilarity) of A) raw abundance and B) presence-absence data of freshwater invertebrate communities per concentration of the neonicotinoid insecticide thiacloprid ($n = 9$; black: 0, red: 0.1, green: 1 and blue: 10 $\mu\text{g/L}$).

Table 7.S1. Average macroinvertebrate taxonomic class abundance (n = 9) one month after the first application of the neonicotinoid thiacloprid. Significant effects of the neonicotinoid on abundance are indicated by linear regression results (tested singly and interactive with ditch position).

Taxonomic Class	Neonicotinoid concentration (µg/L)				Regression
	0	0.1	1	10	
Clitellata	6	9	6	5	<i>NS</i>
Hirudinea	8	3	9	5	$F_{1,32} = 4$, $p < 0.043$ *
Arachnida	5	5	11	25	$F_{1,32} = 33$, $p < 0.001$
Branchiopoda	88	58	66	153	$F_{1,32} = 13$, $p < 0.001$
Insecta	112	126	89	75	$F_{1,32} = 4$, $p < 0.044$
Malacostraca	255	214	139	34	$F_{1,32} = 22$, $p < 0.001$
Maxillopoda	39	21	33	13	$F_{1,32} = 5$, $p = 0.039$
Ostracoda	50	22	9	0	$F_{1,32} = 4$, $p < 0.043$
Bivalvia	3	4	4	3	<i>NS</i>
Gastropoda	168	142	106	157	$F_{1,32} = 14$, $p < 0.001$ *
Rhabditophora	2	3	2	3	<i>NS</i>

The order of Entognatha and Hydrozoa are not shown as abundances were generally lower than 1 individual per ditch. 'NS' = not significant. * interactive with ditch position at the experimental site.

Table 7.S2. Abiotic conditions before and after application of the neonicotinoid thiacloprid expressed as a monthly time-weighted average (N = 9) based on weekly measurements.

Parameter		Neonicotinoid concentration (µg/L)			
		0	0.1	1	10
Conductivity (µS/cm)	Before	684	689	687	684
	After	509	515	511	501
Nitrate (mg/L)	Before	0.34	0.31	0.82*	0.31
	After	0.40	0.54	0.66	0.55
Oxygen (mg/L)	Before	12.2	12.1	12.1	11.9
	After	15.4	15.0	15.0	15.7
pH	Before	8.2	8.1	8.2	8.1
	After	8.6	8.5	8.6	8.8
Phosphate (mg/L)	Before	0.12	0.20	0.15	0.20
	After	0.09	0.12	0.09	0.06
Temperature (°C)	Before	17.8	17.8	17.8	17.7
	After	23.9	23.8	23.9	23.9
Turbidity (NTU)	Before	91.7	96.3	86.0	85.3
	After	115.7	112.3	105.8	93.8

*strongly skewed by one outlier, the average concentration is 0.14 mg/L when the outlier is excluded.

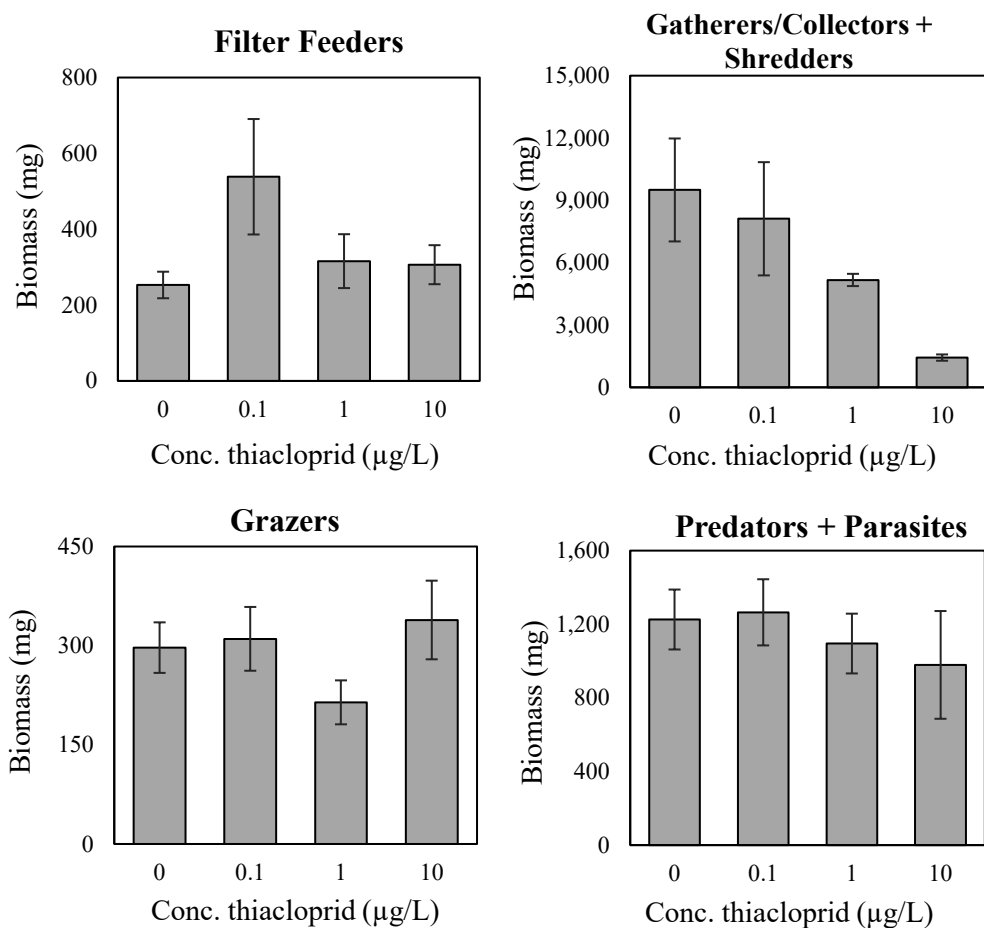


Figure 7.S3. Average biomass ($n = 9$, $\pm$ SE) per invertebrate feeding guild per concentration ('conc.') thiacloprid.



Figure 7.S4. Photograph example of the dominance of floating algal beds. Left: Control, right: 10 $\mu\text{g/L}$ thiacloprid. Photographs were taken one month after the initial thiacloprid spike.

Chapter 8

Discussion

8. General discussion

Neonicotinoids are the most commonly used class of insecticides in the world (Jeschke et al., 2011; Sparks, 2013). Although these modern synthetic pesticides degrade swiftly, have low drift emissions and have a specific mode of action, the concentrations in surface waters all over the world are relatively high (Morrissey et al., 2015). In fact, the insecticides are detected in surface waters across the globe in concentrations that can overlap with toxicity results as obtained from ecotoxicological experiments (Chapter 1.4). This thesis aimed to identify and unravel the impact of neonicotinoid insecticides as well as fertilizers on individual species, aquatic (non-vertebrate) communities, and key functional parameters. Moreover, this thesis showed how the impacts of neonicotinoids manifested in the aquatic system additionally influence the fringing ecosystems via energy (food) fluxes. In combination, my research adds ecological complexity by explicitly covering the abiotic conditions and biotic relationships that are not often understood from extrapolated lab-derived results. Firstly, I examined to what extent fertilizers and food play a role in neonicotinoid toxicity and secondly, I incorporated full-scale ecological complexity in order to examine the impacts of neonicotinoids at the community and ecosystem level.

8.1 Availability of resources shapes neonicotinoid toxicity

Within the first aim of this thesis, it was investigated to what extent food quantity/quality play a role in toxicity at a given neonicotinoid concentration. The main focus was on fertilizers as these are used in concord with neonicotinoids to stimulate yield, and which – as a consequence – are observed in concord in surface waters (Morrissey et al., 2015; Hladik et al., 2018; Leiden University and Rijkswaterstaat-WVL, 2018; Casado et al., 2019; Chen et al., 2019). While being a direct driver of life, fertilizers can also potentially affect toxicity by increasing the energy budget of individuals through the bottom-up food surplus (Halstead et al., 2014), allowing them to cope with toxic stress (as part of the Dynamic-Energy Budget theory, see Nisbet et al., 2010).

Chapter 2 shows that fertilizers indeed reduce the neonicotinoid-toxicity experienced in several test species. At an environmentally relevant neonicotinoid concentration, three out of four test species experienced significant fitness impairment at one or more endpoints. The addition of fertilizer to the aquatic system nullified these toxic responses in all cases. It was hypothesized that the test animals (and others individuals living in the ditch ecosystems) were using stimulated primary production or microbial growth as diet supplement. This increases energy potential, allowing the organisms to cope with the toxic stress (following the DEB-theory, Nisbet et al., 2010). This hypothesis was supported through the finding that phytoplankton was suppressed in ditches that had received a neonicotinoid application (potentially through compensatory feeding, see Alexander et al., 2007; Goedkoop et al., 2010), even when fertilizers were also applied.

In Chapter 3, a similar experimental design was used to investigate these toxicity suppressing effects at a higher trophic level. Blue-tailed damselflies (*I. elegans*), a predatory species, were found to be susceptible to neonicotinoids as they showed reduced performance for all sublethal endpoints investigated (growth, molting, consumption, mobility and emergence). However, most toxicity was only observed when damselflies were allowed to hunt naturally present prey. Damselflies that were fed at *ad libitum* conditions with cultured water fleas showed less and lower toxic responses. These high food quantity/quality conditions of Chapter 2 and 3 share most similarity with standardized ecotoxicological experiments, as these are performed *ad libitum* (e.g. OECD 211, 2012). This highlights the importance of food in modifying chemically induced stress. The impacts of optimal conditions which are used to gain laboratory-derived effect concentration (or threshold) values vs. the often stress-on-stress conditions due to lack of food in field situations are crucial to consider. This food-mediated toxicity of neonicotinoids can have several consequences for freshwater biodiversity and it explains the toxicity in the field observed at current environmental levels of neonicotinoids. First, neonicotinoids are likely more toxic in low-nutrient/food (oligotrophic) environments. Second, within current scopes of reducing nutrient emissions (such as nitrogen as according to

the Gothenburg Protocol, (United Nations, 2012), neonicotinoid toxicity may potentially increase in the future in waters that are currently meso- or eutrophic.

8.2 Toxicity differences between laboratory and field

Some of the neonicotinoid toxic effects found within this thesis differed majorly from results as obtained within standardized risk experiments. Most notable were the results for *D. magna*, the most commonly studied test species neonicotinoid risk assessment in ecotoxicology (Pérez and Beiras, 2010). In Chapter 2, toxicity was observed for growth and reproduction in an *in situ* setup at concentrations that were near 2500 times lower than toxicity results as obtained in laboratory tests (Pavlaki et al., 2011). This was surprising as *D. magna* is recognized as one of the most insensitive invertebrates towards neonicotinoids (see Chapter 1.3). In addition, the experimental setup was comparable to standardized experiments (i.e. OECD 211, 2012) in the sense that cultured daphnids (following the guideline) were studied in an enclosed environment for 21 days. The most notable differences were the use of natural waters (instead of OECD-prescribed exposure medium) and the fact that the daphnids were not fed (instead of *ad libitum*). Not feeding means that the daphnids had to invest energy in collecting food within the ditch water. Additionally, natural food from the field was likely of lower quality than the optimized freshly cultured algae as used in the laboratory. Moreover, within the ditch fluctuations in temperature, oxygen, nutrients, availability of food, predator presence and other factors are common, which also requires additional energy investments to cope with. These mark the main differences between the laboratory and the field; in the approach of standardizing as many factors (thus removing stress) as possible within ecotoxicological risk assessment, we are neglecting the fact that within nature stress is the norm rather than an exception. This is most clearly exemplified in Chapter 3 where toxic pressure in damselflies increased strongly from individuals that were i) caged and fed *ad libitum* to ii) caged and freely feeding on naturally present prey to iii) damselflies that lived freely within a ditch.

Given that different species respond differently to different stress, the cocktail of agrochemicals (insecticide, herbicide and fertilizer) abundant in our ditches affect invertebrate populations in multiple ways. Chapter 4 shows that, while in a relatively simple assemblage (nine species), the effects of secondary mixtures can be estimated from their single effects, this was not the case for tertiary mixtures. Effects go even further than populations as Chapter 6 learns us that both the neonicotinoid thiacloprid and synthetic fertilizers are not only single but also joint drivers of naturally assembled aquatic *communities*. Since mixtures of multiple agrochemicals are commonly observed in freshwaters (Vijver and van den Brink, 2014; Schreiner et al., 2016), it is evident that not only the dose (Paracelsus) but also the environment (both abiotic & biotic conditions) determines the “poison”.

8.3 The missed hazards of neonicotinoids to freshwaters

Chapters 2, 3, 4, 5, 6 and 7 all evaluated toxic effects of neonicotinoids to aquatic invertebrates at globally observed surface water concentrations. Effects ranged from single species (Chapters 2 and 3) to populations (Chapter 4 and 5), communities (Chapters 6 and 7) and even the full-scale ditch ecosystem (Chapter 7). The used test concentrations within this thesis are usually exceeding Dutch environmental norms (according to JG-MKN/MTR norms, see Chapter 1.4, Table 1.1), but do reflect the actual neonicotinoid concentrations found in surface waters. While the proposed norms may be suitably protective for freshwaters, in reality freshwaters are thus not as protected towards the hazards of neonicotinoids.

This thesis showed several times that neonicotinoids and fertilizers did not directly affect invertebrate species richness or α -diversity (Chapter 6 and 7). However, following the ongoing global pattern of changing biodiversity (Blowes et al., 2019), there were marked differences in what species were actually present after disturbance by the agrochemicals (Chapters 6 and 7). From an ecosystem functioning perspective, theoretically, it could be that the new species assembly functions equally well as the initial assembly. However, Chapter 7 showed that neonicotinoid pollution led to stunted integrity of the invertebrate

communities which in turn led to retarded organic material (OM) degradation and the built-up of large masses of free floating algae beds (FLABs). Both these processes obstruct water flow and thus negatively impact one of the main ecosystem services provided by a ditch: the removal of excess water (Highler, 1994).

In addition, Chapter 5 experimentally showed that neonicotinoids are one of the actors of the ongoing insect decline (Hallmann et al., 2017, 2019; Lister and Garcia, 2018; Homburg et al., 2019; Sánchez-Bayo and Wyckhuys, 2019), both in terms of diversity, abundance and sheer biomass. Some of the impacted insect species, such as dragonflies, are predators of plague species (Painter et al., 1996; Sánchez-Bayo and Wyckhuys, 2019). Other impacted insects act as prey for larger predators such as birds and bats. Neonicotinoids thus also potentially negatively impact naturally occurring biological control of pest species. These observed negative impacts on the functioning of the ditch ecosystem were not short-lived. Chapter 6 shows that community structure was still impacted four months after the initial application of the neonicotinoid. Species that accounted for most of the dissimilarity between the control and ditches that received the neonicotinoid were not necessarily the same species that were affected in the short-term. Individual invertebrate species that had never actually been exposed to the neonicotinoid thus still experienced its legacy effects (or indirect effects) through resonating effects within the food-web. In addition, ditches that received the higher doses of the neonicotinoid were still dominated by FLABs 5-6 months after the initial application (Chapter 7; personal observation). This means that recovery of the ditch ecosystem, dependent on the neonicotinoid concentration, only occurs after removal of the toxicant and a significant amount of time. However, in actual freshwaters, neonicotinoids are generally observed to be continuously present (Hladik et al., 2018), especially during the summer months (Chapter 2, Leiden University and Rijkswaterstaat-WVL, 2018), which would thus not allow for recovery to take place. Because of these legacy effects and the continued presence of neonicotinoids, the assumption that recovery will or might take place (as adapted in European authorization of plant protection products; Moermond et al., 2016) relatively shortly after neonicotinoid pulses

(because of their short aquatic half-lives, see Chapter 1.4, Table 1.1) should be reevaluated within the legislation of these agrochemicals.

8.4 Investigating risks in the environment

This thesis showed several times how the environment determines the toxicity of a neonicotinoid through food quality/quantity, agrochemical mixtures, or species interactions. In contrast, within current pesticide risk assessment the environment is generally simplified to such an extent that all these parameters are controlled and standardized. This effectively leads to not observing several neonicotinoid-induced hazards that do occur in the actual environment (see 8.3). Experimenting from the most controlled perspective - and with that: minimal stress - thus leads us to miss major impacts such as insect decline, retarded ecosystem functioning and changes in biodiversity due to species interaction alterations. On the other hand, performing the largescale ecosystem experiments as described in this thesis for different ecosystems and all different pesticides requires an enormous amount of effort and is not feasible. Requirements for performing such experiments must thus be met. This can be met by, for example, post-registration monitoring of pesticide concentrations in surface waters (Vijver et al., 2017) combined with existing ecotoxicological data to indicate potential risks in the environment (see Chapter 1.4). Ultimately, the outcomes of the ecosystem experiments can then be used within legislation policy when suspected risks are either confirmed or disproved.

As natural species interactions were shown to govern the toxicity of neonicotinoids (see Chapter 6 and 7), new statistical methods are also necessary to quantify a potential loss in community structure. Following ecologists, exciting opportunities lay in the adoption of network analysis to evaluate community integrity (see Chapter 7) as this can be used as an indicator for community 'health' and can potentially act as an early-warning signal for ecosystem functioning.

8.5 From test species to ecosystems

Neonicotinoids are currently being phased-out as their usage becomes increasingly restricted (European Commission implementing regulation, 2014a,b,c; European Parliament, 2019). These restrictions came into play for several reasons, most importantly being their adverse effects towards pollinators (EASAC, 2015) and suspected carcinogenic effects. For thiacloprid, the primarily investigated neonicotinoid within this thesis, the evaluation for renewal of approval occurred in 2019 (during the finalization of this thesis; European Parliament, 2019). It was concluded that: “In the area of ecotoxicology, no critical concerns were identified.” This conclusion largely relied on i) results for a single group of test species, being the pollinators and primarily the honey bee (*Apis mellifera*) and ii) a lack of suitable endpoints for aquatic organisms.

In this thesis, it was shown that current environmental concentrations of neonicotinoids pose an actual threat to freshwaters, both in terms of invertebrate biodiversity and ecosystem functioning. Within this thesis, it was also shown that testing with common test species does not reflect the actual environment, and as such, these impacts are not found at standardized laboratory conditions. This has two main reasons; firstly, the environment determines the toxicity, especially through the effects of agrochemical mixtures and the availability of resources. Secondly, large cascading effects within an ecosystem (such as shifting biodiversity, disruption of community networks, insect declines and retarded ecosystem functioning) cannot be estimated or fully comprehended from standardized bioassays and most mesocosm experiments. The inclusion of environmentally realistic testing with natural communities, as performed in this thesis, is thus essential to optimize future research on pesticides-induced impacts in nature.

I therefore plea for the continued and accelerated inclusion of ecological theory within pesticides-induced impact assessment. When an ecological risk is indicated (e.g. by the overlap of environmental levels and toxicity data, see Chapter 1.4), experiments should be conducted that include full natural complexity so that effects on ecosystems can be optimally

estimated and then mitigated. This requires working with naturally formed ecological communities in realistic environments and exposure scenarios, but this does not have to go at the cost of standardization.

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Summary

While freshwaters only cover less than 1% of the Earth's surface, they harbor rich biodiversity representing 10% of all known animal species. However, biodiversity is declining at an unprecedented rate and freshwaters are among the most endangered ecosystems. Within this thesis, I describe the role of pollution, and specifically neonicotinoid insecticides, as an actor of the ongoing biodiversity decline. A current risk of neonicotinoids to freshwaters is indicated by the considerable overlap of global surface water concentrations and toxicity values for invertebrates as observed in ecotoxicological bioassays. While the risks of neonicotinoids were relatively well-studied in these bioassays, there are still many uncertainties to what extent these results are representative for the actual environment. This is because of the many different additional drivers in the field situation that can potentially affect toxicity. Especially the role of synthetic fertilizer, another globally important agrochemical, is investigated in depth in this thesis to evaluate how they might affect neonicotinoid toxicity. In addition, the generally standardized and relatively simple ecotoxicological setups are not suitable for evaluating species interactions and shifts therein with an ecosystem. To investigate the impacts of both additional environmental drivers and of species interactions on the perceived toxicity of neonicotinoids, a new experimental facility was realized, the Living Lab. Here I examined the effects of neonicotinoids and fertilizers to individuals and naturally assembled populations and communities in 36 experimental ditches.

In Chapter 2, we tested how synthetic fertilizers affect the toxic pressure exhibited by the neonicotinoid thiacloprid by applying these agrochemicals to the experimental ditches in a full factorial design. We used an *in situ* caged experimental setup within the ditches using four different aquatic invertebrates species: *A. aquaticus*, *C. dipterum*, *C. riparius* and *D. magna*. Depending on species, the endpoints investigated were survival, growth, reproduction, consumption and emergence. The results showed that current surface water concentrations of thiacloprid affects several of the investigated sublethal endpoints in three out of four species. Effects for growth and reproduction of *D. magna* were even found at concentrations that were 2456 times lower than used in laboratory assessments. However, nutrient enrichment (through fertilizer addition)

nullified this toxicity, likely due to increased primary production that allowed for compensatory feeding. Such compensatory feeding would increase the internal energy budget so that the invertebrates could combat the toxic stress. These differences between lower and higher food quality/quantity regimes explain a large part of the discrepancies between neonicotinoid toxicity results from the laboratory and the field as laboratory assessments are always performed *ad libitum*.

In Chapter 3, we used a similar experimental design (*in situ* caged bioassays) as in Chapter 2 to determine the effects of an environmentally relevant range of the neonicotinoid thiacloprid to the damselfly species *I. elegans*. In addition, we investigated to what extent *ad libitum* feeding regimes play a role in the exhibited toxic expression of this predatory species. Damselflies were either fed with cultured daphnids or allowed to hunt naturally present prey. In addition, each experimental ditch received one emergence trap to monitor emergence of the naturally present *I. elegans* populations. Neonicotinoids were found to affect all sublethal endpoints investigated (growth, molting, consumption, mobility and emergence). However, damselflies that had been fed performed markedly better than those that had been hunting the natural prey as stronger toxicity was observed in the latter group. Emergence of the natural populations also declined strongly with increasing neonicotinoid concentrations. As this species is the most common damselfly in large parts of Europe, it was concluded dragonfly populations can be currently threatened and possibly even declining, following ongoing trends, due to the investigated neonicotinoid.

In Chapter 4, we tested the single and joint effects of an insecticide (the neonicotinoid imidacloprid), a herbicide (terbuthylazine) and synthetic fertilizer to an aquatic assemblage of nine species in a full-factorial mesocosm design with all three agrochemicals. Toxicity results obtained within the experiment were compared to literature in order to approximate whether results from single-species bioassays are accurate in more complex agrochemical mixtures. Single agrochemical and binary mixtures were found to accurately reflect results from single-species bioassays. However, tertiary mixtures, as commonly found in the

environment, were often disparate from cumulative responses to the single agrochemicals. This indicates that non-additive effects of agrochemicals determine the abundance of invertebrates in natural populations.

In Chapter 5, we investigated the role of the neonicotinoid thiacloprid as a potential actor in the ongoing insect decline. An environmentally relevant range of concentrations of the neonicotinoid thiacloprid (0, 0.1, 1 and 10 $\mu\text{g/L}$) was applied to the experimental ditches in order to investigate potential declines in insect emergence. Each ditch was fitted with one emergence trap and these were monitored continuously for the duration of three months on abundance, biomass and diversity of naturally colonized insect populations. Increasing neonicotinoid concentrations strongly declined the abundance of four (Coleoptera, Ephemeroptera, Odonata and Trichoptera) out of five major insect orders. The fifth major insect order, Diptera, experienced smaller declines in abundance, while biomass was more strongly affected. The diversity of Chironomidae, being the most species-rich insect group in freshwaters, declined by 50% at a monthly average concentration of 0.4 $\mu\text{g/L}$ thiacloprid. As this experiment was performed with natural populations and field-relevant neonicotinoid concentrations, it provided causal evidence of thiacloprid being an actor of the ongoing insect decline.

In Chapter 6, the neonicotinoid thiacloprid and synthetic fertilizer were investigated as potential factors that singly and/or jointly structure aquatic invertebrate communities. Like in Chapter 2, both agrochemicals were applied at field-relevant concentrations in the experimental ditches in a full factorial design. Invertebrate communities were sampled and identified one month before, one month after and four months after application. For all different treatments, there were no apparent effects on total invertebrate abundance nor species richness. However, there were marked dissimilarities compared to the control for communities that had been exposed to the neonicotinoid and the fertilizer, but especially for those that had been exposed to the mixture of both. While some toxicity depressing effects of fertilizer were observed, the combined application

of both agrochemicals showed the most dissimilarity to the control overall. These effects were of a long-term nature as dissimilarities were observed for different species four months after the initial application of the agrochemicals. This indicates that there was also a disrupting impact within the food web that induced species transition which might have consequences for ecosystem functioning.

In Chapter 7, the effectiveness of correlation based network analyses (i.e. species co-occurrence networks) to identify the effects of a stressor (the neonicotinoid thiacloprid) to invertebrate communities was examined. Aquatic communities were exposed to a range of field-relevant neonicotinoids concentrations (like in Chapter 5). These communities were then sampled, identified and analyzed as according to Chapter 6. Like in Chapter 6, no effects on α -diversity were found, but we again observed a considerable species transition. With increasing neonicotinoid concentrations, strong losses in structural complexity within the co-occurrence network were found. Structural complexity of the neonicotinoid-impacted communities was weaker or indistinguishable from that of randomly generated communities (i.e. a null model). These losses in structural complexity paired with losses in ecosystem functioning; a piecewise structural equation model indicated large losses in the 'Gatherer-Collector' and 'Shredder' feeding guilds that led to retarded organic matter consumption and the dominance of floating algal beds. These floating algal beds, in turn, suppressed phytoplankton communities. This Chapter thus illustrates how network analyses accurately identify stressor-impacted communities and that the loss of structural complexity can even have strong consequences for the functioning of aquatic ecosystem.

In conclusion, current surface water levels of neonicotinoids have been shown in this thesis to have strong impacts on natural freshwater invertebrate populations, communities and their roles within ecosystems. Such impacts are not found at standardized laboratory conditions where as they are by definition hidden whilst working with single species or simplified species assemblages. The inclusion of environmentally realistic testing with natural communities, as performed in this thesis, is thus

essential to predict the actual risks of agrochemicals in the actual environment. I therefore plea for the accelerated inclusion of ecological concepts and theory to optimize future research on pesticides-induced impacts in nature.

Samenvatting

Zoetwateren bevatten een rijke biodiversiteit met 10% van alle bekende diersoorten terwijl ze slechts 1% van het totale aardoppervlak bekleden. Echter neemt de biodiversiteit momenteel op verschillende locaties in een rap tempo af en zoetwateren behoren tot de meest bedreigde ecosystemen. In dit proefschrift beschrijf ik de rol van vervuiling, specifiek de neonicotinoïde insecticiden, op de huidige biodiversiteitscrisis. Er bestaat een risico van neonicotinoïden in zoetwateren aangezien toxiciteitswaarden voor aquatische ongewervelden en metingen in oppervlaktewateren aanzienlijk overlappen. Terwijl de risico's relatief goed bestudeerd zijn in de zogenoemde bioassays, zijn er nog veel onzekerheden in hoeverre deze resultaten representatief zijn voor de omgeving. Dit is omdat er in de veldsituatie vele verschillende factoren zijn die mogelijk de toxiciteit beïnvloeden. Vooral de rol van kunstmesten, een wereldwijd belangrijk agrochemicaal, en hoe zij mogelijk de toxiciteit van neonicotinoïden beïnvloeden wordt in dit proefschrift onderzocht. In dit proefschrift werd duidelijk dat de huidige ecotoxicologische standaardproeven relatief simpel zijn en dus niet geschikt om soorteninteracties en verschuivingen in biodiversiteit aan te duiden. Om de impacts van omgevingsfactoren en soorteninteracties op neonicotinoïde toxiciteit in kaart te brengen, werd daarom een nieuw experimenteel buitenlaboratorium gerealiseerd, het Levend Lab. Hier heb ik de effecten van neonicotinoïden en kunstmesten op individuen, populaties en gemeenschappen van ongewervelden onderzocht in 36 experimentele sloten.

In Hoofdstuk 2 hebben we getest hoe kunstmesten mogelijk de toxiciteit van een neonicotinoïde beïnvloeden door beide agrochemicaliën in de experimentele sloten aan te brengen in een volledig factorieel ontwerp. We gebruikten een *in situ* kooi experiment met vier verschillende aquatische ongewervelde soorten: *A. aquaticus*, *C. dipterum*, *C. riparius* en *D. magna*. Afhankelijk van de soort, bestudeerden we de eindpunten overleving, groei, voortplanting, consumptie en uitsluipen. De resultaten lieten zien dat huidige oppervlakte concentraties van thiacloprid verschillende subletale eindpunten van drie uit de vier testsoorten beïnvloedde. Effecten op de groei en voortplanting van *D. magna* waren zelfs 2456 maal sterker dan eerder in laboratorium experimenten werden

gevonden. Echter, nutriënten verrijking door middel kunstmest toevoeging hief deze toxiciteit op. Dit was waarschijnlijk door de waargenomen toegenomen primaire productie dat ervoor zorgde dat er voedingscompensatie kon plaatsvinden voor de toxische druk. Deze voedingscompensatie vergrootte waarschijnlijk het interne energie budget in zulke mate dat de ongewervelden de toxische stress konden weerstaan. Deze verschillen tussen lage en hoge voedsel beschikbaarheid/kwaliteit verklaren een groot deel van de tegenstrijdigheid over neonicotinoïde toxiciteitswaarden tussen het laboratorium en het veld omdat laboratoriumproeven altijd *ad libitum* worden uitgevoerd.

In Hoofdstuk 3 hebben we een vergelijkbare experimentele opzet zoals in Hoofdstuk 2 gebruikt (de *in situ* kooien) om effecten van milieurelevante concentraties van het neonicotinoïde thiacloprid op de waterjuffer *I. elegans* te onderzoeken. Daarnaast onderzochten we hoe *ad libitum* voeren een rol speelt in de mate van toxiciteit die geuit wordt op dit roofdier. We voerden een deel van de waterjuffers met gekweekte watervlooien terwijl het andere deel vrij mocht jagen op natuurlijk aanwezige prooien in de sloten. Ook ontving elke experimentele sloot één uitsluitval om het uitsluipen van de natuurlijk aanwezige *I. elegans* populaties te onderzoeken. De neonicotinoïde beïnvloedde alle subletale eindpunten die onderzocht werden (groei, vervellen, consumptie, mobiliteit en uitsluipen) op een negatieve manier. Echter, de waterjuffers die gevoerd waren presteerde duidelijk beter dan diegene die op de natuurlijk aanwezige prooien jaagden aangezien er meer en hogere toxiciteit werd waargenomen in de laatstgenoemde groep. Het aantal uitsluiters van de natuurlijk aanwezige populaties nam ook sterk af met toenemende neonicotinoïde concentratie. Aangezien dit de meest voorkomende waterjuffer soort is in grote delen van Europa concludeerden we dat huidige populaties van Odonata mogelijk een risico lopen of zelfs afnemen (zoals we ook waarnemen in Nederlandse trends) door deze neonicotinoïde.

In Hoofdstuk 4 testte we de afzonderlijke en gezamenlijke effecten van een insecticide (de neonicotinoïde imidacloprid), een herbicide

(terbuthylazine) en kunstmest op een verzameling van negen aquatische ongewervelde soorten in een volledige factorieel ontwerp met de drie agrochemicaliën. De verkregen toxiciteitswaarden werden daarna vergeleken met resultaten uit literatuur om te onderzoeken of zulke resultaten accuraat zijn in meer complexe mixen van agrochemicaliën. Afzonderlijke agrochemicaliën en mixen van twee bleken accuraat de uit literatuur verkregen waarden te reflecteren. Echter, de behandeling met alle agrochemicaliën, zoals vaak wordt waargenomen in oppervlaktewateren, bleek soortenaantallen te bevatten die niet uitgelegd konden worden van de resultaten van afzonderlijke agrochemicaliën. Dit laat zien dat de niet-additieve effecten van agrochemicaliën de abundantie van ongewervelden in natuurlijke populaties kunnen bepalen.

In Hoofdstuk 5 onderzochten we of de neonicotinoïde thiacloprid mogelijk een rol speelt in de huidige afname van insectenstanden. Een milieurelevante reeks van concentraties (0, 0.1, 1 en 10 µg/L) werd aan de experimentele sloten toegevoegd om mogelijke afnames van het aantal uitsluitende insecten te onderzoeken. Boven elke sloot werd één uitsluitval geïnstalleerd om zo het aantal uitsluiters, de biomassa en de diversiteit te bepalen van natuurlijke insecten populaties gedurende drie maanden. Toenemende neonicotinoïde concentraties verlaagden sterk de abundantie van vier (Coleoptera, Ephemeroptera, Odonata en Trichoptera) van de vijf meest voorkomende insecten ordes. De vijfde order, Diptera, nam in lagere mate in abundantie af, terwijl de biomassa juist sterker afnam. Diversiteit van de meest soortenrijke groep in zoetwater, de Chironomidae, nam met 50% af bij een maandelijks gemiddelde concentratie van 0.4 µg/L thiacloprid. Omdat dit experiment werd uitgevoerd met natuurlijke populaties en milieurelevante neonicotinoïde concentraties, leverde deze resultaten causaal bewijs dat thiacloprid een rol speelt in de huidige afname van insectenstanden.

In Hoofdstuk 6 werden de neonicotinoïde thiacloprid en kunstmest onderzocht als potentiële factoren die op zichzelf of samen aquatische ongewervelde gemeenschappen structureren. Vergelijkbaar met hoofdstuk 2 werden ook hier beide agrochemicaliën toegevoegd in milieurelevante concentraties in de experimentele sloten in een volledig

factorieel ontwerp. Ongewervelde gemeenschappen werden bemonsterd en geïdentificeerd één maand voor toevoeging, alsmede één en vier maanden na toevoeging van de agrochemicaliën. Voor alle verschillende behandelingen waren er geen klaarblijkelijke effecten op zowel de totale abundantie van ongewervelden, als de soortenrijkdom. Echter, er waren duidelijk zichtbare verschillen in de aanwezige diversiteit binnen de gemeenschappen die waren blootgesteld aan thiacloprid of kunstmest vergeleken met de controlebehandeling. De gemeenschappen die waren blootgesteld aan de combinatie van beide lieten de meest sterke afwijking van de diversiteit zien. Hoewel enkele toxiciteitsverlagende effecten van kunstmest werden waargenomen, vertoonde de gecombineerde toepassing van beide agrochemicaliën de meeste diversiteitsverschillen met de controle in het algemeen. Deze effecten waren langdurig omdat verschillen voor meerdere soorten werden waargenomen vier maanden na de eerste toepassing van de agrochemicaliën. Dit geeft aan dat er ook een versturende impact was binnen het voedselweb, die de soortovergang veroorzaakte en welke mogelijk gevolgen had voor het functioneren van het ecosysteem.

In Hoofdstuk 7 werd de effectiviteit van op correlatie gebaseerde netwerkanalyses (d.w.z. soorten die tegelijkertijd voorkomen in netwerken) onderzocht om de effecten van een stressor (de neonicotinoïde thiacloprid) op ongewervelde gemeenschappen te identificeren. Aquatische gemeenschappen werden blootgesteld aan een reeks milieurelevante neonicotinoïde concentraties (zoals in Hoofdstuk 5). Deze gemeenschappen werden vervolgens bemonsterd, geïdentificeerd en geanalyseerd volgens Hoofdstuk 6. Net als in Hoofdstuk 6 werden geen effecten op α -diversiteit gevonden, maar er werd opnieuw een aanzienlijke soortenovergang waargenomen. Met toenemende neonicotinoïde concentraties werden sterke verliezen in structurele complexiteit binnen het correlatienetwerk gevonden. Daarnaast was de structurele complexiteit van de door neonicotinoïden getroffen gemeenschappen zwakker of niet te onderscheiden van die van willekeurig gegenereerde gemeenschappen (d.w.z. een nul-model). Deze verliezen in structurele complexiteit gingen gepaard met verliezen in het

functioneren van de ecosystemen; een 'piecewise structural equation model' wees op grote verliezen in de 'Gatherer-Collector' en 'Shredder' voedergilden die leidden tot een verlaagde consumptie van organische stof en tot dominantie van algenbedden. Deze drijvende draadalgen onderdrukten op hun beurt fytoplankton-gemeenschappen. Dit hoofdstuk illustreert hoe netwerkanalyses nauwkeurig de door stressoren beïnvloede gemeenschappen kunnen identificeren en dat het verlies van structurele complexiteit zelfs sterke gevolgen kan hebben voor het functioneren van aquatische ecosystemen.

In conclusie, dit proefschrift toont aan dat de huidige oppervlaktewaterconcentraties van neonicotinoïden sterke effecten hebben op natuurlijke ongewervelde zoetwaterpopulaties, gemeenschappen en hun rol binnen ecosystemen. Dergelijke effecten worden niet gevonden bij gestandaardiseerde laboratorium-omstandigheden omdat ze per definitie verborgen zijn door het werken met afzonderlijke soorten of vereenvoudigde soortensamenstellingen. De opname van milieu-realistische testen met natuurlijke gemeenschappen, zoals uitgevoerd in dit proefschrift, is dus essentieel om de werkelijke risico's van agrochemicaliën in de werkelijke omgeving te voorspellen. Ik pleit daarom voor de versnelde opname van ecologische concepten en theorieën om toekomstig onderzoek naar de effecten van pesticiden in de natuur te optimaliseren.

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Curriculum vitae and publications

Sijbertus Hendrik (Henrik) Barmantlo was born on the second of July, 1991 in Huizen, The Netherlands. After completing his VWO at the Goois Lyceum in Bussum in 2009, he started studying Biology at the University of Amsterdam in 2010. Successfully completing this BSc, he then started his MSc in Biological Sciences with a specialization in Ecology and Evolution in 2013. During this MSc, he performed two scientific internships. The first internship was in the field of conservation genetics at the University of Amsterdam on in- and outbreeding consequences of remnant populations of the primrose *Primula vulgaris*. His second internship was at the Free University Amsterdam on potential effects of climate change on the toxic effects exhibited by metal(loid)s from mine tailings to the soil dwelling organism *Enchytraeus crypticus*. The successful completion of both internships and several courses led to a Cum Laude (with distinction) MSc degree in 2015.

In 2015, he started his PhD research on environmentally realistic ecotoxicological testing of agricultural chemicals at Leiden University, resulting in this thesis. During his PhD research, he co-developed, co-constructed and co-founded together with Dr. Maarten Schrama and Prof. Dr. Ing. Martina G. Vijver the outdoor research facility 'Living Lab', located on the Bio Science Park of Leiden University. This facility was successfully funded through a crowdfunding campaign that he managed together with Dr. Schrama and Prof. Vijver. In addition, he published several publications, taught in multiple courses and supervised a total of 11 students during their internships. During his studies and PhD, he also volunteered as a scout leader from 2010 until 2019.

In January 2020, he started his Post-doc position at the University of Amsterdam to investigate and model the spread and effects of nitrogen deposition around dairy farms and within nature reserves.

List of publications (peer-reviewed)

- 1) Musters, C.J.M., Ieromina, O., **Barmentlo, S.H.**, Hunting, E.R., Schrama, M., Cieraad, E., Vijver, M.G. and van Bodegom, P.M. (2019). Partitioning the impact of environmental drivers and species interactions in dynamic aquatic communities. *Ecosphere* 10(11), e02910.
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