Effects of pesticides on aquatic macrofauna in the field

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CHAPTER 1

GENERAL INTRODUCTION

Biodiversity in European and global context

Biological diversity is essential for the healthy state of our planet. Every species plays an important role in the biosphere and should be protected. Biodiversity adds value to our society by providing various ecosystem services and creating the basis for culture, science, and education (Wall & Nielsen, 2012).

During the last decades, humanity has faced the problem of rising demand for food, energy and fresh water. Because of a growing need for resources, natural ecosystems within the past few years have been modified to a much broader extent than before. Loss of biodiversity is significantly high and often non-reversible in many areas around the world (Millennium Ecosystem Assessment, 2005). High biodiversity loss is observed in developed countries where economic success is associated with a large threat to the natural environment (Millennium Ecosystem Assessment, 2005).

The European Union pays significant attention to issues related to biodiversity and climate change. In 2001, the EU Government started an initiative addressing the problem of nature degradation and set the objective to prevent the decline of biodiversity in Europe by the year 2010 and further (EU Biodiversity Policy, 2015). In 2006, the "*EU Biodiversity Action Plan*", confronting the problem of biodiversity loss in Europe, was introduced. This plan established specific aims towards preventing species extinctions and habitat degradation (European Communities, 2008). The objectives set in the Plan included the preservation of natural habitats, protection of farmland biodiversity, lowering pollution levels, conservation of marine and freshwater life, control over invasive species and climate change (European Communities, 2008). In 2011, the initiative on the reduction of biodiversity degradation in Europe was continued in the form of the new "*EU 2020 Biodiversity Strategy*", setting a target year of 2020 (European Commission, 2011). The new plan continued and extended the goals set in the "EU Biodiversity Action Plan" aiming at "*resource efficient and green economy*" in the European Union (European Commission, 2011).

Much attention in the EU biodiversity policy is given to the protection of freshwater biodiversity (European Communities, 2008). Freshwater occupies 3% of all water on the planet (McMichael, 2014). Accounting for 0.3% of all freshwater, surface water provides a habitat for approximately 6% of all species living on the planet (McMichael, 2014; Dudgeon et al., 2006). The biodiversity of inland waters is an important natural resource of high economic value. At the same time, freshwater biodiversity is subjected to a number of threats. At a global scale, water pollution represents an important negative factor affecting freshwater biodiversity, along with the over-exploitation of water resources, habitat degradation, species invasion, and the modification of flow regimes (Dudgeon et al., 2006). Because various types of disturbances resulting from human activities affect aquatic life, the biodiversity of fresh waters declines at a much higher rate than the biodiversity of terrestrial or marine ecosystems (Ricciardi & Rasmussen, 1999; Strayer & Dudgeon, 2010).

Water quality in the Netherlands

Being the sixth smallest country in Europe by land area (Centraal Bureau voor de Statistiek, 2004), the Netherlands occupies second place in the world by the amount of exported agricultural products (behind the USA in first place) (Netherlands Enterprise Agency, 2013). At the same time, a special feature of the Netherlands is that it is an extremely water-rich country. The total length of ditches in the lower parts of the Netherlands is approximately 300.000 km (Higler, 1989). The necessity of having such intense water coverage is explained by the location of the country: a large area of the Netherlands lies below sea level (Rijkswaterstaat, 2011). A continuously functioning system of ditches and canals connected to pumping stations helps to control water levels and protect the country from floods. In addition, the ditch systems ensure irrigation and drainage of agricultural fields. The first ditch systems were built in the middle of the XIII century, the period corresponding to the beginning of agricultural development in the country (Highler, 1989; Wolff, 1993). Since that time, polder areas represent the most common type of landscape in the Netherlands and drainage ditches are the dominant aquatic ecosystems.

Dense water coverage creates a very special situation in the country in terms of water management. The water is pumped in and out of the ditches constantly, depending on the levels of precipitation and evaporation. This creates a dynamic environment in ditches in terms of hydrology, even though the speed of the water flow in ditches is relatively low. Management activities at the ditch banks (mowing) and removal of aquatic vegetation excess from the ditch (dredging) makes ditches highly disturbed ecosystems. In addition, ditch systems are located in intensively used agricultural areas occupied by pastures and different types of crops. Intense farming close to interconnected ditch systems is often coupled with high pesticide levels in surface waters (Vijver et al., 2008). At the same time, the protection of aquatic biodiversity in inter-connected water systems is very challenging. After all, ditch systems are not isolated bodies of water, and hence environmental managers should control the upstream and downstream reaches, as well as the adjacent land (Dudgeon et al., 2006).

The importance of water quality protection in agricultural areas, where pesticides and fertilizers are used intensively, is highlighted in different environmental policies at the European and national levels. The correct use of plant protection products is considered a very important issue in Europe, starting from the authorization process and ending with the control of pesticide residues in surface waters and - if needed – the development of mitigation strategies. To manage the use of plant protection products accordingly, the EU has developed a joint legislation that applies to all EU Member States: "*Regulation (EC) No 1107/2009 concerning the placing of plant protection products on the market*." The aim of this legislation is to promote consistency in Europe with regard to the use of agricultural chemicals. The final goal of the regulation is to minimize the individual differences in risk assessment of chemicals between EU Member States, so that the legislation in one country can also be recognized in another Member State.

The national government of the Netherlands pays significant attention to the quality of surface waters (Government of the Netherlands, 2015). The local water managers monitor water quality and quantity in the Netherlands (Rijkswaterstaat, 2011). The Dutch office, managing the plant protection and biocidal products, is represented by the CTGB – "College voor de toelating van gewasbeschermingsmiddelen en biociden" (CTGB, 2015). To carry out the impact assessments, the CTGB has developed the Evaluation Manuals. These manuals cover various aspects related to the properties of plant protection products, their fate and the effects on the environment and human health. All tests included in the manuals should be performed according to the standard guidelines, and all evaluation criteria should be met before the product enters the market. The procedure of tiered risk assessment is followed, that begins with the toxicity assessment by simple conservative laboratory tests, and only proceeds to a more complex test setting (higher-tiered assessment) if more information on chemical toxicity is needed. Higher-tiered assessment (toxicity tests with wide ranges of species, microcosm and mesocosm experiments, or test settings in the field) is relatively more expensive than laboratory tests, but provides ecologically relevant information on chemical toxicity enabling field-relevant predictions to be made.

Biodiversity of ditches in the Netherlands

One of the important reasons for such strict monitoring and control over pesticide use in the Netherlands is the fact that agro-ecosystems in the country still contain high biodiversity (for instance, plants, pollinator insects, birds), essential not only for the healthy functioning of crops, but also for the overall high value of the area in terms of species diversity. Many aquatic plant and animal species find their habitats in drainage ditches (Verdonschot, 2012a; Herzon & Helenius, 2008). The number of aquatic invertebrate species in ditches is relatively high even though ditches are affected by physical and chemical disturbances: aquatic biodiversity in ditches was shown to be comparable to that in small lakes (Verdonschot, 2012a). Moreover, the structural composition of aquatic biota influences the hydrological functions of the ditches. For instance, aquatic plants in ditches can reduce the water flow, which increases the retention and degradation of pollutants (Beltman et al., 2004). Aquatic invertebrates represent an important component of the aquatic food web and take part in biochemical cycles in aquatic ecosystems (Herzon & Helenius, 2008; Kristensen & Kostka, 2005).

Despite a large contribution of aquatic biota in ditches to the overall biodiversity of agroecosystems in the Netherlands, to our knowledge not many studies focused on macrofauna inhabiting ditch systems. For instance, the earlier research of Scheffer et al. (1984) and Higler & Verdonschot (1989) identified patterns in the invertebrate community composition in ditches in relation to habitat structure (vegetation structure). The study of Canters et al. (1989) addressed aquatic fauna in ditches of the Netherlands. Higler (1989) described the general trends in water chemistry parameters in ditches. A more recent study of Verdonschot et al. (2012a) described the taxonomic composition of fauna and flora of ditches

in the Netherlands in comparison to that of small lakes. In the other work of Verdonschot et al. (2012b), the relationship between the invertebrate community composition and the structure of aquatic macrophytes was investigated. Verdonschot (2012c) also performed a comprehensive study of ecological processes and biodiversity in ditches.

Compared to the relatively small amount of research on aquatic fauna in ditches, a much larger number of studies have investigated the ditch bank vegetation in the Netherlands, and the effects of associated management practices on the ditch bank vegetation (for instance, Best et al., 1992; Leng et al., 2011ab; Noordijk et al., 2011; Dijk et al., 2013; Blomqvist et al., 2006; Whatley et al., 2014 and others).

Impact of pesticides on biodiversity in ditches

Being indispensable components of Dutch polders, the ditch systems constantly receive chemical loadings from the surrounding agricultural fields (amongst all, pesticides and nutrients). Pesticides can enter the aquatic environment through different pathways. Pesticides are in most cases applied in agricultural fields by spraying. After spraying, pesticides undergo several processes: volatilization, spray drift and adsorption by the crop and soil (Van Linden et al., 2008). The largest proportion of pesticides sprayed is assumed to end up on soil and crops. Pesticides deposited onto soil can leach through the root zone into the surface and ground water (Van Linden et al., 2008). This leaching, together with the inflow from field drainage systems, leads to the contamination of surface and ground waters (Van Linden et al., 2008). The direct drift of pesticides to water systems during the spraying process accounts for the largest percentage of pesticide emissions to the environment in the Netherlands (up to 70%) (De Zwart, 2003). Elevated concentrations exceeding the environmental quality standards are found in many ditches and larger waterbodies in the Netherlands (Vijver et al., 2008).

Such high chemical input creates unfavorable conditions for ditch fauna because many invertebrate and vertebrate species living in ditches are highly sensitive to pesticides. It was shown in previous research conducted in controlled settings that pesticides may affect population density, reproduction rate, and the birth and mortality rates of invertebrates (Hanazato, 2000). At the ecosystem level, pesticides may produce changes in the structure of aquatic communities. Hanazato (2000) observed such effects of pesticides on aquatic ecosystems, as the lengthening of the food chain accompanied by lowered energy transport between different components of the food web. Several studies focused on the effects of chemicals on aquatic invertbrates in semi-field conditions (for instance, Van den Brink, 1996; Wijngaarden et al., 1996, 2004). In semi-field experiments of Van Wijngaarden et al. (2004) pesticide mixtures (applied at the concentrations of up to 5% of the spray drift emission) did induce negative effects on invertebrate communities in ditches. However, to our knowledge no study other than ours focused on the effects of pesticides on aquatic macrofauna in the field drainage ditches of the Netherlands.

Flower growing in the Netherlands and research area

The flower bulb growing region located in the province South Holland (the Netherlands) that represents a highly productive agricultural area. Flower cultivation makes the country famous around the world for its beautiful flowers and other floricultural products (such as bulbs, potted plants, foliage) (Dinham, 2008). Floricultural production occupies a large part of the agricultural land in the Netherlands (Dinham, 2008).

Flower diseases induced by pests and the growth of weeds are controlled by pesticides, including insecticides, herbicides and fungicides (Jansma et al., 2002). As a result of environmental policy aiming to diminish the use of plant protection products in the Netherlands by the year 2010, the use of chemicals in agriculture has lowered two fold already by the year 2000 when compared to the previous two decades (Van Eerdt, 2007). Consequences of such policy measures were the reduction of chemical emissions and overall improvement of environmental quality (Van Eerdt, 2007). For instance, the percentage of pesticide measurements in surface water exceeding MTR (Maximum Tolerable Risk) decreased by 75% between 1988 and 2009. The percentage of locations at which msPAF (Potentially Affected Fractions of species) was higher than 5%, diminished by 58% during the same period (De Snoo & Vijver, 2012). In the subsequent years, however, an increase in pesticides use was observed again (De Snoo & Vijver, 2012). Even though, as a general trend, the use of chemicals on the national scale reduced during the previous decades, it should be noted that the amounts of chemicals applied varies greatly between the crops. The amount of pesticides applied in bulb crops in 2012 was 54.4 kg/ha (Centraal Bureau voor de Statistiek, data from 2014). This amount was much higher when compared to the other crops. For instance, in 2012 pesticide use in champignon and glass house vegetable growing was lower than in bulb crops: 0.3 kg/ha and 12.1 kg/ha respectively (Centraal Bureau voor de Statistiek, data from 2014). Only in rose, chrysanthemum and lily fields, the amount of pesticides applied in 2012 was higher than in bulb crops (106.2 kg/ha, 70.7 kg/ha and 134.6 kg/ha, respectively) (Centraal Bureau voor de Statistiek, data from 2014).

In addition to pesticides, nitrogen- and phosphorus- containing fertilizers are also applied extensively in flower fields (Jansma, 2002). The total use of nitrogen-containing fertilizers in the Netherlands in 2013 was 8 million kg, compared to 11 million kg used in 2000. The amount of phosphorus-containing fertilizers applied in flower fields was lower: 3 million kg applied in 2013 compared to 4 million kg applied in the year 2000 (Centraal Bureau voor de Statistiek, data from 2015). Even though an overall reduction of fertilizer use has been observed in the country, nutrients applied at the agricultural fields can enter surface waters and cause various effects to aquatic life. Janse & Van Puijenbroek (1998) found that excessive nutrient loads in ditches produced shifts in the structural composition of aquatic macrophytes. Thus, a shift from submerged macrophytes towards floating macrophytes dominated by *Lemna sp.* was observed (Janse & Van Puijenbroek, 1998). Such shifts initiate a chain of consequences to aquatic life: duckweed dominance causes

shading and reduces light availability for algae and invertebrates. Excessive algae growth leads to lowered dissolved oxygen concentrations. This results in direct and indirect effects to aquatic faunal organisms. Nutrients therefore constitute an important factor likely to affect aquatic biota in addition to pesticides.

The research area selected for the current study covered polders located in the flower bulb growing area of the Netherlands. The area represents a typical example of agricultural area, where different farming activities are performed throughout the year. All crops in the research area are grown in a close proximity (approximately 25 cm to 2 m) to ditches. In addition, the research area is covered with open crops (while most of flowers, e.g. 69% are grown in glass houses) (Dinham, 2008). Therefore, chemicals applied in the fields can enter water systems directly after spraying through spray drift. In addition, watersheds in the nature reserve next to the polder area were sampled as control sites.

Context dependency concept

As an independent field of science, ecotoxicology aims to identify the effects of toxicants on the environment across different levels of biological organization: sub-organisms, organism, populations, communities, and ecosystems. Understanding the effects of toxicants on the higher levels of biological organization is associated with high uncertainty because toxicants explain only a part of the overall variation in communities, while the remaining variation can be attributed to other natural abiotic and biotic factors, intrinsic to ecosystems. Clements et al. (2012) introduced the theoretical framework of the context-dependency approach in ecotoxicology, which introduces abiotic and biotic factors into the assessment of toxicant effects on communities.

In relation to pesticides, previous studies showed that factors, such as the type of ecosystem, location, weather, and environmental conditions, might all affect the toxicity of pesticides in the aquatic environment (Maund et al., 1999). The importance of the food web structure in shaping responses of aquatic invertebrates to toxicants was also demonstrated by several studies. For instance, the interaction of organisms within one population was found to be an important factor affecting the responses of the Trichoptera populations to the pesticide fenvalerate (Liess, 2002). Beketov & Liess (2006) found that predation pressure intensified the adverse effects of esfenvalerate on the zooplankton species *Artemia sp.* A similar result was observed in the study of Liess (2013), in which the aquatic insect *Culex pipiens* was more sensitive to the insecticide thiacloprid under predating pressure than without the presence of the predator. Species interactions, such as predation or competition, were shown to be important factors affecting the responses of aquatic invertebrates to pesticides in several studies, for instance, in Trekels et al. (2010) and in Foit et al. (2012).

The sensitivity of organisms to pesticides and their potential to recover from toxic stress is largely determined by the species functional characteristics (species traits) (Pof, 1997). The trait-based approach in community ecology was introduced a few decades ago.

The species trait approach was used to study the effects of various types of disturbances (for instance, farming types, metal pollution, cargo ship traffic, eutrophication and climate change, land use, and nutrient pollution) on invertebrate communities (Magbanua et al., 2010; Statzner et al., 2010; Vandewelle et al., 2010; Doledec, 2006). Recently, the species trait approach was recognized as an important tool in ecotoxicology. Baird et al. (2008) introduced the concept of TERA (trait-based ecological risk assessment). Rubach et al. (2011) demonstrated the potential of the species trait approach in ecotoxicological research. With regard to pesticides, the studies incorporating the species trait approach into pesticide effect assessment are scarce. For instance, Liess & Van der Ohe (2005) introduced the SPEAR index to identify the effects of pesticides on invertebrate communities. This index includes the species traits that determine the sensitivity of species to pesticides: generation time, dispersal ability, the presence of aquatic life stage and its sensitivity to toxicants (Liess & Van der Ohe, 2005). In the research of Rubach et al. (2010), the sensitivity of the arthropoda taxa was related to concentrations of organophosphate, carbamate and pyrethroid pesticides using species traits. Even though several initiatives aiming to link pesticides and species traits of aquatic invertebrates were undertaken, more research is needed to understand the effects of pesticides on the species trait composition of aquatic biota in the field, taking into account multi-stressor conditions of natural ecosystems.

Thesis aims

The overall aim of the thesis was to study the effects of pesticides on aquatic macrofauna in the field. There is a large amount of research addressing the effects of pesticides on aquatic species under control or semi-field conditions. Yet, the studies focusing on pesticide effects on aquatic biota in the complex field setting are scarce. Ditch systems located in the agricultural area of the Netherlands represent an example of highly dynamic aquatic ecosystems influenced by chemical and physical disturbances, and provide a good setup to study the effects of pesticides on aquatic biota in the field.

The main aims of research were:

1. To study the temporal variation in pesticide concentrations and macrofauna diversity in ditches of the flower growing region of the Netherlands over the period 1975 - 2010
2. To quantify what proportion of the total variance in the community composition of aquatic macrofauna can be explained by pesticides, environmental factors (nitrate, nitrite, phosphate, dissolved organic carbon, dissolved oxygen, temperature, and macrophyte coverage), presence of other biota and time, and to investigate the predictive power of the species trait approach in quantifying pesticide effects on aquatic macrofauna in the field
3. To study the effects of pesticides combined with environmental factors on aquatic invertebrates exposed in situ in ditches bordering flower bulb fields
4. To study the combined effects of the insecticide imidacloprid and nutrient limitation on the aquatic invertebrate *Daphnia magna*

Thesis outline

Chapter I. In this chapter, the major threats to freshwater biodiversity are discussed. An overview of the environmental issues in the Netherlands is given, with respect to water quality and agricultural practices (flower growing in particular). The importance of aquatic biodiversity with regard to nature conservation and ecosystem functioning, and the adverse effects of pesticides on aquatic ecosystems are discussed. An overview of the current challenges in risk assessment of pesticides (context dependency of pesticide effects and species trait considerations) is given.

Chapter II. In this chapter, annual trends in pesticide concentrations in surface waters (expressed as toxic units and concentrations of individual pesticides) and aquatic macrofauna diversity (expressed as Shannon diversity index) in ditches of the flower growing area of the Netherlands over the period 1975 - 2010 are analyzed.

Chapter III. In this chapter, the effects of pesticides on freshwater macrofauna are investigated, taking into account environmental factors, the presence of other biota and temporal variation. To this purpose, field work was performed in the intensively used agricultural area (the flower bulb growing region of the Netherlands). Sampling and taxonomic identification of aquatic macrofauna, measurements of water chemistry parameters and pesticide concentrations in ditches bordering flower bulb fields was carried out during two consecutive years. A variance partitioning procedure, based on the partial redundancy analysis (pRDA), was applied to divide the total variance in macrofauna community composition into the variance explained by pesticides, environmental factors (nitrate, nitrite, phosphate, dissolved organic carbon, dissolved oxygen, temperature, and macrophyte coverage), the presence of other biota, time (seasonal and annual variation), shared variance between different factors and unexplained variance.

Chapter IV. In this chapter, the potential of species trait approach in quantifying the effects of pesticides on aquatic macrofauna in the field is investigated. To this aim, macrofauna data previously collected in the field was analyzed (described in Chapter II). Each macrofauna taxon was classified according to the 54 trait modalities of nine species traits. After that, a variance partitioning procedure was applied to divide the variance in trait community composition into the variance explained by pesticides, environmental factors, time, shared variance between different factors, and unexplained variance. In addition, redundancy analysis (RDA) was performed to identify the relationships between species trait modality distributions, pesticides and environmental factors.

Chapter V. In this chapter, population responses of *Daphnia magna*, *Chydorus sphaericus* and *Asellus aquaticus* to pesticides in contaminated ditches around bulb fields were studied, taking into account environmental factors (nitrate, nitrite, phosphate, dissolved organic carbon, dissolved oxygen, and temperature). The responses of aquatic invertebrates to pesticides and environmental factors were ivestigated by means of in situ bioassays deployed in ditches adjacent to flower bulb fields.

Chapter VI. In this chapter, the responses of *Daphnia magna* to the insecticide imidacloprid in combination with food quality levels (expressed as the carbon: phosphorus ratio of algae cells) were investigated. To study the combined effects of imidacloprid and food limitation, *D. magna* juveniles exposed to different concentrations of imidacloprid were supplied with algae of varying nutritional quality.

Chapter VII. In this chapter, the results of the thesis are discussed within the scientific and social context. The implication of the study results to water management and environmental policy is highlighted.

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CHAPTER 2

TEMPORAL VARIATION IN PESTICIDE CONCENTRATIONS AND FRESHWATER MACROFAUNA DIVERSITY IN DITCHES OF THE FLOWER GROWING AREA OF THE NETHERLANDS

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Introduction

The Netherlands is known worldwide for flower production. In 2011, the total export of horticultural products from the Netherlands (including seeds, ornamentals, plants, vegetables, fruits, nuts, spices, vegetables and fruits) reached 20.9 billion Euro (Centraal Bureau voor de Statistiek, 2012). The export of ornamentals (that includes flowers and flower bulbs), and other plants in 2011 accounted for 8.1 billion Euro (Centraal Bureau voor de Statistiek, 2012) and was one of the largest in the world. To ensure good quality of floricultural products, pesticides and fertilizers are applied in flower fields to enrich the soil, and control pests and weeds. The Netherlands is also a water-rich country covered with ditches and canals. These water systems possess a relatively high aquatic biodiversity. Intensive agriculture in close proximity to these open water systems creates difficulties in the control and management of surface water quality.

The national-level monitoring of pesticide levels in surface waters is conducted by water management organizations. The collected data is further evaluated in the so-called Pesticide Atlas (see http://www.bestrijdingsmiddelenatlas.nl/) (De Snoo et al., 2006). This tool visualizes the locations where pesticide concentrations were measured and allows comparison of actual pesticide concentrations with various water quality standards, analysis of time trends, and relating pesticide data to land use types.

The pesticide levels in surface waters often exceed the Maximum Permissible Concentration (MPC) in ditches located in the flower growing area of the Netherlands (province South Holland) (Vijver et al., 2008; De Snoo & Vijver, 2012). However, as an overall trend, the surface water quality in the Netherlands with respect to pesticides tends to increase, even though at many sites high pesticide concentrations are still found (Vijver et al., 2008). Whether this overall improvement of the water quality is reflected in the actual performance of aquatic biota in ditches, has not yet been investigated.

In the current study, we aimed to analyze the long-term trends in pesticide concentrations (over the period 1975 - 2010) and aquatic macrofauna diversity (over the period 1983 - 2010) in ditches of the flower growing area of the Netherlands. The research questions were: 1) did water quality in the flower growing area of the Netherlands improve over the previous decades with respect to pesticide residues in surface water? 2) did aquatic macrofauna diversity in ditches increase over time? To address the research questions, we analyzed a database obtained from the Water Board Rijnland. The database included concentrations of pesticides and macrofauna diversity data collected in the flower growing area. We hypothesized that pesticide levels decreased over the previous decades because chemicals with more specific modes of action have been produced by the chemical industry, so that pesticides can be applied at lower concentrations. In addition, less persistent chemicals have been produced in the last decades. We also hypothesized that macrofauna diversity increased over the previous decades, as a result of the improved water quality (reduced pesticide concentrations in surface waters) and all policy measures undertaken to control and reduce pollution of surface waters.

Materials and methods

Description of database

A database containing pesticide concentrations and macrofauna diversity in ditches of the flower growing area of the Netherlands (province Southern Holland) was obtained from the Water Board "Hoogheemraadschap Rijnland" (Leiden, the Netherlands). The area monitored by the Water Board lies mostly on sandy soil, with a smaller patch of light clay and peat, according to the soil maps presented in "Grondsoortenkaart" 2006 - Simplified Soil Map of the Netherlands (Wageningen UR – Alterra, 2006). The majority of sampling sites where pesticides were measured are located in the flower growing area (characterized by sandy and light clay soil types). In the current study, pesticide data only from the flower growing area was analyzed. Sampling sites where macrofauna data was collected are located in the flower growing area, peat and nature reserve areas (characterized by sandy soil type). In the current study, macrofauna data collected in the flower bulb growing area and the nature reserve was analyzed.

Pesticides data

Pesticide concentrations were measured at 92 locations in the research area over the period 1975 - 2010. The overall number of pesticides (including pesticides and their degradation products) analyzed was 109 and the total number of pesticide measurements done was 33560. Table 1 contains the list of pesticides analyzed, their EC50 values (derived in a 48 hour test with *D. magna*, endpoint immobility), time periods at which pesticides were measured in water samples, and the total number of measurements done for each pesticide.

Table 1. List of pesticide measured in water samples, with corresponding EC50 values (based on immobility test with *D. magna* 48 h) and reference to EC50 values.

Name of the compound	EC50, µg/L	Reference	Measurement years	Number of measurements
1.2 dichloropropane	55900	OECD SIDS (2003)	1987-1992	57
2.4 dichlfeenazijnzuur	*N*[4]	*N*	1988-1995	63
2.meth4chlfenazijnzuur	*N*	*N*	1996-2002	133
2.meth4chlfenboterzuur	*N*	*N*	2003-2006	124
2.meth4chlfenpropionzuur	*N*	*N*	1991-2004	131
2-nitrophenol	210[5]	EPA (1980)	1998	2
3-methyl-4-nitrophenol	12000	OECD SIDS (1994)	1997-1998	10
aldicarb	420	PPDB	1990-2010	264
aldicarb-sulfon	250	Reference 1[7]	1999-2010	261
aldicarb-sulfoxide	800	Reference 1[7]	1999-2002	78
adrin	28	PPDB	1980-2005	417

Table 1. List of pesticide measured in water samples, with corresponding EC50 values (based on immobility test with *D. magna* 48 h) and reference to EC50 values. (*Continued*)

Name of the compound	EC50, µg/L	Reference	Measurement years	Number of measurements
aminomethylphosphonic acid	691000	Traas & Smit (2003)	2001-2005	192
atrazine	85000	PPDB	1990-2010	187
bentazone	64000	PPDB	1994-2004	166
Benzothiazole	19	Reference 2[8]	1997	2
bitertanol	4460	PPDB	2002-2010	79
butocarboxim	3200	PPDB	1999-2002	78
butocarboximsulfoxide	*N*	*N*	1999-2002	78
captafol	3340	PPDB	1999	12
captan	7100	PPDB	1987-1999	105
carbaryl	6.4	PPDB	1999-2002	78
carbendazim	150	PPDB	1987-2010	1990
carbofuran	9,4	PPDB	1997-2010	342
chlorbromuron	5800	PPDB	2003-2010	58
chlorfenvinphos	0.25	PPDB	1995-2010	293
chlorpropham	2600	PPDB	1992-2010	297
chlorothalonil	84	PPDB	1994-2010	93
chloridazon	132000	PPDB	1990-2010	1595
chloroxuron	2950	PPDB	2001-2010	61
cyanazine	49000	PPDB	1998	1
DDD[1]	9	PPDB	1980-2005	383
DDE[2]	1	PPDB	1980-2005	384
DDT[3]	5	PPDB	1980-2005	384
o.p'-DDD	9	PPDB	2001-2005	17
p.p'-DDD	9	PPDB	2001-2005	17
o.p'-DDE	1	PPDB	1983-2005	29
p.p'-DDE	1	PPDB	2001-2005	18
o.p'-DDT	5	PPDB	2001-2005	18
p.p'-DDT	5	PPDB	2001-2005	18
desethylatrazine	5100[6]	Ralston-Hooper et al. (2009)	1995	1
dichlobenil	6200	PPDB	1980-2004	462
1.3-dichloropropene	3600	PPDB	1991-1992	16
dichlorvos	0.19	PPDB	1990-2010	236
dieldrin	250	PPDB	1980-2005	416
diethyltoluamide	75000	PPDB	2001-2010	198
difenoconazole	770	PPDB	2001-2002	2
dimethoate	2000	PPDB	1987-2010	119

Table 1. List of pesticide measured in water samples, with corresponding EC50 values (based on immobility test with *D. magna* 48 h) and reference to EC50 values. (*Continued*)

Name of the compound	EC50, µg/L	Reference	Measurement years	Number of measurements
dinoseb	240	PPDB	1990-1997	5
diuron	5700	PPDB	1999-2010	1370
dinitrosol	1100	PPDB	1990-2010	76
alpha-endosulfan	440	PPDB	1980-2005	340
endrin	4.2	PPDB	1980-2005	417
ethiofencarb	220	PPDB	1999-2010	132
ethofumesate	14000	PPDB	2001-2010	166
ethylenethiourea	21600	PPDB	1992-1993	21
fenamiphos	1.9	PPDB	1999-2010	56
fluazinam	220	PPDB	1998-1999	13
flutolanil	6800	PPDB	1997-2010	1668
folpet	680	PPDB	1999	12
furalaxyl	39000	PPDB	1999-2004	16
glyphosate	40000	PPDB	2001-2005	192
heptachlor	42	PPDB	1980-2005	350
heptachlor epoxide	240	PPDB	1980-2005	350
hexachlorobenzene	500	PPDB	1975-2005	552
alpha-hexachlorocyclohexane	1000	IPCS INCHEM (1991)	1975-2005	491
beta-hexachlorocyclohexane	500	IPCS INCHEM (1991)	1975-2005	491
gamma-hexachlorocyclohexane	1600	PPDB	1975-2005	560
HTI	*N*	*N*	1996-1997	28
imazalil	3500	PPDB	1999-2010	185
imidacloprid	85000	PPDB	1999-2010	1051
iprodione	660	PPDB	1997-2010	183
isoproturon	580	PPDB	2001-2010	335
lenacil	8400	PPDB	1997-2010	55
linuron	310	PPDB	1998-2010	234
metalaxyl	28000	PPDB	1999-2008	52
metamitron	5700	PPDB	2004-2010	1076
methabenzthiazuron	30600	PPDB	2002-2007	3
methiocarb	8	PPDB	1999-2010	261
methiocarb methoxy sulfone	180000	PPDB	1999-2010	261
methomyl	7.6	PPDB	1999-2010	261
methyl isothiocyanate	76	PPDB	1990-1998	73
metolachlor	23500	PPDB	1997-2008	2
metoxuron	215600	PPDB	1997-2010	314

Table 1. List of pesticide measured in water samples, with corresponding EC50 values (based on immobility test with *D. magna* 48 h) and reference to EC50 values. (*Continued*)

Name of the compound	EC50, µg/L	Reference	Measurement years	Number of measurements
oxamyl	319	PPDB	1997-2010	263
2,3',4,4',5-pentachlorobiphenyl	*N*	*N*	1980-1987	45
parathion-ethyl	2,5	PPDB	2001-2008	101
parathion-methyl	7.3	PPDB	1987-2010	230
pencycuron	300	PPDB	1999-2005	103
permethrin	0.6	PPDB	1999	1
pirimicarb	17	PPDB	1995-2010	199
pirimiphos-methyl	0.21	PPDB	1997-2010	292
prochloraz	4300	PPDB	1992-2008	342
procymidone	1800	PPDB	1994-2008	297
propham	23000	PPDB	1997	3
propachlor	7800	PPDB	1997-2002	16
propoxur	150	PPDB	1992-2010	325
propyzamide	5600	PPDB	2001-2010	81
prosulfocarb	510	PPDB	1999-2004	110
pyrimethanil	2900	PPDB	1999-2002	9
simazine	1100	PPDB	1990-2010	359
tebuconazole	2790	PPDB	2004-2008	7
terbuthylazine	21200	PPDB	1997-2010	68
thiram	11	PPDB	1990-1992	16
tolclofos-methyl	48000	PPDB	1987-2010	384
toluenesulfonamide	210000	OECD SIDS (2002)	1997-2004	2
tolylfluanid	190	PPDB	2001-2004	101
tri-allate	91	PPDB	2001	1
trichostatin	*N*	*N*	1998	1
vinclozolin	3650	PPDB	1987-2010	238

[1] DDD - dichlorodiphenyldichloroethane
[2] DDE - dichlorodiphenyldichloroethylene
[3] DDT - dichlorodiphenyltrichloroethane
[4] *N* - EC50 was not found
[5] EC50 value derived in 24 h test with daphnids
[6] EC50 value derived in 96 hour test with *Hyalella Azteca*, age below 7 days, test conditions: freshwater, temperature 25 ºC
[7] Ministerie van Landbouw, Natuurbeheer en Visserij (1998)
[8] Ministry of the Environment (Environmental Health Department, Environmental Risk Assessment Office)

Because different pesticides were measured in water samples during the period 1975 - 2010, the annual variation in concentrations of individual pesticides over this time period could not be analyzed. To analyze the change in pesticide levels in surface waters over the period 1975 - 2010, toxic units (TU) were calculated for each sample. TU were calculated as follows:

$$\sum_{i=1}^{n} TUi = \frac{\mathrm{Ci}}{EC50, D.magna},$$

Where, TU_i is the toxic unit of the pesticide *i, Ci* - is the concentration (µg/L) of the pesticide *i*; and EC50 - the corresponding Effect Concentration (48 hours) of *D. magna* exposed to substance *i* (µg/L). When the concentration of a pesticide was below the limit of detection, half of the detection limit was used in the analysis. We used EC50 values because those values could be made available for 101 out of 109 compounds. We know that NOEC (No Observed Effect Concentration) for *D. magna* derived in a 21 day test is a more sensitive parameter for TU calculation, but only a limited NOEC data was found in literature (59 out of 109 compounds). Logarithms of toxic units (base 10) for each sample were then plotted versus time. The total number of compounds analyzed each year, the number of compounds analyzed per sample, and the total number of samplings per year were also calculated and plotted versus time. The relationship between the toxic units and the number of pesticides measured per sample was analyzed by means of regression analysis. Additionally, to account for the different number of pesticides measured over the years 1975 - 2010, TUs were normalized by the number of pesticides measured per sample.

As a next step, sampling locations at which log TU exceeded zero (meaning that the concentration of at least one pesticide was higher than the EC50 value and therefore provided a potential risk to aquatic biota) were identified and analyzed separately. Pesticides contributing mostly to TU exceedances were identified, and concentrations of these individual pesticides were plotted versus time. In addition, pesticides mostly measured in samples (measured more than 250 times), measured until (and including) the year 2010, with concentrations exceeding detection limits in at least 15% of the measurements, were identified. Concentrations of these pesticides were plotted versus time and analyzed with linear regression. The authorization dates of these pesticides were retrieved and added to the graphs.

Macrofauna data

The macrofauna dataset covered the period 1983 - 2010. The total number of macrofauna samples collected over time was 84: 74 samples were collected in the ditches of the flower bulb area and 10 samples were collected in the nature reserve. We calculated the Shannon diversity (H) index for macrofauna collected in two areas according to the formula:

$$H' = \sum_{i=0}^{n} pi \times \ln(pi),$$

where p*i* is the proportion of species *i* relative to the total number of species (p_i). The relationship between Shannon diversity index and time was analyzed with linear regression, separately for macrofauna collected in flower bulb growing area and nature reserve. The difference in Shannon diversity indices between the flower growing area and the nature reserve was analyzed with the t-test assuming the equal variance.

Most of the macrofauna and pesticide data were collected not consistently over time and sampling site. For this reason, causal relationships between pesticide levels in ditches and macrofauna diversity could not be analyzed.

Non-metric Multi-Dimensional Scaling (MDS) based on Bray-Curtis similarity matrix was applied to macrofauna species data to visualize similarities in macrofauna species composition between the different years. All macrofauna samples were divided in five groups according to the sampling periods: 1975 - 1979, 1980 - 1985, 1986 - 1990, 1991 - 1999, 2000 - 2010 (these time intervals corresponded to the main periods of TU change over time). The difference in macrofauna species composition between the groups of samples was tested by the analysis of similarity (ANOSIM) test. Before the analysis, macrofauna data were log(x+1) transformed. Multivariate analysis was performed in PRIMER Software (Clarke & Gorley, 2006).

Results and discussion

Annual variation in pesticide concentrations

The total number of pesticides analyzed in water samples per year increased over time and reached a maximum of 60 - 80 in 2002 - 2005, compared to 4 compounds measured in 1975 - 1979 (Figure 1A). The number of pesticide samples analyzed per year increased simultaneously with the number of compounds analyzed (Figure 1B): from 5 - 50 compounds analyzed in 1974 - 1998 until 200 - 250 analyzed in 2000 - 2006. This means that in 2000 - 2006 the probability to detect high pesticide concentrations in water was higher than in 1974 – 1998, due to the higher measurement frequency. The number of pesticides measured per sample also tended to increase over time with a maximum observed in 2000 - 2004. However, during the years 2000 - 2010 this number varied between 5 and 50 (Figure 1C).

Figure 1D shows toxic units plotted versus time. The lowest TUs were found in 1975 - 1980. Nevertheless, this statement should be considered with care because in 1975 - 1979 only four pesticides (hexachlorobenzene, alphahexachlorocyclohexane, beta hexachlorocyclohexane and gamma-hexachlorocyclohexane) were analyzed in water samples (Figure 1D). In 2006 – 2010, the TU largely remained below zero. This means that pesticide concentrations did not exceed the EC50 for *D. magna*.

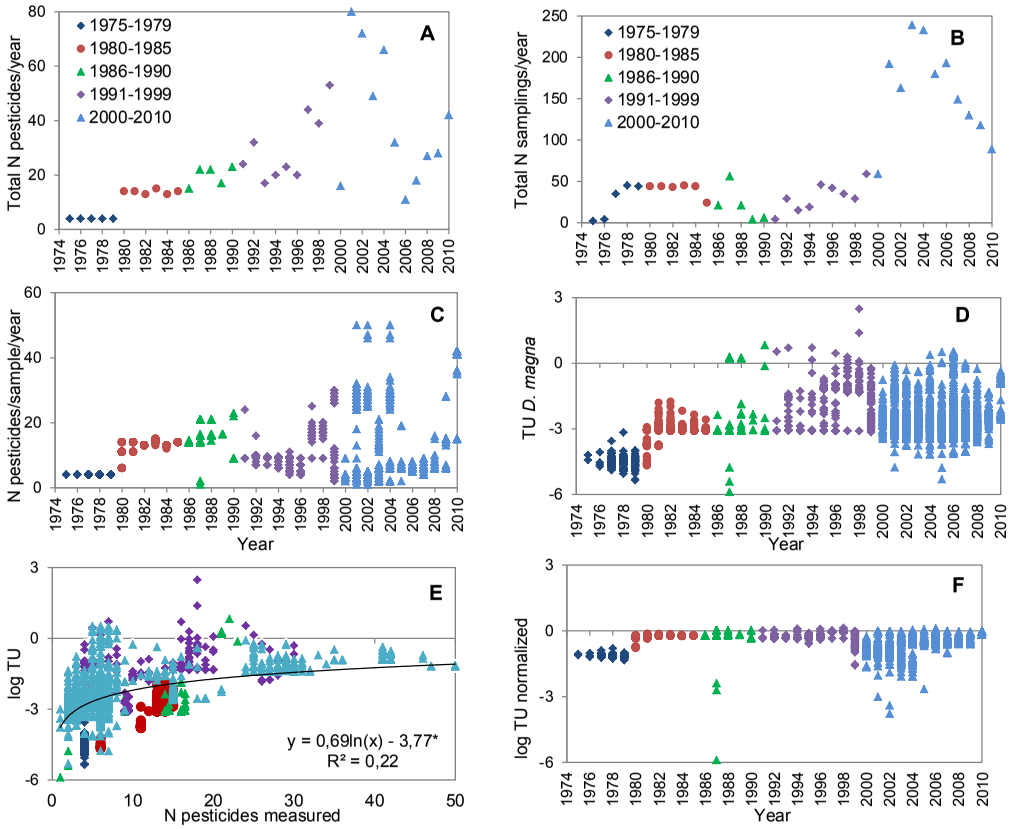

Figure 1. Total number (N) of pesticides measured in water samples per year (A), total number of samplings per year (B), number of pesticides measured in one sample per year (C), Toxic Units (D) plotted versus time, regression analysis between toxic units and the number of pesticides measured per sample (E), Toxic Units normalized by the number of pesticides measured per sample plotted versus time (F) * statistically significant at $p<0.05$

TU values in 1980 - 1987 were two log units higher than in 1975 - 1980. Starting from the year 1980, highly toxic pesticides (to *D. magna*) were measured in water samples, along with other less toxic compounds. Such toxic pesticides included DDT and its degradation products (DDE and DDD), eldrin, pirimicarb, dichlorvos (starting from year 1990), chlorfenvinphos (starting from 1995), pirimiphos-methyl and carbofuran (starting from 1997), permethrin, fenamiphos, carbaryl and methomyl (Table 1). The presence of these compounds in water samples starting from the year 1980 has led to a significant increase in TU values in the years 1980 - 1981 (Figure 1D). However, even though EC50 values of these compounds are very low (vary in the range 0.19 µg/L – 9.4 µg/L), their individual concentrations in surface waters rarely exceeded the EC50 values. The highest values of TU were found at a number of locations in 1987 - 1997 and 2003 - 2007. Pesticides that contributed mostly to the high TU (exceeding 1) were pirimiphos-methyl, carbendazim (in years 1987 - 2007), dichlorvos and chlorfenvinphos (in years 1994 - 1996). Among these pesticides, pirimiphos-methyl was one of the pesticides found at high concentrations in surface waters on the national scale in years 2003 - 2004, and was mainly linked to land use types of floriculture and greenhouse production (Vijver et al, 2008).

Most of the TU exceedings in the current study were observed in the periods 1987 - 1997 and 2003 - 2007. When pesticides contributing to TU exceedances were analyzed on an individual compound basis, it was found that concentrations of pirimiphos-methyl and carbendazim in surface waters of the study area revealed a significant negative trend over time (Figure 2). Concentrations of dichlorvos and chlorfenvinfos were not plotted versus time because most of their concentrations (up to 94%) remained below the limit of detection.

Concentrations of the other frequently measured pesticides revealed a significant declining trend over time (for instance, tolclophos-methyl, imidacloprid, chloridazon, isoproturon, chlorpropham, diuron, simazine, metoxuron). Concentration of prochloraz did not change significantly over time. Even though exceedances of EC50 values for several pesticides were found, the TU at most of the sampling sites remained below 1: out of 2505 sampling sites at which pesticides were measured, the TU exceeded 1 at 80 sites, what makes 3.2% of all locations.

As can be seen in Figure 1D, TU increased simultaneously with the number of pesticides analyzed per sample. When the number of pesticides measured per sample was plotted versus TU, a significant positive trend was found (Figure 1E), suggesting a dependence of TU on the number of compounds measured per sample. Figure 1F shows normalized TU plotted versus time. The normalized TU remained stable between 1974 and 1998, followed by a decrease in 1998 - 2010 with lowest values observed in 2000 - 2004 (Figure 1F).

Annual patterns in macrofauna diversity

Figure 3 shows the Shannon index of macrofauna diversity plotted versus time. The difference in Shannon diversity indices between the two areas was not statistically

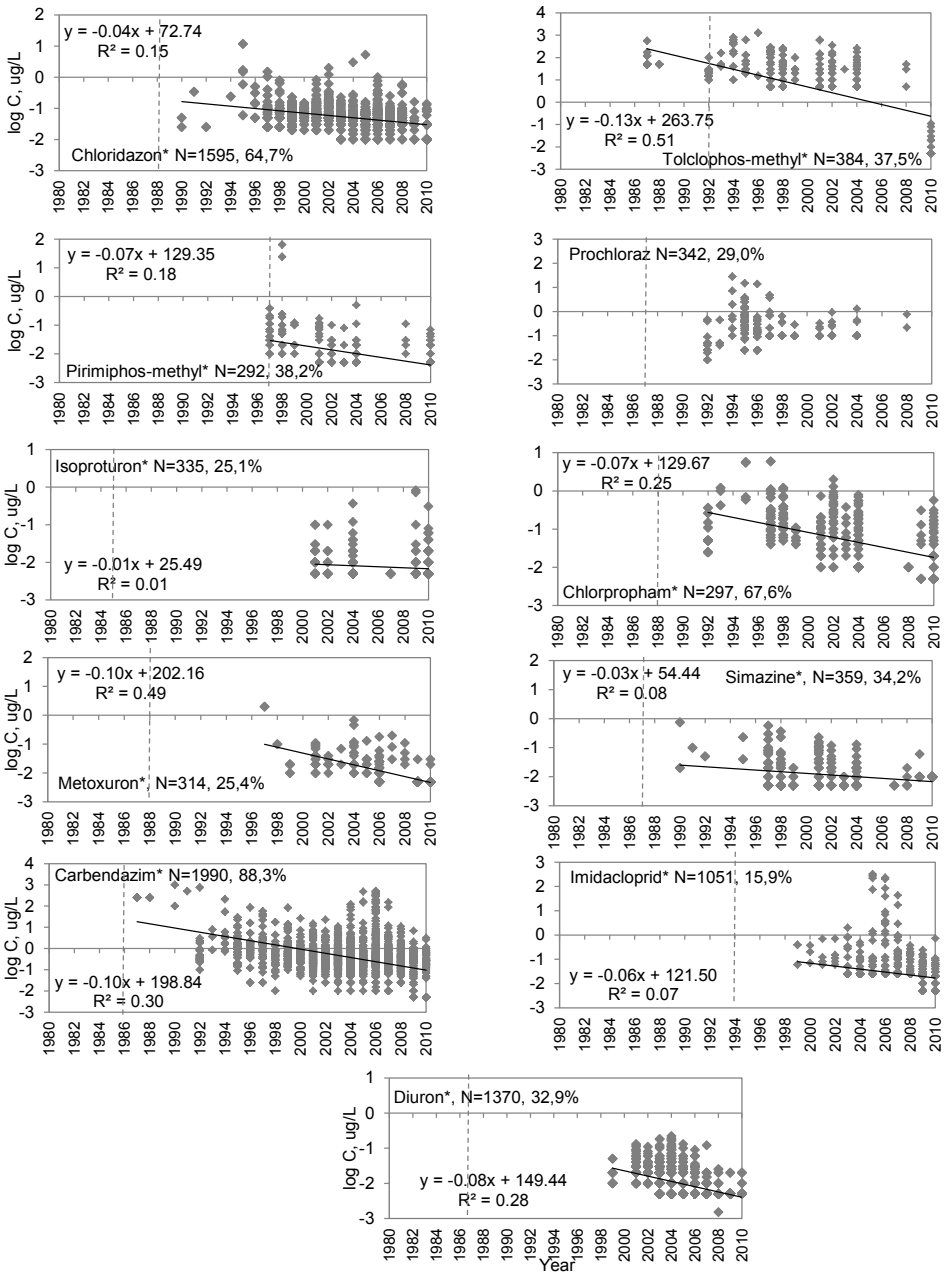

Figure 2. Concentrations of most frequently measured pesticides (logarithms with base 10, log C) plotted versus time, number of measurements done for each pesticide (N), and the percentage of measurements in which pesticide concentration exceeded the limit of detection (%). The dashed line corresponds to pesticide authorization date. *the regression line and equation are shown in case when relationship between pesticide concentration and time was statistically significant, as identified by regression analysis ($p<0.05$)

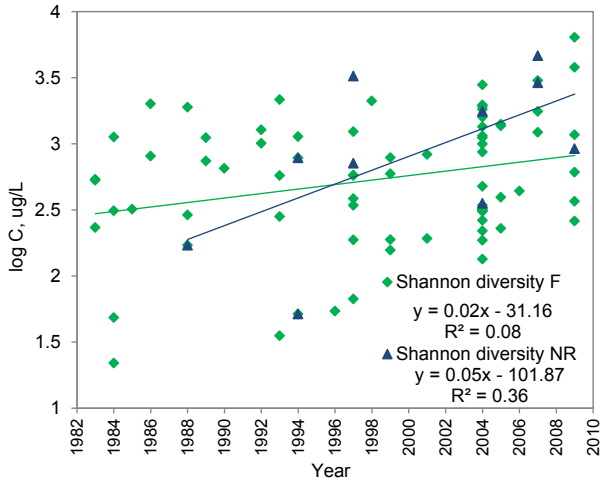

Figure 3. Shannon index of macrofauna diversity in ditches next to flower fields (green points) and nature reserve (blue points) plotted versus time. Regression lines and equations are shown. F = sampling sites in the flower growing area (p = 0.017), NR = sampling sites in the nature reserve (p = 0.066)

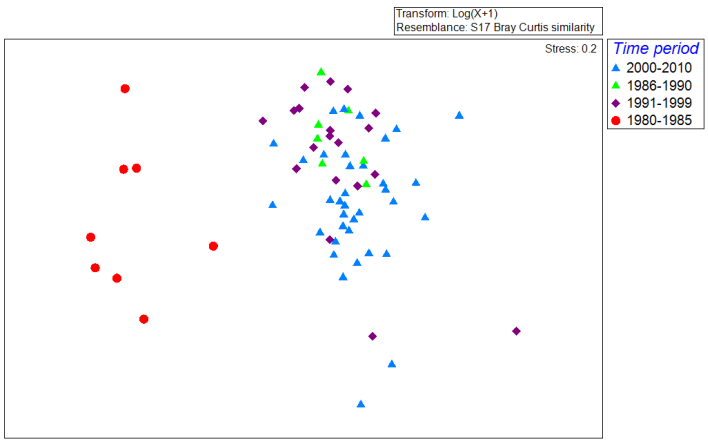

Figure 4. Non-metric multidimensional scaling of the macrofauna species composition Different colors correspond to different sampling periods. Summary of ANOSIM analysis testing the differences in macrofauna community composition between the sampling periods is given in Table 2.

Table 2. Results of ANOSIM analysis testing the differences in macrofauna community composition between sampling periods

Groups tested	R statistic	Significance level %
2000-2010 vs 1986-1990	0.078	23.8
2000-2010 vs 1991-1999	0.204	0.2*
2000-2010 vs 1980-1985	0.896	0.1*
1986-1990 vs 1991-1999	-0.076	72.0
1986-1990 vs 1980-1985	0.852	0.2*
1991-1999 vs 1980-1985	0.821	0.1*

*statistically significant at $p<0.05$

significant, as identified by the t-test. The Shannon index of macrofauna diversity in both the flower growing area and the nature reserve tended to increase over time ($p=0.017$ and $p=0.066$ respectively).

An MDS plot of macrofauna species composition is presented in Figure 4. Samples collected in 1980 - 1985 formed a distinct cluster, suggesting dissimilar macrofauna species composition in these years compared to other years (Figure 4). The possible explanation is that macrofauna in 1980 - 1985 was characterized by lower species diversity compared to the following years (Figure 3). ANOSIM analysis revealed significant difference between macrofauna species composition in 1980 - 1985 and that in later years (Table 2). Similarly, macrofauna community composition in 1991 - 1999 was significantly different from that in 2000 - 2010.

Conclusions

In the current study, we could not quantitatively link pesticide levels in surface waters and macrofauna diversity because pesticide and macrofauna data were not collected consistently over time and sampling site. Hence, it was not possible to perform a correlative analysis between pesticides levels in ditches and macrofauna diversity. To infer causal relationships between pesticide levels and the performance of aquatic biota, further field research is needed.

Addressing the two research questions, our results are the following. 1) Pesticide levels in ditches of the flower growing area changed over the previous decades. Toxic Units normalized by the number of pesticides measured per sample remained stable between 1974 and 1998, followed by decrease in 2000 – 2010, with the minimum values observed in years 2000 - 2004. Concentrations of the most frequently measured pesticides (like tolclophos-methyl, imidacloprid, chloridazon, isoproturon, chlorpropham, diuron, simazine, metoxuron) decreased over time confirming our starting hypothesis, while concentrations of

other pesticides (like prochloraz) remained stable over time. Carbendazim and pirimiphos-methyl contributed mostly to the exceedances of toxic units in recent years. 2) Macrofauna diversity in ditches of the flower growing area and watersheds of nature reserve increased over time. Macrofauna species composition in 1983 - 1985 was significantly different from that in the later years.

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CHAPTER 3

THE CONTRIBUTION OF PESTICIDES TO THE VARIANCE IN COMMUNITY COMPOSITION OF AQUATIC MACROFAUNA IN THE FIELD

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Under review

Abstract

Ditches surrounding agricultural fields in the Netherlands serve predominantly the function of flood control, and in addition accommodate aquatic plant and animal species. The studies addressing the effects of pesticides on aquatic biota in the field are scarce. The current study aimed to assess the contribution of pesticides along with other factors to the total variance in community composition of aquatic macrofauna in ditches next to flower bulb fields. Macrofauna samples and environmental data were collected during two consecutive years (2011 - 2012) in ditches next to flower bulb fields and pastures. Watersheds in the nature reserve area next to the polders were sampled as control sites. Data was analyzed with the variance partitioning procedure based on the redundancy analysis (RDA). The total variance in macrofauna community composition was divided into the variance explained by pesticides, environmental factors, the presence of other biota, time, shared variance, and unexplained variance. The total explained variance reached 22.6%. The largest proportion of explained variance (10.1%) was attributed to environmental factors, followed by pesticides (5.4%) and time (4.8%). When each macrofauna group was analyzed separately, the presence of other biota and environmental factors explained the largest proportion of variance in most of the macrofauna groups. Results of the study indicate that environmental factors, biotic interactions and temporal variation influence freshwater macrofauna considerably along with pesticides. We suggest that environmental managers should consider the multiple stressor context of aquatic ecosystems.

Keywords: abiotic factors, biotic factors, freshwater macrofauna, pesticides, RDA

Introduction

Ditches are the representative aquatic ecosystems in the Netherlands and next to their direct function of water level control also have high aquatic biodiversity (Verdonschot et al., 2012). Drainage ditches contain high numbers of aquatic plant and animal species, as well as semi-terrestrial species. Macrofauna in turn plays an important role in the food chain and biochemical cycles in the aquatic ecosystem. Thus, the presence of macrofauna in the sediment enhances the microbial nitrogen cycle by bioturbation. Bioturbation facilitates the transport of inorganic and organic nitrogen between sediment and water (Laverock et al., 2011; Kristensen & Kostka, 2005). This way macrofauna takes part in the processes of nitrification and denitrification, which in turn link nutrients in water to microbial communities in sediments (Stief, 2013). Macrofauna living in the water column feed on unicellular algae and bacteria, consuming fixed nitrogen and controlling the nitrogen pool in the ecosystem (Stief, 2013).

To protect aquatic biodiversity and the ecosystem functions it performs, it is important to understand the effects of chemicals on aquatic biota in the field. The most important reason is that in the realistic environment various abiotic and biotic factors influence the fate of pesticides and the performance of aquatic organisms. Several studies underlined that it is important to consider ecological parameters in ecotoxicological studies (Liess et al., 2003; Clements et al., 2012; Maund et al., 1997). Policy guidelines also emphasized that complexity within the biological communities, and the presence of multiple stress factors in natural ecosystems represents one of the challenges in ecological risk assessment (SCENIHR, SCHER, SCCS, 2012). A number of studies did include ecological factors in the assessment of pesticide effects on aquatic biota in the field. For instance, in the study of Berenzen et al. (2005) the effects of pesticides on aquatic invertebrates in freshwater streams were analysed in combination with environmental factors. Martin et al. (2012) studied the responses of aquatic invertebrates to pesticide runoff accounting for physico-chemical and hydrological parameters, and the vegetation coverage. Bollmohr et al. (2011) studied the effects of pesticides along with environmental factors on benthic communities in estuary. However, to our knowledge, the effects of pesticides on aquatic biota in the field, in combination with abiotic, biotic factors and time, have not been studied before.

In the present study we aimed to quantify what proportion of the total variance in aquatic macrofauna community composition (including crustaceans, annelids, molluscs, fish and insects) can be explained by pesticides, environmental factors (water chemistry parameters and macrophyte coverage), presence of other biota and time. To answer these questions, macrofauna sampling, measurements of water chemistry parameters and pesticide concentrations in ditches of the Dutch polders with intensive flower bulb crops were done during two consecutive years (2011 - 2012). Variance partitioning based on the redundancy analysis (RDA) was used to rank the explanatory factors (pesticides, environmental factors, biota, time and shared variance) with respect to the amount of explained variance.

Materials and methods

Research area

The research area is located in the flower bulb growing area of The Netherlands. There is an elevation gradient in the area: the height above sea level decreases gradually from the nature reserve (the highest site is located 4.26 - 4.5 m above the sea level) towards the polders (the lowest site is located -0.49 m to -0.25 m below the sea level). The nature reserve area is located on the northern part of the polder, so that no contamination comes from the north and north-west side. The water flows mainly in the South-West direction partly due to the pumping of the water coordinated by the water management board "Rijnland" and partly by the natural elevation gradient. A detailed description of the research area is given in Ieromina et al. (2014).

Sampling sites

During the year 2011, macrofauna and water chemistry samples were collected at 14 sites within the area: two sites in watersheds of the nature reserve, two sites in ditches alongside pastures and ten sites in ditches alongside flower bulb fields. Sampling was performed during the period April – November 2011 six times in order to account for seasonal fluctuation in water chemistry parameters and macrofauna life cycles. This period corresponds to the main phase of agricultural activities in the area. The same sites were sampled again in 2012, with four additional sites (two watersheds of nature reserve and two ditches next to pastures), located next to the polder area, sampled 4 times a year. In total 148 macrofauna and water chemistry samples were collected. Coordinates of the sampling sites are given in the Supplemental Data (Table S1).

Pesticide and nutrient measurements

Concentrations of pesticides were measured by Omegam Laboratoria BV. Pesticides were measured according to the standard guidelines (GC-MS and LC-MS/MS analysis). Nutrients were measured according to the following guidelines: NEN 6663 for phosphate and NEN-EN-ISO 13395 for nitrate and nitrite. Water samples for pesticide measurements were collected in watersheds of nature reserve (site D1) and in ditches of the flower bulb area (P1, P2, P3, F1, F2, F3, F5, F6, F8, and F10). Pesticide and nutrient concentrations were found to be below detection limits in samples collected in the nature reserve site D1. Therefore, pesticide and nutrient concentrations were assumed to be below detection limits in other nature reserve sites D2, D3 and D4. Sampling for pesticide and water chemistry measurements and macrofauna sampling were done during the same day. If the concentration of a pesticide was below its limit of detection, half of the detection limit was used in the data analysis (Clarke, 1998). The overview of environmental parameters and pesticide concentrations at the sampling sites is given in the Supplemental Data, (Tables S2 and S3).

Measuring water chemistry parameters

The following water chemistry parameters were measured: temperature (°C), dissolved oxygen (DO, mg/L), pH and conductivity (mS). Temperature and Oxygen were measured with a Z521 Consort Oxygen meter. pH was measured with a Greisinger electronic pH-meter. Conductivity was measured with a Eijkelkamp Agriresearch Equipment conductivity-meter. DOC measurements were done with a non-dispersive infrared detector (NDIR). In addition, the percentage of water surface covered with floating macrophytes was estimated.

Macrofauna sampling and determination

Macrofauna samples were collected with a dipping net (mesh size of 500 µm). The dipping net with a 0.25 m opening was dragged over a total length of 5 m of the upper part of the sediment layer (depth 3-5 cm within the sediment layer). Therefore, 20 sampling units (each sampling unit was 0.25 m×0.25 m) in total were collected from dominating habitats according to the method described in Keizer-Vlek et al. (2011) and Vlek et al. (2006), hence resulting in a multi-habitat sampling strategy.

Larger organisms (for instance: Gastropoda, Coleoptera) were identified in the field or photographed for further identification. After all sampling units were collected, macrofauna samples were rinsed and transferred to plastic sample jars. Samples were preserved with 70% ethanol directly after sampling. Samples were washed in the laboratory, sorted and identified to the lowest taxonomic level feasible. Latin names for species, genus, family, order and class were verified in ITIS (the Integrated Taxonomic Information System, http://www.itis.gov/). The level of identification for each taxonomic group is given in the Supplemental Data (Table S4).

Statistical analysis

Principal component analysis (PCA) of macrofauna community composition

Principal component analysis (PCA) was performed on macrofauna abundance data on the level of order to identify variation patterns in the macrofauna community composition and visualize groups of sampling sites containing similar macrofauna taxa. This analysis was done for macrofauna collected from all sampling locations (N = 145) and separately for macrofauna collected from sampling sites where pesticide concentrations were measured (N = 79). Prior to analysis, all biological data were transformed using the Hellinger transformation (Legendre & Gallagher, 2001). In all multivariate analysis, data were centered by species and not centered by sample (Leps & Smilauer, 2003).

Selecting explanatory variables for Redundancy Analysis (RDA)

Variance partitioning based on RDA was applied to divide the total variance in macrofauna community composition into different components (Borcard et al., 1992; Leps & Smilauer, 2003). Four groups of explanatory variables were defined: pesticides, environmental factors, time, and the presence of other biota. A list of explanatory variables included in each

component of variance is given in Table 1. Variance partitioning analysis was based on the data from sampling sites at which pesticide concentrations were measured (N = 79). In previous studies, the percentage of explained variance obtained in canonical analysis is denoted as R^2 (Peres-Neto et al., 2006). Similarly, in the current manuscript we imply canonical R^2 when referring to the percentages of explained variance.

Table 1. List of response variables and explanatory variables included in four variance components

Response variables	Components of variance	Explanatory variables included in variance components
Total species composition	Pesticides (P)	Chlorprofam, pirimiphos-methyl, tolclophos-methyl, carbendazim, imidacloprid, isoproturon, imazalil, methiocarb, ethiofencarb
	Environmental factors (E)	Temperature, dissolved oxygen, dissolved organic carbon, phosphate, nitrite, nitrate, macrophyte coverage
	Time (T)	number of the year, number of the month
Species composition of different macrofauna groups (orders Hemiptera, Diptera, Ephemeroptera, Trichoptera, Odonata, Coleoptera, Gasterosteiformes, Haplotaxida, Diplostraca, Basommatophora, Heterostropha, Veneroida, Neotaenioglossa, Rhynchobdellida)	Biota (B)*	Hemiptera, Diptera, Ephemeroptera, Trichoptera, Plecoptera, Odonata, Coleoptera, Lepidoptera, Collembola, Megaloptera, Gasterosteiformes, Cypriniformes, Rhynchobdellida, Haplotaxida, Tricladida, Isopoda, Cyclopoida, Diplostraca, Amphipoda, Arguloida, Anostraca, Mysida, Basommatophora, Heterostropha, Architaenioglossa, Neotaenioglossa, Veneroida, Ostracoda, Acari

*When each macrofauna group was analysed separately, additional biota component of variance was included in the analysis

Table 2. Summary of variance partitioning analysis

Response variable	Estimated component of variance	Explanation	Calculation procedure
Total macrofauna	Total Variance	all variance	assumed to be 100%
community composition	P∪E∪T	total explained variance	all groups of variables (P, E, T) included as explanatory variables
	residual variance	unexplained variance	100% - P∪E∪B
	P|E∪T	variance explained by pesticides only	pesticides included as explanatory variables, environmental factors and time-covariables
	E|P∪T	variance explained by environmental factors only	environmental factors included as explanatory variables, pesticides and time-covariables

Table 2. Summary of variance partitioning analysis (*Continued*)

Response variable	Estimated component of variance	Explanation	Calculation procedure
	T|E∪P	variance explained by time only	time included as explanatory variable, environmental factors and pesticides-covariables
	P∩E∩T	shared variance by P, E and T	P∪E∪T -P|E∪T - E|P∪T- T|E∪P (equation 1)
	P*	all variance explained by pesticides	pesticides included as explanatory variables, no covariables
	E*	all variance explained by environmental factors	environmental factors included as explanatory variables, no covariables
	T*	all variance explained by time	time included as explanatory variable, no covariables
	T∩E	shared variance between time and environmental factors	P∩E∩T - (P - P|E∪T)
	T∩P	shared variance between time and pesticides	P∩E∩T - (E - E|P∪T)
	P∩E	shared variance between pesticides and environmental factors	P∩E∩T - (T - T|E∪P)
	PTE	joined variance between P, T and E	P∩E∩T- T∩E - T∩P - P∩E
Composition of different macrofauna groups	P∪E∪T∪B	total explained variance	all groups of variables (P, E, T, B) included as explanatory variables
	P|E∪T∪B	variance explained by pesticides only	pesticides included as explanatory variables, environmental factors, biota and time-covariables
	E|P∪T∪B	variance explained by environmental factors only	environmental factors included as explanatory variables, pesticides, biota and time-covariables
	T|E∪P∪B	variance explained by time only	time included as explanatory variables, environmental factors, biota and pesticides-covariables
	B|E∪P∪T	variance explained by biota only	biota included as explanatory variables, environmental factors, time and pesticides-covariables
	P∩E∩T∩B	shared variance by P, E, T and B	P∪E∪T∪B - P|E∪T∪B - E|P∪T∪B - T|E∪P∪B - B|E∪P∪T (equation 2)

*including variance shared with other factors

Response variable datasets consisted of the total macrofauna community composition and of the composition of different macrofauna groups on the level of order (Table 2).

The variance in total macrofauna community composition was divided into five components: variance explained by pesticides (P|E∪T), environmental factors (E|P∪T), time (T|P∪E), shared variance between pesticides, environmental factors and time (P∩E∩T), and residual (unexplained) variance (Table 2, Supplemental Data, Figure S1).

The variance in each macrofauna group (Hemiptera, Diptera, Ephemeroptera, Trichoptera, Odonata, Coleoptera, Gasterosteiformes, Haplotaxida, Diplostraca, Basommatophora, Heterostropha, Veneroida, Neotaenioglossa, and Rhynchobdellida) was divided into six components: variance explained by pesticides (P|E∪T∪B), environmental factors (E|P∪T∪B), the presence of other biota (abundance of other macrofauna orders except the order being analysed) (B|E∪P∪T), time (T|E∪P∪B), shared variance between pesticides, environmental factors, biota and time (P∩E∩T∩B), and unexplained variance.

Data transformation prior RDA

The number of explanatory variables in each component of variance was different (Table 1). Generally, the number of explanatory variables included in RDA model affects the model outcome: the explained variance increases even when additional variables that only contain noise (i.e. are not related to the response variable) are included (Freedman, 1983, Peres-Neto et al., 2006). To account for different number of explanatory variables in variance components, a PCA was performed on each set of explanatory variables, and sample scores of the first four Principal Components (PC) axes were included in RDA as explanatory variables. Results of PCA on pesticide, environmental factors, biota, and time data datasets can be found in Supplemental Data, Tables S5 and S6. In addition, the correlation between PC sample scores was checked prior the RDA. The correlation coefficient between PC scores was below 0.5.

Missing values in the water chemistry dataset were estimated based on the average values of the variable calculated from the samples collected at the same date/sampling site (Leps & Smilauer, 2003). In addition, we performed PCA on datasets with and without missing values to test if PCA results differed between the two datasets. The results of PCA on datasets with and without estimated missing values were similar (Supplemental Data, Tables S5 and S6). Therefore, further analysis (RDA on PC sample scores) was based on the dataset with estimated missing values, because otherwise the software would replace the missing values with zeros.

Variance partitioning procedure

The Total Variance (TV) in macrofauna community composition represented the sum of unconstrained and constrained eigenvalues. The total explained variance (P∪E∪T for total macrofauna community and P∪E∪T∪B for different macrofauna groups) represented the amount of variance explained by all variance components, or the sum of the constrained eigenvalues. The total explained variance was obtained by constructing a RDA in which all groups of explanatory variables (PC sample scores obtained from PCA

on pesticide, environmental factor, time, and biota datasets) were included in the analysis as explanatory variables.

The percentage of variance explained by pesticides was estimated by constructing a partial RDA in which pesticide data (PC sample scores obtained from PCA on pesticide dataset) were included in the analysis as explanatory variables, and PC sample scores obtained from PCA on environmental, time and biotic datasets were included in the analysis as covariables (Table 2). Similar procedure was repeated to quantify the percentages of variance explained by environmental factors, time and biota. Proportion of variance in macrofauna community composition shared by different factors was estimated based on equations 1 and 2 presented in Table 2. In addition, Ezekiel's R^2 adjustment was applied to the estimated explained variance according to the formula:

$$R2adjusted = 1 - \frac{n - 1}{n - p - 1}(1 - R2)$$ **(Peres-Neto** et al., 2006).

where n = number of samples, p = number of explanatory variables, R^2 = percentage of explained variance. In the manuscript we refer to R^2 adjusted. Multivariate analysis was performed in Canoco software version 4.5 (Lepš & Šmilauer, 2003).

Results

Macrofauna community composition

The order Diptera contained the highest number of species followed by the order Coleoptera (Supplemental Data, Table S4). As a result of Principal Component Analysis (PCA), first four PC explained 61.6% of variance in macrofauna abundance on the level of order (based on the macrofauna data from sampling sites where pesticide concentrations were measured, N = 79) (Supplemental Data, Table S7). Diplostraca contributed mostly to the PC1, Ephemeroptera – to PC2 and Diptera – to PC3. High abundances of crustaceans Diplostraca, annelid Haplotaxida, gastropods Basommatophora and Heterostropha were associated with ditches adjacent to flower bulb fields (Figure 1). On the other hand, watersheds of nature reserve contained high numbers of insects Ephemeroptera, Odonata, Diptera, and Trichoptera. A similar result was found when PCA was performed on macrofauna data collected from all sampling sites (N = 145) (Supplemental Data, Figure S2, Table S8). Average abundances of macrofauna on the level of order and standard deviations are given in Supplemental Data, Table S9.

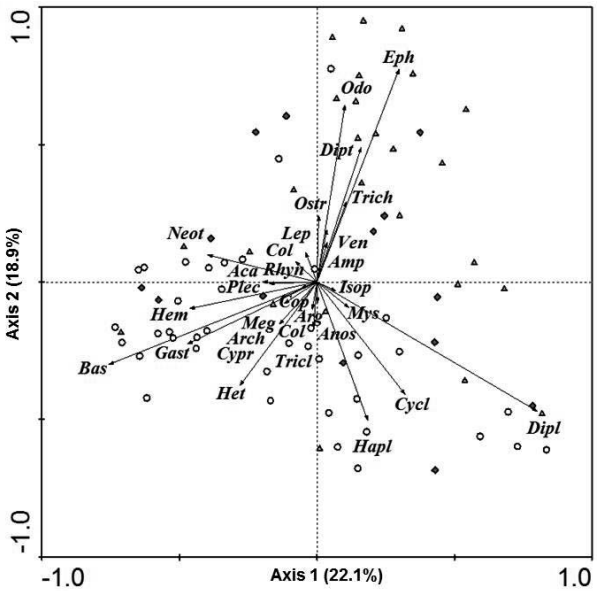

Figure 1. Principal component analysis of Hellinger-transformed macrofauna abundance on the level of Order (N=79). Hem = Hemiptera, Dipt = Diptera, Eph = Ephemeroptera, Trich = Trichoptera, Plec = Plecoptera, Odo = Odonata, Col = Coleoptera, Lep = Lepidoptera, Meg = Megaloptera, Colm = Collembola, Gast = Gasterosteiformes, Cypr = Cypriniformes, Rhyn = Rhynchobdellida, Hapl = Haplotaxida, Tricl = Tricladida, Isop = Isopoda, Ostr = Ostracoda, Cycl = Cyclopoida, Dipl = Diplostraca, Cop = Copepoda, Amp = Amphipoda, Arg = Arguloida, Anos = Anostraca, Mys = Mysida, Bas = Basommatophora, Het = Heterostropha, Ven = Veneroida, Neot = Neotaenioglossa, Arch = Architaenioglossa, Aca = Acari, Tricl = Tricladida. Triangular = sites in watersheds of nature reserve, Circle = sites in ditches next to flower fields, Diamond = sites in ditches next to pastures

Table 3. Components of variance estimated for total macorfauna community composition: total explained variance (P∪E∪T), residual variance, variance explained by pesticides (P|E∪T), environmental factors (E|P∪T), time (T|E∪P), shared variance between pesticides, environmental factors and time (P∩E∩T), shared variance between pesticides and environmental factors (P∩E), pesticides and time (P∩T), time and environmental factors (T∩E), joined variance between three components (TPE). Presented are the percentages of explained variance (R^2) and R^2 adjusted by Ezekiel's transformation (in italic)

Response group	P∪E∪T	Residual variance	P|E∪T	E|P∪T	T|E∪P	P∩E∩T	P∩E	P∩T	T∩E	TPE
Macrofauna community composition	19	81	4.7	8.6	4.2	1.5	1.1	0.3	0.2	-0.1
	22.6	*77.4*	*5.4*	*10.1*	*4.8*	*1.6*	*1.1*	*0.2*	*0.04*	*-0.3*

Variance partitioning of macrofauna community composition

When the total community composition was analyzed, the explained variance reached 22.6% (Table 3). Environmental factors contributed mostly to the explained variance (10.1%), followed by pesticides (5.4%) and time (4.8%). Shared variance between all factors was 1.6% (Table 3).

From all macrofauna groups analyzed, the percentage of explained variance was the highest for Rhynchobdellida and Ephemeroptera (59.2% and 51.8%, respectively). The variance in Diplostraca was least explained least by RDA model (total explained variance was 12.5%) (Table 4). All components explained between 1% and 17% of the total variance in macrofauna, except for the time (the percentage of variance in Ephemeroptera explained by time reached 21%) (Table 4).

Discussion

Macrofauna community composition in the research area

For most of the macrofauna taxonomic groups, the number of species found in the current study was in line with previous field studies performed in the Netherlands (Keizer-Vlek et al., 2011). The following orders showed the highest similarity with the study of Keizer-Vlek et al. (2011) in terms of the number of taxa: Bivalvia, Trichoptera and Diptera (Supplemental Data, Table S4).

As seen in PCA plots (Figure 1, Supplemental Data, Figure S2), higher densities of insect larvae Trichoptera, Odonata, Ephemeroptera and Diptera were associated with nature reserve. Based on the analysis of the water chemistry dataset, concentrations of dissolved oxygen were generally higher in watersheds of nature reserve than in agricultural ditches. While the levels of nutrients and pesticides in water were considerably lower in the nature reserve (Supplemental Data, Tables S2 and S3). Therefore, high water quality of the nature reserve favored sensitive insect species. The high sensitivity of insect larvae to pesticides observed in our study complies with previous findings (Berenzen et al., 2005; Liess & Von der Ohe, 2005).

On the other hand, abundances of insects Hemiptera, gastropods Bassomatophora and Heterostropha, crustacean Diplostraca, annelids Rhynchobdellida and Haplotaxida were larger in ditches of agricultural area. Similarly, large abundances of Gastropoda, Hirudinea and Oligochaeta in contaminated waters were found in the study of Berenzen et al. (2005). Species from these taxonomic groups are described as generally tolerant to organic pollution (Hilsenhoff 1987, 1988; Murdoch et al., 1996). Previous study of Armendáriza et al. (2012) suggested that nutrients increase the biomass of bacteria and algae used by Oligochaeta

Table 4. Components of variance estimated for macrofauna groups: total explained variance (P∪E∪T∪B), residual variance, variance explained by pesticides (P|E∪T∪B), environmental factors (E|P∪T∪B), biota (B|E∪P∪T), time (T|E∪P∪B) and shared variance (P∩E∩T∩B). Presented are the percentages of explained variance (R^2) and R^2 adjusted by Ezekiel's transformation (in italic)

Response group	P∪E∪T∪B	Residual variance	P|E∪T∪B	E|P∪T∪B	B|E∪P∪T	T|E∪P∪B	P∩E∩T∩B
Rhynchobdellida	49.5	50.5	14	9.1	4.8	0.7	20.9
	59.2	*40.8*	*16.6*	*10.7*	*5.6*	*0.6*	*24.9*
Ephemeroptera	43.3	56.7	1.5	9.3	6.4	18.2	7.9
	51.8	*48.2*	*1.6*	*11.0*	*7.5*	*21.6*	*9.3*
Odonata	35.3	64.7	1.4	13.4	14.2	2.3	4
	42.2	*57.8*	*1.5*	*15.9*	*16.8*	*2.6*	*4.6*
Neotaenioglossa	34.1	65.9	4.9	12.8	4.4	3.5	8.5
	40.7	*59.3*	*5.7*	*15.2*	*5.1*	*4.0*	*10.0*
Heterostropha	30.6	69.4	8.1	6.7	7.9	4.6	3.3
	36.5	*63.5*	*9.5*	*7.8*	*9.3*	*5.3*	*3.8*
Gasterosteiformes	29.7	70.3	10.2	5	4.3	2.3	7.9
	35.4	*64.6*	*12.0*	*5.8*	*5.0*	*2.6*	*9.3*
Basommatophora	25.3	74.7	5.9	6.2	8	1.5	3.7
	30.2	*69.8*	*6.9*	*7.2*	*9.4*	*1.6*	*4.2*
Haplotaxida	18.5	81.5	6	7.9	3	0.1	1.5
	22.0	*78.0*	*7.0*	*9.3*	*3.4*	*-0.1*	*1.6*
Hemiptera	17.6	82.4	2.9	6.9	3.5	2.3	2
	20.9	*79.1*	*3.3*	*8.1*	*4.0*	*2.6*	*2.2*
Veneroida	15.8	84.2	1.1	6.1	8.7	5.7	-5.8
	18.8	*81.2*	*1.1*	*7.1*	*10.2*	*6.6*	*-7.2*
Trichoptera	14.6	85.4	1.9	4	8.2	0.8	-0.3
	17.3	*82.7*	*2.1*	*4.6*	*9.6*	*0.8*	*-0.6*
Diptera	13.7	86.3	1.9	6.2	3.9	3.7	-2
	16.2	*83.8*	*2.1*	*7.2*	*4.5*	*4.2*	*-2.6*
Coleoptera	13.3	86.7	1.5	2.5	5	2	2.3
	15.8	*84.2*	*1.6*	*2.8*	*5.8*	*2.2*	*2.6*
Diplostraca	10.6	89.4	1.5	3.7	3.9	0.5	1
	12.5	*87.5*	*1.6*	*4.2*	*4.5*	*0.4*	*1.0*
Average R^2 adjusted	30.0	70.0	5.2	8.4	7.2	3.9	4.5

as a food source. This way, nutrients induce positive effect on Oligochaeta abundance (Armendáriza et al., 2012).

Variance partitioning of macrofauna community composition

When variance partitioning was applied to the total macrofauna community composition, the overall explained variance reached 22.6%. Other field studies reported similar percentages of variance in biological communities explained by different factors. For instance, in Zuellig et al. (2012), the total variance in freshwater algae, fish, and invertebrate communities explained by between-site variance and time was ~30%. The variance in macroinvertebrate community explained by environmental and spatial factors reached ~ 25% in the study of Heino et al. (2012). Out of 22.6% of total explained variance found in our study, the largest proportion of variance (10.1%) was attributed to environmental factors, followed by pesticides (5.4%), and time (4.8%).

First, our results suggest that environmental factors induce the largest effect on macrofauna community composition. Previous studies emphasized the importance of environmental factors in shaping community composition of aquatic biota. For instance, in the study of Larsen et al. (2012), environmental factors were more important than species interactions in structuring fish and invertebrate communities. In the study of Zuellig et al. (2012), environmental factors dominated the inter-annual variance in shaping invertebrate community. Water chemistry parameters vary significantly in ditches next to flower bulb fields. For instance, the average phosphate and nitrate concentration in ditches varied from 0.03 mg/L to 4.10 mg/L and from 0.05 to 0.6 mg/L, respectively. Average DOC levels varied between 48 mg/L and 290 mg/L (Supplemental Data, Table S2). DOC and nutrients relate to food availability for aquatic macrofauna and limit the performance of many aquatic species. Large variation in these important parameters can possibly explain the high contribution of environmental factors to the total variance in macrofauna community composition. As a second conclusion, the contribution of pesticides to the total variance in macrofauna community composition was two times lower then the contribution of environmental factors (5.4%). There are not many studies quantifying the contribution of toxicants to the variance in community composition of aquatic biota. In the study of De Zwart (2006), the toxicants explained 3% of the total variance in fish communities inhabiting rivers, relative to 28% of variance explained by water chemistry parameters and 16% of variance explained by habitat characteristics. Similarly to our study, environmental factors dominated toxicants in structuring community composition of aquatic biota. Third, we observed a relatively high contribution of the time to the total variance (4.8%) that can be explained by seasonal variation in macrofauna community composition. Shared variance between different components of variance explained up to 1.6% of the total variance in macrofauna community composition. Shared variance can be possibly explained by correlation between different factors. For instance, it is documented that the fate of pesticides in the aquatic environment is largely dependent on environmental conditions (Maund et al.,

1997). In addition, nutrients co-occur with pesticides due to their similar origin: pesticides and fertilizers are applied together at the bulb fields.

The average percentage of total explained variance for all macrofauna groups was 30.0%. On average, biota and environmental factors components explained the largest percentage of variance in different macrofauna groups, followed by pesticides, shared variance between all four components and time. This result can possibly be explained by the importance of biotic interactions and site-specific environmental conditions in structuring macrofauna community composition. RDA procedure yielded a negative value for shared variance for Diptera, Trichoptera and Veneroida (Table 4). Such a result for shared variance means that the groups of variables separately explain the variance in the response variable better than when combined together (Legendre & Legendre, 2012).

Variance partitioning based on the redundancy analysis allowed us to quantify the contribution of different field-relevant factors to the total variance in macrofauna community composition. Results of the study indicated that in most of the macrofauna groups, the contribution of environmental factors and presence of other biota to the total variance exceeded the contribution of pesticides, or was equally important.

Conclusions

The entire aquatic macrofauna community composition was highly dependent on environmental factors that made a twofold higher contribution to the total explained variance than pesticides. Based on our results we can conclude that the responses of macrofauna community to pesticides in the field are largely dependent on environmental factors. Policy guidelines developed to protect surface water and preserve aquatic biodiversity should include multi-stressor assessments at tiered levels, taking into account abiotic factors, habitat features, biotic interactions, as well as the differences in responses of distinct macrofauna groups due to their varying ecological preferences.

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Supplemental information

Table S1. Coordinates of the sampling sites and the number of samples collected at each site

Site Code	Land use area	Coordinates	Year sampled	N samples
MF1	Flower field	52°17'26.94"N, 4°30'51.04"O	2011-2012	10
MF2	Flower field	52°17'35.36"N, 4°29'54.25"O	2011-2012	10
MF3	Flower field	52°16'28.54"N, 4°29'36.05"O	2011	6
MF4	Flower field	52°16'46.93"N, 4°29'44.32"O	2011-2012	10
MF5	Flower field	52°15'55.66"N, 4°28'27.94"O	2011-2012	10
MF6	Flower field	52°15'13.06"N, 4°28'40.95"O	2011-2012	10
MF7	Flower field	52°15'10.38"N, 4°28'16.64"O	2011-2012	10
MF8	Flower field	52°15'6.26"N, 4°27'53.95"O	2011-2012	10
MF9	Flower field	52°14'44.08"N, 4°27'12.15"O	2011	6
MF10	Flower field	52°15'39.93"N, 4°27'49.28"O	2011-2012	10
MP1	Grassland	52°17'14.80"N, 4°29'32.03"O	2011-2012	10
MP2	Grassland	52°16'38.09"N, 4°29'2.23"O	2011-2012	10
MP3	Grassland	52°21'15.76"N, 4°35'56.81"O	2012	4
MP4	Grassland	52°19'35.52"N, 4°34'28.78"O	2012	4
MD1	Dunes	52°17'36.31"N, 4°29'43.92"O	2011-2012	10
MD2	Dunes	52°17'32.42"N, 4°29'39.89"O	2011-2012	10
MD3	Dunes	52°21'45.23"N, 4°33'21.05"O	2012	4
MD4	Dunes	52°20'45.65"N, 4°34'42.81"O	2012	4

Table S2. Mean and standard deviation (in italic) of water chemistry parameters measured at the sampling sites

Site	T, °C	pH	Conductivity, mS	DO, mg/L	DOC, mg/L	Phosphate, mg/L	Nitrite, mg/L	Nitrate, mg/L
D1	15.25	8.07	249.92	6.17	141.23	0.03	0.01	0.05
	4.71	*1.13*	*220.21*	*2.32*	*132.99*	*0.01*	*0.00*	*0.07*
D2	15.10	7.55	401.12	5.24	187.43	0.03	0.01	0.05
	5.50	*0.47*	*297.15*	*2.40*	*204.33*	*0.01*	*0.00*	*0.07*
D3	15.58	10.54	622.25	6.26	63.33	0.03	0.01	0.05
	3.38	*5.34*	*50.49*	*2.50*	*32.32*	*0.01*	*0.00*	*0.07*
D4	15.05	10.05	617.50	6.40	48.27	0.03	0.01	0.05
	3.27	*4.70*	*51.80*	*2.00*	*5.57*	*0.01*	*0.00*	*0.07*
P1	15.85	7.75	612.59	4.83	163.70	2.46	0.03	0.13
	5.08	*0.40*	*306.96*	*1.64*	*128.36*	*2.48*	*0.03*	*0.19*
P2	14.73	7.69	726.77	5.01	181.97	0.91	0.02	0.19
	4.35	*0.48*	*376.44*	*2.21*	*119.18*	*0.44*	*0.01*	*0.24*
P3	15.78	7.56	760.75	6.37	87.27	*N*	*N*	*N*
	5.87	*0.55*	*66.09*	*5.82*	*68.13*			
P4	14.73	7.46	729.50	5.45	86.88	*N*	*N*	*N*
	5.25	*0.31*	*92.42*	*2.36*	*60.85*			
F1	14.48	7.66	547.54	4.37	215.42	1.83	0.04	0.15
	3.57	*0.31*	*418.78*	*1.67*	*144.03*	*1.38*	*0.01*	*0.08*
F2	14.77	7.68	467.32	3.71	193.96	0.78	0.02	0.06
	3.83	*0.42*	*351.88*	*1.43*	*217.22*	*0.99*	*0.01*	*0.07*
F3	14.74	7.90	450.25	4.67	286.40	2.01	0.05	0.23
	5.86	*0.74*	*634.63*	*2.14*	*112.96*	*1.58*	*0.04*	*0.19*
F4	15.23	8.09	607.27	4.45	290.97	*N*	*N*	*N*
	4.73	*0.62*	*359.19*	*1.91*	*360.86*			
F5	15.67	7.98	712.42	4.76	152.58	1.12	0.04	0.18
	4.46	*0.65*	*358.91*	*2.10*	*124.94*	*0.44*	*0.02*	*0.33*
F6	16.02	8.20	752.94	5.34	233.32	4.10	0.03	0.33
	4.50	*0.99*	*403.71*	*1.61*	*144.41*	*3.24*	*0.03*	*0.49*
F7	15.02	7.99	816.43	5.20	173.37	*N*	*N*	*N*
	4.29	*0.67*	*408.01*	*1.92*	*137.78*			
F8	15.35	8.11	851.24	6.10	179.27	1.96	0.07	0.60
	4.43	*0.75*	*425.48*	*1.47*	*164.93*	*0.79*	*0.07*	*0.66*
F9	14.52	8.13	617.86	4.60	247.11	*N*	*N*	*N*
	5.11	*0.99*	*536.43*	*2.70*	*126.32*			
F10	15.28	7.98	743.59	5.58	153.43	1.48	0.01	0.05
	5.09	*0.69*	*370.61*	*1.72*	*103.93*	*1.32*	*0.01*	*0.04*

N = the parameter was not measured at this sampling site

Table S3. Mean and standard deviation (in italic) of pesticide concentrations (µg/L) measured at the sampling sites

	Chloor	PirM	TolM	Prochloraz	Carb	Ethfc	Imzl	Imdc	Ispr	Meth
D1	0.010	0.005	0.005	0.100	0.010	0.025	0.005	0.024	0.005	0.010
	0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
D2	0.010	0.005	0.005	0.100	0.010	0.025	0.005	0.024	0.005	0.010
	0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
D3	0.010	0.005	0.005	0.100	0.010	0.025	0.005	0.024	0.005	0.010
	0	*0*	*0*	*0.045*	*0*	*0*	*0*	*0*	*0*	*0*
D4	0.010	0.005	0.005	0.100	0.010	0.025	0.005	0.024	0.005	0.010
	0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
P1	0.030	0.035	0.005	0.100	0.650	0.025	0.005	0.046	0.005	0.010
	0.045	*0.064*	*0*	*0*	*1.259*	*0*	*0*	*0.047*	*0*	*0*
P2	0.019	0.030	0.007	0.100	0.124	0.025	0.005	0.026	0.005	0.010
	0.018	*0.066*	*0.005*	*0*	*0.173*	*0*	*0*	*0.002*	*0*	*0*
P3	0.010	0	0.005	0.100	0.460	0.025	0.005	0.030	0.005	0.010
	0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
F1	0.025	0.005	0.005	0.100	0.110	0.025	0.005	0.025	0.005	0.010
	0.021	*0*	*0*	*0*	*0.042*	*0*	*0*	*0*	*0*	*0*
F2	0.010	0.005	0.006	0.100	0.168	0.025	0.005	0.029	0.005	0.010
	0	*0*	*0.003*	*0*	*0.259*	*0*	*0*	*0.008*	*0*	*0*
F3	0.040	0.007	0.005	0.100	0.186	0.025	0.005	0.025	0.005	0.010
	0.037	*0.003*	*0*	*0*	*0.097*	*0*	*0*	*0*	*0*	*0*
F5	0.016	0.005	0.012	0.100	0.069	0.025	0.014	0.065	0.005	0.010
	0.018	*0*	*0.014*	*0.000*	*0.063*	*0*	*0.016*	*0.118*	*0*	*0*
F6	0.017	0.183	0.007	0.100	0.287	0.025	0.005	0.053	0.006	0.010
	0.021	*0.241*	*0.005*	*0*	*0.263*	*0*	*0*	*0.075*	*0.002*	*0*
F8	0.017	0.005	0.006	0.162	0.277	0.025	0.005	0.138	0.005	0.010
	0.021	*0*	*0.002*	*0.198*	*0.687*	*0*	*0*	*0.359*	*0*	*0*
F10	0.018	0.005	0.005	0.100	0.373	0.025	0.005	0.029	0.005	0.010
	0.020	*0*	*0*	*0*	*0.798*	*0*	*0*	*0.010*	*0*	*0*
LOD (µg/L)	0.02	0.01	0.01	0.2	0.02*	0.05	0.01	0.05*	0.01	0.02

*LOD of carbendazim and imidacloprid for samples collected in autumn 2012 was 0.01 µg/L
Chlor = chlorprofam, Pir-meth = pirimiphos-methyl, Tolc-meth = tolclophos-methyl, Carb = carbendazim, Ethiofen = ethiofencarb, Imidacl = imidacloprid, Ispr = isoproturon, Proch = prochloraz, Imaz = imazalil Meth = methiocarb, LOD = limit of detection

Table S4. The level of identification for each macrofauna Class, the total number of individuals counted in each Order and the number of taxa identified in each Order

Class	Taxonomic identification level	Order	Total N individuals	Total N taxa
Insecta	Species (73.6%), genus	Hemiptera	4217	18
	(22.7%), family (0.4%), order	Diptera	4308	43
	(1.0%)	Ephemeroptera	9078	6
		Trichoptera	220	12
		Plecoptera	6	1
		Odonata	336	13
		Coleoptera	982	25
		Lepidoptera	10	2
		Collembola	308	3
		Megaloptera	1	1
Actinopterygii	Species (100%)	Gasterosteiformes	883	2
		Cypriniformes	32	5
Clitellata	Species (98.7%), genus	Rhynchobdellida	148	4
	(0.6%)	Haplotaxida	4736	3
		Tricladida	31	1
Malacostraca	Species (99.0%), genus	Isopoda	656	1
	(0.9%)	Mysida	22	1
		Amphipoda	240	3
Ostracoda	Order (100%)	Class Ostracoda	202	1
Maxillopoda	Genus (0.45%), order	Cyclopoida	2323	1
	(67.9%), subclass (31.6%)	Subclass Copepoda	1081	1
		Arguloida	17	1
Branchiopoda	Species (8.2%), genus	Diplostraca	72523	4
	(91.8%)	Anostraca	4	1
Mollusca	Species 81.43%), genus	Basommatophora	6785	10
	(18.56%)	Architaenioglossa	13	1
		Veneroida	1485	3
		Heterostropha	4293	3
		Neotaenioglossa	1281	3
Arachnida	Suborder (100%)	Subclass Acari	212	1
Total	Species (32.8%), genus (63.2%), order (2.3%)		116433	174

Table S5. Summary of PCA on pesticide, environmental and macrofauna data. Presented are cumulative percentages of variance explained by the first four Principal Components (PC)

PC axes	1	2	3	4
Pesticides	81.7	91.9	96.5	99.4
Env Estimated*	56	78.2	88.2	94.3
Env Not estimated*	56.8	79.5	90	94.7
Time	100	0	0	0
Hemiptera	22.8	43.1	55	65.5
Diptera	23.9	43.3	54.1	64.6
Ephemeroptera	24.9	41.5	53.1	63.8
Trichoptera	22.5	41.6	52.4	62.4
Odonata	22.3	40.9	52	61.8
Coleoptera	22.4	41.6	52.7	62.6
Gasterosteiformes	22.1	41.3	52.6	62.2
Haplotaxida	24.2	43.5	55.3	65.7
Diplostraca	23.7	39.3	51.5	62.8
Basommatophora	22	40.3	52.2	63.1
Heterostropha	23.2	42.6	54.3	64.3
Veneroida	23	42.4	53.8	63.9
Neotaenioglossa	22.5	42.2	53.6	63.6
Rhynchobdellida	22.1	41	52.1	61.9

*PCA on environmental dataset was performed including (Env Estimated) and excluding (Env Not estimated) the estimated values of water chemistry parameters

Table S6. Summary of PCA on pesticide and environmental data. Presented are cumulative percentages of variance explained by the first four Principal Components (PC), percentages of variance explained by each PC (Individual %) and component loadings.

Dataset	PC axis	1	2	3	4
Pesticide data	Cumulative % Pesticides	81.7	91.9	96.5	99.4
	Individual %	81.7	10.2	4.6	2.9
	Ethiofencarb	0	0	0	0
	Chlorprofam	-0.007	-0.074	-0.037	-0.053
	Pirimiphos-methyl	0.369	-0.055	0.928	0.013
	Tolclophos-methyl	0.042	-0.029	0.118	-0.025
	Prochloraz	-0.040	-0.029	-0.022	0.999
	Carbendazim	1.000	-0.003	-0.022	0.001
	Imazalil	-0.058	-0.025	-0.008	-0.021
	Imidacloprid	0.035	0.999	0.025	0.009
	Isoproturon	0.088	-0.054	0.015	-0.037
	Methiocarb	0	0	0	0
Environmental data*	Cumulative % Estimated	56	78.2	88.2	94.3
	Individual %	56	22.2	10	6.1
	Temperature	0.090	-0.324	0.006	-0.340
	Dissolved oxygen	0.111	-0.349	0.041	-0.877
	DOC	-0.421	0.879	-0.184	-0.121
	Phosphate	0.054	0.409	0.908	-0.044
	Macrophyte coverage	0.986	0.163	-0.044	-0.006
	Nitrite	0.070	0.226	0.458	0.250
	Nitrate	-0.025	0.212	0.381	0.277
Environmental data	Cumulative % Not estimated	56.8	79.5	90	94.7
	Individual %	56.8	22.7	10.5	4.7
	Temperature	0.167	-0.302	0.131	0.827
	Dissolved oxygen	0.131	-0.172	-0.241	-0.460
	DOC	-0.416	0.883	-0.206	0.059
	Phosphate	-0.041	0.440	0.889	-0.054
	Macrophyte coverage	0.985	0.172	-0.028	0.002
	Nitrite	-0.008	0.219	0.454	-0.070
	Nitrate	-0.078	0.210	0.343	-0.427

*PCA on environmental dataset was performed including (Estimated) and excluding (Not estimated) the estimated values of water chemistry parameters

Table S7. Summary of PCA on the Hellinger-transformed macrofauna abundance on the level of Order (N=79): cumulative percentage of variance explained by the first four Principal Components (PC) and component loadings

PC Axis	1	2	3	4
Cumulative %	22.1	40.9	51.9	61.6
Hemiptera	-0.463	-0.097	-0.160	-0.438
Diptera	0.161	0.490	0.701	-0.210
Ephemeroptera	0.299	0.774	-0.427	0.256
Trichoptera	0.107	0.292	0.438	0.018
Plecoptera	-0.173	-0.006	-0.036	-0.039
Odonata	0.101	0.644	0.017	-0.198
Coleoptera	-0.017	-0.100	0.242	-0.235
Lepidoptera	-0.041	0.107	-0.119	0.211
Megaloptera	-0.133	-0.059	-0.024	0.032
Collembola	-0.079	0.074	-0.031	-0.092
Gasterosteiformes	-0.471	-0.226	-0.040	-0.496
Cypriniformes	-0.119	-0.078	0.158	-0.178
Rhynchobdellida	-0.017	0.000	-0.187	0.233
Haplotaxida	0.184	-0.503	0.392	0.536
Tricladida	-0.013	-0.164	0.164	0.053
Isopoda	0.069	-0.032	-0.222	0.338
Ostracoda (Class)	0.007	0.242	0.190	-0.228
Cyclopoida	0.320	-0.408	0.027	0.014
Diplostraca	0.802	-0.471	-0.254	-0.189
Copepoda (Subclass)	-0.058	-0.022	-0.123	0.006
Amphipoda	0.036	0.143	0.380	0.033
Arguloida	-0.060	-0.089	0.016	0.064
Anostraca	0.002	-0.076	0.065	0.013
Mysida	0.116	-0.094	0.123	0.270
Basommatophora	-0.757	-0.300	-0.250	-0.025
Heterostropha	-0.280	-0.377	-0.027	0.556
Veneroida	0.036	0.191	-0.125	0.172
Neotaenioglossa	-0.398	0.098	-0.229	0.375
Architaenioglossa	-0.137	-0.154	0.024	0.029
Acari (Subclass)	-0.198	0.000	0.078	-0.185

Table S8. Summary of PCA on the Hellinger-transformed macrofauna abundance on the level of order (N=145): cumulative percentage of variance explained by the first four Principal Components (PC) and component loadings

PC Axis	1	2	3	4
Cumulative %	9.8	17.7	24.6	31.2
Hemiptera	9.4	17.3	24.8	31.2
Diptera	-0.056	0.030	0.248	0.255
Ephemeroptera	0.741	0.145	0.055	-0.063
Trichoptera	0.419	0.224	-0.173	-0.569
Plecoptera	0.684	0.205	0.017	0.248
Odonata	-0.118	0.404	0.676	-0.106
Coleoptera	0.557	-0.111	0.059	-0.537
Lepidoptera	0.346	0.182	0.163	0.200
Megaloptera	0.201	-0.012	-0.160	-0.267
Collembola	-0.193	0.546	0.555	-0.175
Gasterosteiformes	0.030	0.021	0.541	-0.175
Cypriniformes	-0.209	-0.104	0.380	0.303
Rhynchobdellida	-0.012	-0.039	0.042	0.000
Haplotaxida	-0.413	0.389	-0.207	-0.076
Tricladida	-0.022	0.278	-0.029	0.149
Isopoda	-0.272	-0.004	-0.202	0.145
Ostracoda(Class)	-0.120	0.506	-0.389	-0.203
Cyclopoida*	0.221	-0.218	0.312	-0.114
Diplostraca	-0.287	0.462	-0.328	-0.043
Copepoda(Subclass)	-0.041	0.676	-0.232	-0.005
Amphipoda	0.157	0.224	-0.089	0.254
Arguloida	0.516	0.369	-0.235	0.282
Anostraca	-0.056	-0.223	0.190	0.042
Mysida	0.070	-0.038	-0.001	0.352
Basommatophora	-0.226	0.128	0.133	-0.149
Heterostropha	-0.305	0.136	0.123	-0.024
Veneroida	-0.149	0.359	-0.051	-0.045
Neotaenioglossa	0.211	-0.023	-0.135	-0.593
Architaenioglossa	-0.072	0.301	0.073	-0.150
Acari(Subclass)	-0.062	-0.186	-0.011	0.088

Table S9. Average values and standard deviations (in italic) of macrofauna abundances on the level of order at the sampling sites. Abbreviations can be found in Figure 1.

	Hem	Dipt	Eph	Trich	Plec	Odo	Col	Lep	Meg	Col	Gast	Cypr	Rhyn	Hapl
D1	18.8	31.9	119.1	0.3	0.0	5.4	0.8	0.1	0.0	15.8	2.2	0.1	0.4	7.4
	12.6	*71.4*	*106.6*	*0.9*	*0.0*	*5.6*	*1.0*	*0.3*	*0.0*	*49.3*	*3.5*	*0.3*	*1.0*	*23.1*
D2	21.2	106.5	286.6	2.7	0.0	23.1	5.7	0.0	0.0	3.5	0.1	0.0	0.0	7.4
	29.6	*104.5*	*487.7*	*3.5*	*0.0*	*32.3*	*9.7*	*0.0*	*0.0*	*9.4*	*0.3*	*0.0*	*0.0*	*23.4*
D3	5.8	435.3	5.8	135.3	0.0	0.5	18.3	0.0	0.0	0.0	0.5	0.0	0.0	44.8
	5.7	*757.8*	*3.6*	*181.3*	*0.0*	*1.0*	*25.4*	*0.0*	*0.0*	*0.0*	*0.6*	*0.0*	*0.0*	*64.2*
D4	9.8	8.3	52.0	0.5	0.0	0.0	167.5	0.0	0.0	0.0	4.0	0.0	0.0	38.5
	10.7	*6.3*	*31.1*	*0.6*	*0.0*	*0.0*	*333.7*	*0.0*	*0.0*	*0.0*	*8.0*	*0.0*	*0.0*	*51.9*
P1	44.1	7.5	39.1	0.6	0.2	0.8	2.2	0.0	0.3	0.9	6.3	0.1	0.9	45.8
	56.8	*8.4*	*34.4*	*1.1*	*0.6*	*1.3*	*3.8*	*0.0*	*0.9*	*1.5*	*9.3*	*0.3*	*1.1*	*98.6*
P2	37.8	4.5	45.1	0.4	0.4	0.5	1.0	0.0	1.7	1.9	1.2	0.0	1.0	13.4
	41.3	*9.2*	*69.5*	*0.7*	*1.3*	*0.8*	*1.5*	*0.0*	*5.0*	*5.0*	*2.8*	*0.0*	*1.6*	*15.8*
P3	20.5	3.5	132.5	1.3	0.0	5.0	2.5	1.0	0.3	0.3	1.0	1.0	0.3	17.5
	19.1	*2.6*	*80.8*	*1.9*	*0.0*	*5.0*	*3.3*	*2.0*	*0.5*	*0.5*	*1.4*	*1.4*	*0.5*	*12.6*
P4	11.5	1.5	34.0	0.0	0.0	0.5	3.0	0.8	0.0	0.8	0.5	0.0	2.0	50.0
	11.1	*0.6*	*44.0*	*0.0*	*0.0*	*1.0*	*4.8*	*1.5*	*0.0*	*1.0*	*1.0*	*0.0*	*2.3*	*57.7*
F1	49.0	6.2	12.8	0.1	0.1	0.3	1.2	0.0	0.0	0.1	2.1	0.7	0.0	24.2
	48.2	*12.3*	*12.3*	*0.3*	*0.3*	*0.7*	*1.4*	*0.0*	*0.0*	*0.3*	*6.0*	*1.3*	*0.0*	*26.7*
F2	27.5	3.3	18.6	1.2	0.0	0.0	1.3	0.1	0.2	0.1	6.3	0.0	0.9	13.1
	63.0	*8.1*	*32.0*	*3.5*	*0.0*	*0.0*	*3.8*	*0.3*	*0.4*	*0.3*	*13.0*	*0.0*	*1.4*	*20.2*
F3	1.7	39.5	7.5	0.3	0.0	0.0	2.2	0.0	0.0	0.0	0.7	0.0	0.7	30.7
	2.3	*59.3*	*8.8*	*0.8*	*0.0*	*0.0*	*3.3*	*0.0*	*0.0*	*0.0*	*1.2*	*0.0*	*1.0*	*32.0*
F4	19.5	23.1	21.7	0.0	0.0	0.1	0.9	0.0	1.6	1.7	3.8	0.1	4.4	10.4
	27.4	*65.1*	*28.3*	*0.0*	*0.0*	*0.3*	*1.1*	*0.0*	*4.7*	*4.7*	*4.0*	*0.3*	*6.0*	*15.5*
F5	16.0	1.4	26.8	0.0	0.0	0.0	0.8	0.0	0.0	0.0	9.4	0.0	0.7	69.5
	17.5	*2.0*	*33.1*	*0.0*	*0.0*	*0.0*	*1.1*	*0.0*	*0.0*	*0.0*	*27.6*	*0.0*	*1.1*	*83.1*
F6	47.8	6.9	7.5	1.7	0.0	0.0	2.4	0.0	0.1	3.6	2.6	0.1	1.2	38.6
	63.3	*12.9*	*8.5*	*3.9*	*0.0*	*0.0*	*3.4*	*0.0*	*0.3*	*11.4*	*3.7*	*0.3*	*2.1*	*55.4*
F7	33.2	26.7	58.8	0.3	0.0	0.3	0.7	0.0	0.0	1.7	33.3	0.0	0.3	128.6
	48.8	*62.3*	*53.6*	*0.9*	*0.0*	*0.9*	*0.9*	*0.0*	*0.0*	*5.4*	*70.1*	*0.0*	*0.5*	*240.2*
F8	32.2	4.5	31.0	0.1	0.0	0.1	1.6	0.0	0.2	1.0	12.3	1.3	0.7	11.0
	28.5	*5.8*	*28.9*	*0.3*	*0.0*	*0.3*	*2.9*	*0.0*	*0.6*	*3.2*	*22.2*	*4.1*	*0.7*	*11.9*
F9	51.2	5.3	194.3	0.5	0.0	1.0	0.0	0.0	0.5	0.0	2.8	0.5	3.3	11.8
	36.4	*5.1*	*250.8*	*0.8*	*0.0*	*2.4*	*0.0*	*0.0*	*0.8*	*0.0*	*3.7*	*1.2*	*5.4*	*21.8*
F10	19.1	2.4	30.2	0.1	0.0	0.1	1.9	0.0	0.1	0.0	4.2	0.1	1.2	6.8
	12.4	*3.9*	*38.2*	*0.3*	*0.0*	*0.3*	*3.2*	*0.0*	*0.3*	*0.0*	*8.8*	*0.3*	*2.6*	*13.2*

Tricl	Isop	Ostr	Cycl	Dipl	Cop	Amp	Arg	Anos	Mys	Bas	Het	Ven	Neot	Arch	Aca
0.0	1.0	0.2	0.3	69.7	0.0	0.1	0.0	0.0	0.0	24.7	0.3	61.1	1.1	0.0	1.1
0.0	*3.2*	*0.6*	*0.7*	*131.0*	*0.0*	*0.3*	*0.0*	*0.0*	*0.0*	*28.2*	*0.7*	*127.2*	*3.1*	*0.0*	*1.9*
0.0	0.0	18.0	0.5	22.0	0.0	0.0	0.0	0.0	0.0	18.0	21.4	32.9	2.6	0.0	0.8
0.0	*0.0*	*40.5*	*1.6*	*44.1*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*38.0*	*51.7*	*66.8*	*6.9*	*0.0*	*1.1*
0.0	0.0	0.0	0.5	28.0	0.0	31.0	0.0	0.0	0.0	7.0	18.3	0.8	9.3	0.0	6.5
0.0	*0.0*	*0.0*	*1.0*	*56.0*	*0.0*	*20.7*	*0.0*	*0.0*	*0.0*	*10.9*	*20.8*	*1.5*	*15.3*	*0.0*	*9.3*
0.0	12.5	0.0	0.5	324.3	0.0	4.5	0.0	0.0	0.0	7.0	8.3	0.0	0.3	0.0	0.0
0.0	*13.0*	*0.0*	*1.0*	*273.5*	*0.0*	*4.2*	*0.0*	*0.0*	*0.0*	*2.9*	*15.8*	*0.0*	*0.5*	*0.0*	*0.0*
0.0	2.1	2.0	3.2	223.7	7.8	0.1	0.6	0.0	0.9	27.9	24.7	9.9	6.3	0.1	1.0
0.0	*3.1*	*6.3*	*5.5*	*455.1*	*16.8*	*0.3*	*1.1*	*0.0*	*1.4*	*22.4*	*28.8*	*24.4*	*10.9*	*0.3*	*1.6*
0.2	2.9	0.0	2.1	1032.7	86.5	0.6	0.1	0.0	1.0	35.7	17.5	5.1	4.6	0.0	3.4
0.6	*4.6*	*0.0*	*2.8*	*3241.5*	*271.8*	*1.9*	*0.3*	*0.0*	*1.8*	*52.6*	*16.9*	*5.2*	*4.8*	*0.0*	*8.0*
0.0	5.8	0.0	5.0	290.3	0.0	5.5	0.0	0.0	0.0	10.5	7.8	1.0	8.3	0.0	2.8
0.0	*5.1*	*0.0*	*7.1*	*274.8*	*0.0*	*6.4*	*0.0*	*0.0*	*0.0*	*6.6*	*12.3*	*1.4*	*7.4*	*0.0*	*5.5*
0.0	12.8	0.0	0.3	104.8	0.0	2.0	0.0	0.0	0.0	47.3	9.3	3.8	8.5	0.0	0.5
0.0	*5.4*	*0.0*	*0.5*	*196.9*	*0.0*	*2.2*	*0.0*	*0.0*	*0.0*	*41.7*	*15.2*	*4.1*	*7.7*	*0.0*	*0.6*
0.4	2.0	0.0	6.5	155.1	1.0	1.8	0.1	0.3	5.5	12.5	24.5	5.3	2.4	0.0	4.3
1.3	*2.3*	*0.0*	*9.3*	*311.6*	*3.2*	*2.9*	*0.3*	*0.9*	*10.9*	*18.6*	*23.4*	*9.7*	*3.0*	*0.0*	*6.0*
0.0	1.7	0.0	0.6	25.3	1.2	0.3	0.0	0.0	2.4	63.1	47.8	10.1	31.3	0.0	0.2
0.0	*1.7*	*0.0*	*1.3*	*47.7*	*3.8*	*0.5*	*0.0*	*0.0*	*4.1*	*49.3*	*57.4*	*11.1*	*27.9*	*0.0*	*0.4*
0.3	2.0	0.0	10.0	6.5	0.0	0.2	0.0	0.0	0.8	19.2	17.0	1.5	2.2	0.0	0.8
0.8	*2.1*	*0.0*	*15.8*	*9.5*	*0.0*	*0.4*	*0.0*	*0.0*	*1.3*	*23.0*	*19.9*	*2.8*	*2.7*	*0.0*	*2.0*
1.3	2.3	0.0	46.1	1197.5	0.0	0.4	0.3	0.0	0.4	32.4	38.7	4.3	2.0	0.0	0.0
4.1	*4.6*	*0.0*	*62.4*	*1684.6*	*0.0*	*1.3*	*0.9*	*0.0*	*1.0*	*24.8*	*29.1*	*6.4*	*3.9*	*0.0*	*0.0*
0.1	2.6	0.0	78.7	782.0	0.0	0.2	0.0	0.0	0.3	38.8	83.8	3.5	5.2	1.2	0.7
0.3	*2.6*	*0.0*	*225.7*	*1321.4*	*0.0*	*0.4*	*0.0*	*0.0*	*0.7*	*36.2*	*96.1*	*6.4*	*6.3*	*3.8*	*1.3*
0.5	3.5	0.0	7.0	60.1	0.3	0.0	0.6	0.0	0.1	50.9	17.4	0.4	0.4	0.0	0.6
1.6	*10.0*	*0.0*	*15.0*	*117.6*	*0.9*	*0.0*	*1.3*	*0.0*	*0.3*	*60.7*	*18.7*	*0.7*	*0.7*	*0.0*	*1.1*
0.1	3.0	0.0	8.5	331.7	0.3	0.0	0.0	0.1	0.1	98.8	20.1	5.7	4.2	0.0	0.1
0.3	*7.2*	*0.0*	*21.8*	*629.9*	*0.9*	*0.0*	*0.0*	*0.3*	*0.3*	*212.5*	*16.0*	*11.3*	*5.7*	*0.0*	*0.3*
0.0	2.5	0.0	7.9	19.5	4.5	0.4	0.0	0.0	0.9	77.0	25.5	1.9	8.7	0.0	1.5
0.0	*4.2*	*0.0*	*12.4*	*42.8*	*14.2*	*1.3*	*0.0*	*0.0*	*2.8*	*66.4*	*27.4*	*2.4*	*10.8*	*0.0*	*3.2*
0.0	32.5	0.0	77.3	1345.2	10.8	3.7	0.0	0.0	0.0	50.8	22.7	6.5	9.5	0.0	2.7
0.0	*47.7*	*0.0*	*106.8*	*1452.2*	*16.8*	*5.4*	*0.0*	*0.0*	*0.0*	*34.3*	*22.2*	*4.2*	*17.0*	*0.0*	*3.4*
0.3	8.3	0.0	10.4	2155.0	0.0	0.6	0.0	0.0	0.0	130.6	70.1	2.0	35.2	0.0	1.5
0.9	*9.6*	*0.0*	*22.6*	*4252.7*	*0.0*	*1.3*	*0.0*	*0.0*	*0.0*	*149.5*	*84.5*	*2.3*	*43.9*	*0.0*	*2.9*

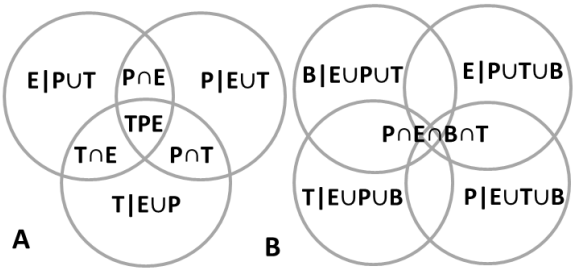

Figure S1. Visual representation of variance components estimated for total macrofauna species composition (A) and each macrofauna group on the level of order (B)

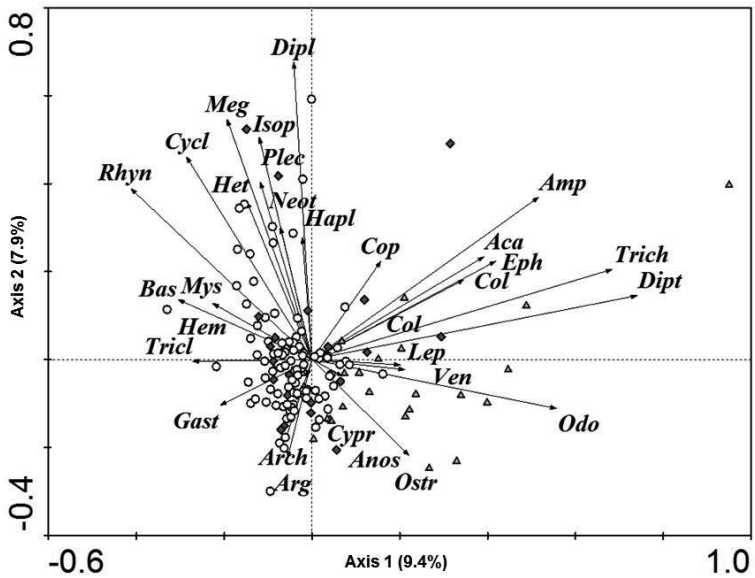

Figure S2. Principal component analysis of Hellinger-transformed macrofauna abundance on the level of Order (N=145). Abbreviations can be found in Figure 1.

CHAPTER 4

VARIANCE IN TRAIT MODALITY DISTRIBUTION OF AQUATIC MACROFAUNA EXPLAINED BY PESTICIDES IN THE FIELD

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Abstract

Analyzing functional characteristics of species helps to understand the impacts of various stressors on aquatic communities in the field. This research aimed to study the effects of pesticides combined with other environmental factors (temperature, dissolved oxygen, dissolved organic carbon, coverage with floating macrophytes, phosphate, nitrite, nitrate), and time (seasonal and annual variation) on the trait modality distribution of aquatic macrofauna. To address the research aim, field work was performed in the flower bulb growing area of the Netherlands characterized by intensive agricultural activities. Field work included sampling of macrofauna in ditches next to flower bulb fields followed by taxonomic identification, measurements of physico-chemical parameters and pesticide concentrations. Each taxon was classified into the trait modalities of nine traits (feeding mode, respiration mode, locomotion type, diapause form, reproduction mode, life stage, voltinism, saprobity, maximum body size). Relationships between trait modality distribution per trait, pesticides, and environmental factors were analyzed with redundancy analysis (RDA). A variance partitioning based on the redundancy analysis (RDA) was applied to divide the total variance in trait modality distribution per trait into the variance explained by pesticides, environmental factors and time. On average, the largest proportion of variance in the trait composition was explained by environmental factors (11.2%) followed by time (7.2%) and pesticides (2.2%). To obtain a mechanistic understanding of community responses to pesticides, trait composition of aquatic communities should be analyzed explicitly, in combination with taxonomic composition.

Keywords: aquatic community, traits, chemical stress, environmental factors, pesticides

Introduction

Traditionally, the responses of biotic communities to human-induced disturbances have been evaluated based on the taxonomic approach, i.e. estimating the species diversity or the performance of selected indicator species (Mouillot et al., 2006). During the recent decades, the use of trait approaches, i.e. characterizing communities according to the functional characteristics, gained an increasing interest. The reason is that functional traits are supposed to provide an insight in the mechanisms underlying community responses to disturbances (Poff, 1997; Statzner et al., 2010). Information obtained using trait-based approaches can be extrapolated to a broader range of species, geographical zones and ecotypes (Baird et al., 2008; Dolédec et al., 2006; Charvet et al., 2000). These approaches have been successfully developed for a wide array of plant (for instance, Engelhardt 2006; Quétier et al., 2007; Suding et al., 2008) and animal communities, e.g. invertebrates (for instance, Poff et al., 2006; Charvet et al., 2000; Dolédec et al., 2006; Vieira et al., 2006; Magbauna et al., 2010; Menezes et al., 2010; Statzner et al., 2010; Ippolito et al., 2012).

The trait approach has been also adopted by ecotoxicologists. Liess & Von Der Ohe (2005) developed a trait indicator of community responses to pesticides. Ippolito et al. (2012) modelled the effects of pesticides on the trait composition of invertebrate communities. Rubach et al. (2010) linked traits of aquatic invertebrate species to pesticides. Yet, the applicability and the predictive potential of the trait approach in quantifying the effects of pesticides on aquatic communities in the multi-stressor field conditions require further understanding.

A number of key drivers may influence the performance of aquatic biota in water systems around agricultural areas. First, the use of pesticides in the agricultural fields results in the presence of pesticide mixtures in surface waters. Therefore, aquatic biota may be affected by mixtures of pesticides. Second, nutrients (phosphorus and nitrogen) are commonly applied in the fields to enhance yields and are often transported to surface waters in relatively large amounts along with pesticides (EPA, 2012; Tilman et al., 2002). Nutrients affected the responses of aquatic invertebrates to pesticides in the laboratory and semi-field conditions (Alexander et al., 2013; Ieromina et al., 2014a,b). Third, other physico-chemical parameters are highly variable in surface waters around agricultural fields. As an example, transportation of sediment particles from agricultural fields to surface waters has been shown to increase the levels of suspended/dissolved solids and dissolved organic carbon (DOC) in water (Ruark et al., 2009; Neung-Hwan et al., 2013). Various drivers concurrently affect the aquatic biota in agricultural areas. However, to which extent these drivers affect trait composition, i.e., the distribution of the modalities per trait within the community, remains poorly understood. As far as we know, no research has tried to distinguish the effects of pesticides on the trait modality distribution of aquatic communities from the effects of environmental factors and time.

This study therefore aimed 1) to analyze relationships between trait modality distributions, environmental factors and pesticides, and investigate the functional traits of aquatic communities in ditches adjacent to flower bulb fields and in watersheds of nature reserve 2) to quantify what proportion of the total variance in trait composition of aquatic macrofauna community can be explained by pesticides, other environmental factors and time. Traits likely to respond to chemical stress, such as traits related to the external exposure (feeding mode, life stage), internal sensitivity (respiration mode, maximum body size), population recovery (locomotion type, diapause form, voltinism, reproduction mode) (as classified by Rubach et al., 2011) and ecological tolerance (saprobity) were analyzed. We hypothesized that pesticides explain a larger proportion of variance in community trait composition than other environmental factors and time, because traits directly relate to the mechanisms of pesticide effects on communities and therefore are expected to be a sensitive indicator of pesticide pollution.

Materials and methods

Macrofauna sampling, measurements of environmental parameters and pesticide concentrations

Field work was performed in a freshwater ditch system located in the flower bulb growing region of the Netherlands. Samples were collected from a total of 16 sites in the research area repeatedly in the period April-November 2011 - 2012: 12 sites located in ditches next to flower bulb fields and pastures, and 4 sites located in watersheds of a nature reserve close to the flower bulb growing area. A detailed description of the research area, macrofauna sampling strategy, and taxonomic identification level for each group, procedures for measurements of physico-chemical water parameters and pesticide concentrations, as well as basic data on macrofauna community composition and abundances, water chemistry and pesticide concentrations can be found in O. Ieromina et al. (submitted). In brief, macrofauna samples were collected using a dipping net dragged over the total length of 5 m using multihabitat sampling strategy. The following water chemistry parameters were monitored: temperature (T, ^{o}C), dissolved oxygen (DO, mg/L), pH, conductivity (mS), dissolved organic carbon (DOC, mg/L). Coverage with floating macrophytes (Macr) (expressed in percentage) was estimated to account for habitat structure. Measurements of the concentrations of phosphate ($PO4^{-}$), nitrite ($NO2^{-}$), nitrate ($NO3^{-}$) and pesticides commonly applied in bulb fields (chlorprofam, pirimiphos-methyl, tolclophos-methyl, carbendazim, ethiofencarb, imidacloprid, isoproturon, imazalil, methiocarb, and prochloraz) were performed in the OMEGAM laboratory (Amsterdam, the Netherlands) using the standardized protocols.

Assigning trait modalities

First, each species was classified into the preselected trait modalities of nine traits: feeding mode, locomotion type, diapause form, voltinism, reproduction mode, life stage, respiration mode, body size and saprobity (Table 1). Trait data were found in the online database *www.freshwaterecology.info* (Schmidt-Kloiber & Hering, 2012 accessed in the years 2012 - 2014, last accessed 04.04.2014) or in literature. If a species was characterized by more than one modality of a trait, each of these modalities was assigned a coefficient ranging from 0 to 1, depending on how the given modality is represented in this species. For instance, the trait "feeding mode" included 7 modalities: deposit feeding, predating, grazing, shredding, filter feeding, gathering, and parasite types of feeding. If a species feeds 80% by grazing and 20% by predation, then the modality "grazing" was assigned a coefficient 0.8 and modality "predation" was assigned a coefficient 0.2. If a species was characterized by one modality of a trait, this modality was assigned a coefficient of 1, and the other modalities of this trait were assigned a coefficient of 0. If trait data for a given species was not found in literature, the information on the other species from the same genus or family was used to estimate the missing data. If a larger taxonomic unit was analyzed (macrofauna was identified to the genus, family or order levels), the trait data most characteristic of this taxonomic group was included in the analysis. As a result, a matrix containing trait modality coefficients for each species was obtained.

Second, the trait data was weighted by the biomass. For that, the data on the maximum body size for each species was found in literature. After that, each trait modality coefficient of the given species was multiplied by the maximum body size and by the abundance of this species.

Table 1. List of traits and trait modalities analyzed in this study

Trait category	Trait*	Trait modality	Abbreviation
Physiological	Feeding mode	deposit feeders	FDep
		predators	FPred
		grazers	FGraz
		shredders	FShred
		filter feeders	FFIlt
		gatherers and/or collectors	FGath
		parasites	FPar
	Respiration mode	gill respiration	RGill
		aerial respiration (hydrostatic vesicle)	RAir
		plastron	RPlas
		tegument respiration	RTeg

Table 1. List of traits and trait modalities analyzed in this study (*Continued*)

Trait category	Trait*	Trait modality	Abbreviation
Dispersal	Locomotion type	scatting	LScat
		diving	LDiv
		sprawling, walking	LWalk
		sessile	LSess
		burrowing	LBur
	Diapause form	egg and/or statoblast	ResEgg
		cocoons	ResCoc
		houses against desiccation	ResHous
		diapause and/or dormancy	ResDiap
		quiescence	ResQui
		none	ResNone
Life history	Reproduction mode	ovoviviparity	ROviv
		free isolated eggs	RFreeE
		fixed clutches	RFixCl
		free clutches	RFreeCl
		clutches in vegetation	RClVeg
	Life stage	pupa	Pupa
		larvae	Larv
		adult	Ad
	Voltinism	semivoltine	Sev
		bivoltine	Biv
		multivoltine	Mult
		univoltine	Uni
		trivoltine	Triv
		flexible	Flex
Ecological	Saprobity	xenosaprob	Xeno
		oligosaprob	Oligo
		beta-mesosaprob	Beta
		alpha-mesosaprob	Alpha
		polysaprob	Poly
Morphological	Maximum body size	0.05 cm - 1 cm	0.05-1
		1cm - 2 cm	1-2
		2 cm - 5 cm	2-5
		5 cm – 10 cm	5-10

*Traits and trait modalities were selected based on the literature data: Rubach et al., 2011; Magbauna et al., 2010; Statzner et al., 2010; Vieira et al., 2006; Ippolito et al., 2012; Charvet et al., 2000

Statistical analysis

The relationships between the trait modality distribution per trait, environmental factors, and pesticides were analyzed with redundancy analysis (RDA). Trait modalities per trait weighed by biomass were included in the analysis as response variables, while the concentrations of individual pesticides (chlorprofam, pirimiphos-methyl, tolclophos-methyl, carbendazim, ethiofencarb, imidacloprid, isoproturon, imazalil, methiocarb, and prochloraz) and environmental factors (temperature, DO, DOC, nitrate, nitrite, phosphate, macrophyte coverage) were explanatory variables. Time (the number of the month and the number of the year) was included in the analysis as covariable. The number of explanatory variables (17) was lower than the number of objects (79) fulfilling the requirement of RDA.

According to Legendre & Birks (2012), before the ordination analysis, data need to be examined with regard to the symmetry of data distribution (normality) and the difference in measurement units between variables. Therefore, prior to RDA, the skewness (the symmetry of distribution), the kurtosis (the shape of the distribution), and Shapiro-Wilk test (normality of distribution) were calculated for each variable. These tests showed that the data were asymmetric and not normally distributed. To increase the normality, data were log (x+1) transformed. As a result, skewness and kurtosis values decreased. For many parameters, skewness reached the range between -2 and 2, which corresponds to the univariate normal distribution (George & Mallery, 2010). Log-transformation also removed the effect of measurement units. Results of RDA analysis were presented in triplots. Only explanatory variables having high correlation with the first two ordination axis (correlation coefficient above 0.2) were displayed in RDA triplots (similar to the study of Wesolek et al., 2010). Significance of the first RDA axis and the significance of the sum of all canonical axes per trait were tested by a Monte Carlo permutation test (based on 999 unrestricted permutations). Eigenvalues, F-ratios and p-values were derived. If the first ordination axis was not significant for a given trait ($p>0.1$), the RDA plot for this trait was not shown.

To quantify the percentage of variance in trait composition explained by pesticides, environmental factors and time, the variance partitioning based on pRDA was applied, following the method described in Borcard et al. (1992). The total variance was divided into five components: variance explained by pesticides, other environmental factors, time, shared variance between three components and unexplained (residual) variance. Variance partitioning was performed following the procedure described in Ieromina et al. (submitted). The variance components differed by the number of explanatory variables. According to Freedman (1983), the number of explanatory variables affects the explained variance (R^2). To account for the different number of explanatory variables in variance components, first a Principal Component Analysis (PCA) was performed on each explanatory variable dataset. After that, sample scores of the first four Principal Components (PC) were derived and included in the RDA as explanatory variables. In addition, Ezekiel adjustment was applied

to the R^2 (according to Peres-Neto et al., 2006). In the manuscript, we refer to R^2 adjusted. Before the multivariate analysis, data were centered by species, and not centered by sample (Lepš & Šmilauer, 2003). Normality tests were performed in SPSS software (Version 21, IBM Corp. Released 2012). Multivariate analysis was performed in Canoco software v.4.5 (Lepš & Šmilauer).

4 Results

Linking trait modalities, pesticides and environmental factors

The relationships between the trait modalities, pesticides and environmental factors are shown in RDA triplots (Figure 1 and 2). Figure S1 (Supplemental Data) shows the relative contribution of traits modalities per trait for each sampling site. The first ordination axis for traits locomotion type and maximum body size was not significant, and RDA triplots for these traits are not shown.

High biomass of predators was associated with watersheds of the nature reserve (Figure S1). The biomass of animals breathing through a plastron was negatively correlated to the concentrations of phosphate and tolclophos-methyl. Gill respiration was characteristic of agricultural ditches (Figure S1). Biomass of macrofauna in their pupa and larvae life stage negatively correlated to phosphate, while the biomass of adults was positively correlated to nitrite and negatively to the dissolved oxygen (Figure 2). Large biomass of macrofauna in the larvae and pupa life stage was associated with watersheds of the nature reserve (Figure 2, S1). Semivoltine animals were typical for agricultural ditches (Figure S1). The biomass of animals reproducing by clutches in vegetation was positively correlated to isoproturon and was found in high amounts in agricultural ditches (Figure 2, S1). Biomass of animals reproducing by free clutches, ovoviviparity and fixed clutches was negatively correlated to tolclophos-methyl, chlorpropham and phosphate (Figure 2). The biomass of animals having diapause form was negatively correlated to phosphate (Figure 2). High biomass of these animals was associated with the nature reserve (Figure 2, S1). In contrast, the biomass of animals without a diapause form and a resistance form of egg or statoblast was positively correlated to macrophyte coverage and chlorprofam, respectively (Figure 2). Biomass of xenosaprobic species was positively correlated to the dissolved oxygen (Figure 2).

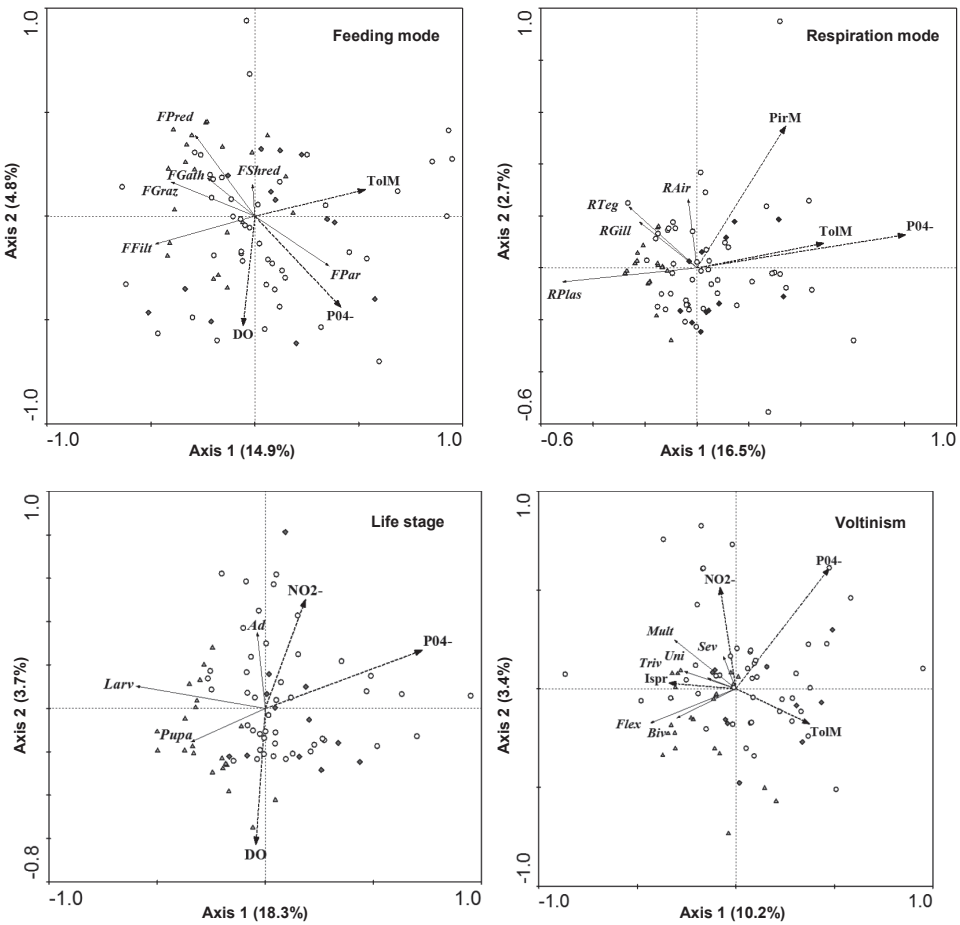

Figure 1. Redundancy analysis triplot of macrofauna trait distribution per trait in relation to pesticides and environmental factors (for traits feeding mode, respiration mode, life stage, voltinism). Abbreviations for trait modalities can be found in Table 1. Dashed lines represent pesticides and environmental factors, solid lines represent trait modalities. Chlor = chlorprofam, PirM = pirimiphos-methyl, TolcM = tolclophos-methyl, Imid = imidacloprid, Ispr = isoproturon. Triangular = sites sampling sites in watersheds of nature reserve, circles = sampling sites in ditches next to flower fields, diamonds = sampling sites in ditches next to pastures.

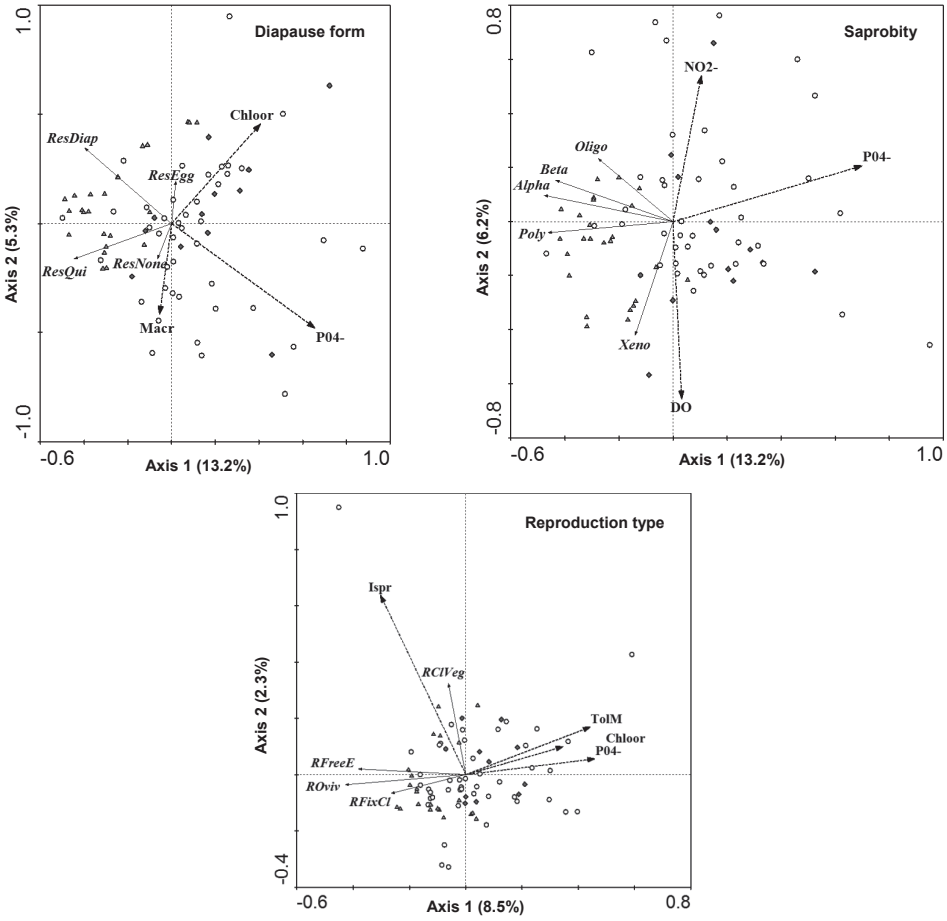

Figure 2. Redundancy analysis triplot of macrofauna trait distribution per trait in relation to pesticides and environmental factors (for traits diapause form, saprobity, reproduction type). Legend can be found in Figure 1.

Table 2. Summary of Monte Carlo test identifying the significance of the first canonical axis and the significance of all canonical axes in RDA, as presented in Figures 1 and 2

Trait		Test of significance of the first canonical axis	Test of significance of all canonical axes
Diapause form	Eigenvalue	0.132	0.222
	F-ratio	11.2	1.4
	p-value	0.011*	0.057**
Reproduction type	Eigenvalue	0.125	0.165
	F-ratio	11.4	1.1
	p-value	0.049*	0.371
Saprobity	Eigenvalue	0.138	0.218
	F-ratio	11.0	1.3
	p-value	0.057**	0.128
Respiration	Eigenvalue	0.165	0.211
	F-ratio	13.4	1.2
	p-value	0.056**	0.197
Feeding mode	Eigenvalue	0.149	0.224
	F-ratio	12.2	1.4
	p-value	0.025*	0.069**
Voltinism	Eigenvalue	0.102	0.187
	F-ratio	7.8	1.1
	p-value	0.063**	0.318
Aquatic life stage	Eigenvalue	0.183	0.239
	F-ratio	14.7	1.4
	p-value	0.064**	0.115
Locomotion type	Eigenvalue	0.126	0.193
	F-ratio	9.7	1.1
	p-value	0.298	0.363
Body size	Eigenvalue	0.363	0.201
	F-ratio	10.1	1.1
	p-value	0.416	0.384

*p<0.05
**p<0.1

Table 3. Components of variance estimated for macorfauna trait composition: total explained variance (P∪E∪T), residual variance, variance explained by pesticides (P|E∪T), environmental factors (E|P∪T), time (T|E∪P) and shared variance (P∩E∩T). Presented are the explained variance (R^2) and R^2 adjusted by Ezekiel's transformation (in italic)

Trait	PUEUT	Residual variance	P|EUT	E|PUT	T|PUE	P∩E∩T
Diapause form	28.0	72.0	2.3	10.9	7.8	7.0
	31.5	*68.5*	*2.5*	*12.2*	*8.7*	*7.8*
Reproduction type	25.1	74.9	1.0	5.1	14.4	4.6
	28.2	*71.8*	*1.0*	*5.6*	*16.1*	*5.1*
Saprobity	23.2	76.8	2.5	12.8	6.0	1.9
	26.1	*73.9*	*2.7*	*14.3*	*6.7*	*2.0*
Respiration	22.3	77.7	2.1	10.2	5.5	4.5
	25.1	*74.9*	*2.2*	*11.4*	*6.1*	*5.0*
Feeding mode	21.0	79.0	1.5	13.8	5.4	0.3
	23.6	*76.4*	*1.6*	*15.5*	*6.0*	*0.2*
Voltinism	19.3	80.7	2.3	8.0	6.1	2.9
	78.3	*2.5*	*8.9*	*6.8*	*3.1*	*21.7*
Aquatic life stage	19.2	80.8	1.7	12.4	5.9	-0.8
	21.6	*78.4*	*1.8*	*13.9*	*6.5*	*-1.0*
Locomotion type	18.1	81.9	1.6	7.7	5.1	3.7
	20.3	*79.7*	*1.7*	*8.6*	*5.6*	*4.1*
Body size	15.2	84.8	3.5	9.0	1.9	0.8
	17.1	*82.9*	*3.8*	*10.0*	*2.0*	*0.8*
Average R^2 adjusted	23.9	76.1	2.2	11.2	7.2	3.0

Variance partitioning of trait modality distribution

The average total explained variance in trait community composition was 23.9% with the minimum of 17.1% found for trait body size and the maximum of 28.0% found for trait diapause form (Table 2). On average, the largest proportion of variance was explained by environmental factors (11.2%, varying in the range 5.6% - 15.5%), time (7.2%, varying in the range 2.0% - 16.1%), followed by the shared variance between three components (3.0%, varying in the range 0.8% - 7.8%) and pesticides (2.2%, varying in the range 1.0% - 3.8%) (Table 2).

Discussion

The effects of pesticides and other environmental factors on the trait modality distribution

The trait modality distribution of aquatic macrofauna was influenced by pesticides and environmental factors (Figure 1 and 2). Remarkably, nutrients affected the distribution of all trait modalities. Such high importance of nutrients in structuring aquatic macrofauna can be possibly explained by the presence of nutrient gradients in surface waters, as a result of relatively high fertilizer application at the flower bulb fields (Centraal Bureau voor de Statistiek, 2015). Previous studies highlighted the importance of nutrient gradients in structuring aquatic biota in freshwater ecosystems. Verdonschot (1992) showed that nutrients along with the acidity and the extent of droughts determine the differences in macrofauna community structure in small ponds in the Netherlands. As found in the study of Scheffer et al. (2002), after the nutrient enrichment, the vegetation structure in a freshwater ecosystem (independently of its type) becomes dominated by phytoplankton. As a result, water turbidity increases, while dissolved oxygen concentration and the amount of light decrease. Subsequently, nutrients induce direct and indirect effects on aquatic biota.

High biomass of predators was found in watersheds of the nature reserve (Figure 1). Predators represent the upper level of the food chain and depend on organisms of lower trophic levels in terms of food source. The effects of stressors on organisms of lower trophic levels are likely to be negatively reflected in the performance of predators. The highly disturbed environment of agricultural ditches was not favorable for predators that were found in high amounts in clean waters of nature reserve.

The biomass of plastron–breathing animals was negatively correlated to nutrients and pesticides. This result suggests a high dependence of plastron-breathing invertebrates, exchanging oxygen and carbon dioxide in a thin layer of air around the body (Flynn & Bush, 2008), on the water quality. In contrast, the biomass of aerial-breathing insects using a hydrostatic vesicle for respiration was not strongly correlated to pesticides and environmental factors. Species using a hydrostatic vesicle obtain the air from an air bubble attached to the body (Database http://www.freshwaterecology.info/), and therefore do not fully depend on the chemical composition of the water for respiration. The biomass of animals with tegument respiration (through the body surface) was also not correlated to pesticides and environmental factors. The high biomass of animals with gill respiration was characteristic of agricultural ditches.

The biomass of pupa was positively correlated to dissolved oxygen and negatively to nutrients, suggesting that the clean waters of the nature reserve were suitable to accommodate macrofauna at this sensitive life stage. Macrofauna having a semivoltine reproduction cycle was associated with ditches of the agricultural area. In contrast, Díaz et al. (2008) reported that semivoltinism is a characteristic feature of an undisturbed environment. As a possible explanation, mollusks (semivoltine species) generally tolerant to organic pollution were

found at high numbers in agricultural ditches. The reproduction by clutches in vegetation was associated with agricultural ditches, where high macrophyte abundance created favorable conditions for reproduction of species fixing egg clutches.

Variance partitioning

The variance partitioning results revealed that the environmental factors other than pesticides explained the largest proportion of variance for five out of nine traits. The environmental factors had the largest contribution (15.5%) to the variance in the trait feeding mode. This can be explained by the high dependence of food availability for macrofauna on environmental conditions. For the remaining four traits, time constituted the most important factor. Time accounted for the largest proportion of variance in reproduction type (16.1%). This can be explained by the seasonal succession of macrofauna exhibiting different reproduction types throughout the year.

In contrast to our hypothesis, pesticides explained the smallest proportion of variance in trait composition from all factors. A similar result was observed in the analysis of the taxonomic composition of macrofauna from the same research area (Ieromina et al., submitted). Trait modality distribution of several traits differed between agricultural ditches and watersheds of the nature reserve. This result suggests that macrofauna in agricultural ditches could possibly adapt to toxic stress. As found in our study, traits more typical to agricultural ditches (gill and aerial respiration, reproduction by clutches in vegetation, semivoltinism) possibly helped to ensure resilience of the macrofauna community to pesticides. In contrast, traits of the nature reserve (predation, xenosaprobity, presence of diapause form) were characteristic features of undisturbed environment.

The current study showed that the trait composition of aquatic communities next to agricultural fields is influenced by pesticides and other environmental factors. Understanding the responses of community composition to disturbances is important with regard to biodiversity conservation (Vandewalle et al., 2010). Species diversity and ecosystem resilience are closely related, and the resilience increases with the number of species within each functional group (Cleland, 2011; Ives & Carpenter, 2007; Naeem, 1998). As a general rule, communities containing high species diversity better withstand natural or anthropogenic disturbances than communities containing low species diversity, because communities with more species are likely to have a large variety of functional characteristics (trait modalities) that facilitates adaptation of such communities to changing conditions (Cleland, 2011). Like Fischer et al. (2006) concluded, conservation measures should aim at preserving species diversity within and between different functional groups.

Conclusions

Trait-based approaches are increasingly used to assess and monitor the health of ecosystems. As found in our study, the trait modality distribution differed between ditches next to flower

bulb fields and nature reserve. Pesticides did not induce a larger effect on the community trait composition than environmental factors, in contrast to the initial hypothesis. However, our data indicates that the presence of an excess of nutrients and pesticides affects the distribution of certain macrofauna trait modalities (related to feeding, respiration, reproduction, ecological tolerance), hinting that macrofauna traits can potentially be used as a tool to monitor the ecological status of aquatic ecosystems in environmental assessment practices.

Acknowledgments

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Supporting information

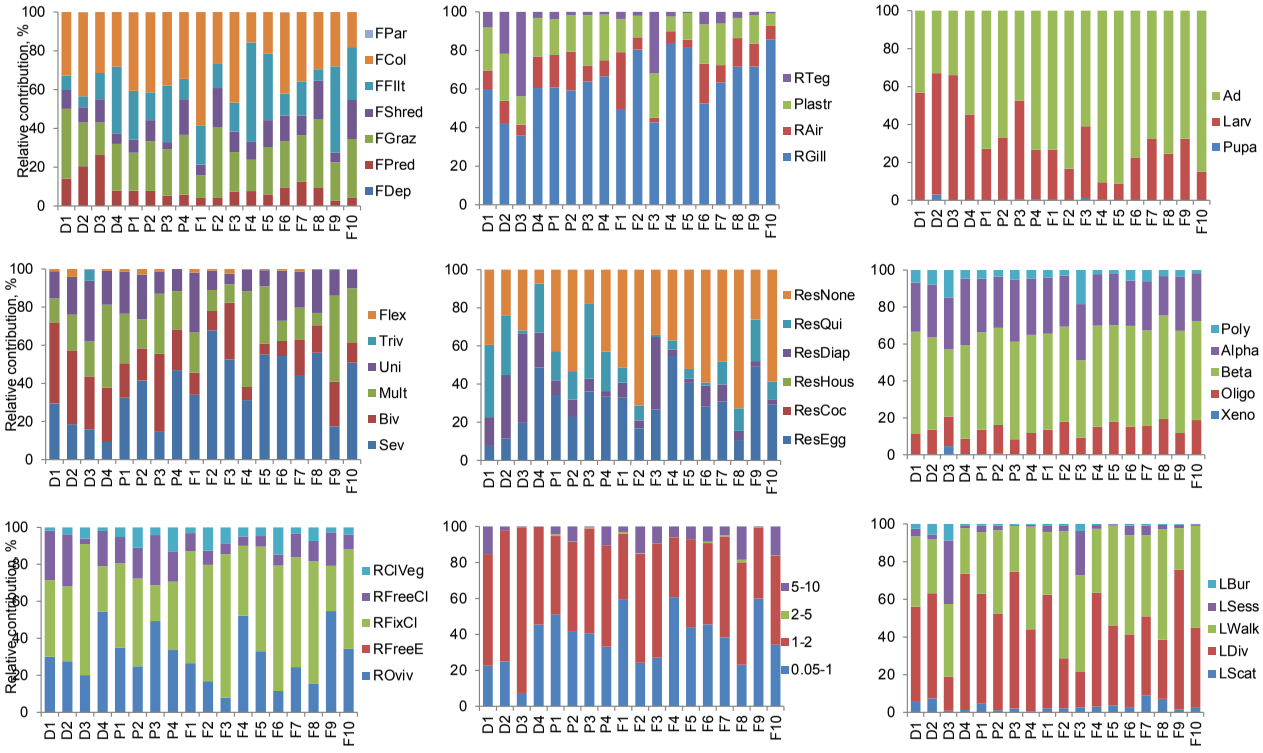

Figure S1. Relative contribution of trait modalities per trait for each study site. For legend, see Table 1. D = sites in watersheds of nature reserve, P = sites in ditches next to pastures, F = sites in ditches next to flower bulb fields

CHAPTER 5

POPULATION RESPONSES OF *DAPHNIA MAGNA*, *CHYDORUS SPHAERICUS* AND *ASELLUS AQUATICUS* IN PESTICIDE CONTAMINATED DITCHES AROUND BULB FIELDS

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Abstract

The goal of this study was to investigate the effects of ambient concentrations of pesticides combined with abiotic factors on the key aquatic species *Daphnia magna*, *Chydorus sphaericus* and *Asellus aquaticus* by means of 21 days field exposure experiments. In situ bioassays were deployed in ditches around flower bulb fields during spring and autumn 2011 - 2012. The results showed that phosphate was the most variable parameter followed by pesticides expressed as toxic units, as the main factors explaining the differences between sites. Variation in reproduction and growth of cladoceran *D. magna* was largely explained by nutrients. Dissolved oxygen contributed mostly to variations in reproduction of *C. sphaericus*, while dissolved organic carbon contributed to variations in growth of the detrivore *A. aquaticus*. Abiotic stressors rather than pesticides contribute significantly to the performance of aquatic invertebrates in the field and should be explicitly considered when evaluating effects of pesticides on aquatic organisms.

Keywords: macroinvertebrates, ditch system, pesticides mixtures, abiotic factors, in situ bioassays

Introduction

Cultivation of flower bulbs in the Netherlands accounts for 93% of the total world flower bulb production (Jansma et al., 2002). Maintaining balance between high yields and low risk to the environment is a main purpose of the environmental policy in the Netherlands (Van Eerdt et al., 2007). The amount of pesticides used in bulb crops has reduced in 1985 – 1990, however despite this fact the amount of chemicals applied in flower bulb fields remains relatively high: 41.9 kg/ha of pesticides were for instance used in the bulb crops in 2008 (Centraal Bureau voor de Statistiek, data from 2008). This number is considerably higher compared to pesticide use in other crops: e.g. 3.2 kg/ha was used in 2008 in open ground vegetable cultivation (Centraal Bureau voor de Statistiek, data from 2008). Pesticides applied in flower bulb crops can enter ditches surrounding agricultural fields through different routes: direct spray, leaching from the soil, runoff and spillage from pesticide containers (Van Wijngaarden et al., 2004). Another important emission route is leakage from the baths where bulbs are disinfected by fungicide treatments before planting to prevent infestation of the bulbs with fungal infections, such as botrytis (Van Kan, 2005). These emissions lead to contamination of surface waters with pesticides. Pesticide concentrations in ditches exceed water quality standards at many locations in the Netherlands (Vijver et al., 2008).

To increase the soil fertility, nitrogen and phosphate are also applied extensively in bulb crops in the form of dairy manure and fertilizers. The maximum permitted amounts of nitrogen and phosphate allowed to be used in flower bulb cultivation in 2002 were 265 kg/ha and 85 kg/ha, respectively (Jansma et al., 2002). However, excessive application of nutrients subsequently leaking to surface waters, results in overall deterioration of water quality leading to eutrophication and adverse effects on aquatic biota.

The ecological effects of ambient concentrations of mixtures of pesticides in ditches surrounding arable fields are poorly studied. Studies have focused mainly on the environmental fate of pesticides in ditches (Renaud et al., 2008; Wan et al., 2006) and on measures to reduce risks of pesticide transferal from the agricultural fields to the surrounding water bodies (De Snoo & De Wit, 1998, Margoum et al., 2006). Ecological effects on aquatic biota in the field are not easy to link to pesticide concentrations because of the uncertainties arising from interactions between pesticides and abiotic factors that results in high data complexity. In situ experiments have proven to reduce the uncertainty in the extrapolation of laboratory data to field responses, as they are a step closer to the realistic field situation (Burton et al., 2005; Schulz, 2003; Domingues et al., 2008; Rand, 2004; Arts et al., 2006; De Jong & Udo de Haes, 2001). To our knowledge, not many studies have focused on the effects of diffusive pesticide contamination on aquatic invertebrates in ditches. Therefore, our study aimed to evaluate responses of aquatic invertebrates to ambient concentrations of a mixture of pesticides in combination with abiotic factors (nutrients, DOC, dissolved oxygen, temperature) in ditches next to flower bulb fields. Although individual pesticides levels were expected to be below critical values for invertebrates, we hypothesized that mixtures

of pesticides combined with abiotic factors will induce significant effects on invertebrates. The cladocerans *D. magna* and *C. shpaericus* were expected to be more vulnerable to pesticide contamination than *A. aquaticus* because of their generally higher sensitivity to toxicants. The aquatic isopod *A. aquaticus* in turn was expected to be the most resistant species to pesticides because it is well established in literature as a species highly tolerant to pollution (Hynes, 1960). Nitrogen and phosphate were expected to contribute significantly to the effects on cladocerans *D. magna* and *C. shpaericus* because nutrients were shown to be important factors affecting growth and reproductive performance of cladocerans (Elser et al., 2001; Urabe et al., 1997; Seidendorf et al., 2010).

Materials and methods

Research area

The research area is located on the territory of two polders (see Figure 1). The research area is intensively used for flower bulb growing, mainly hyacinths, lilies, daffodils and tulips. In addition, there are several patches of pastures and grasslands. Flowers in the area are grown on sandy-rich soil, which makes the leaching of pesticides to the surface- and groundwater considerably high. The ditch sediment in the area consists mostly of medium and fine sand.

Seasonal crop rotation results in the application of different pesticides used continuously in the period February - November. Several pesticides are applied to protect a single crop against pests. Hence, mixtures of pesticide residues were expected to be found in the surface waters. In situ experiments of 21 day duration were deployed in spring and autumn during the years 2011 and 2012 (4 experiments in total).

On the North and North–East the research area is surrounded by sandy dunes, a nature reserve area located above sea level (Supplemental Data Figure S1). There is a natural elevation gradient in the two polder areas in South–West direction: a gradual decrease in the height above sea level from sandy dunes towards the polders (Supplemental Data Figure S1).

The water in polder 1 originates from two sources: 1) from the sandy dunes area West of the polder via the ground water and 2) from the channels that are connected to the lake Oosterduinse Meer (Figure 1) via a water pump. Water from polder 1 is supplied to polder 2 North through two inlets: part of the water enters the high elevation area around site P1, and the main part of the water on the Southern side of the polder via the emergency outlet. In polder 2 North water flows in the South–West direction mainly by a natural gradient. Site F4 is located just outside polder 2 South below the sea level and is the lowest experimental site.

In the research area, in situ experiments were deployed at eight sites in the polder area: six ditches adjacent to flower bulb fields (F1, F2, F3, F4, F5, F6) and two ditches adjacent to pastures surrounded by flower fields (P1 and P2). Two control sites were located in the sandy dunes area North of the polders (D1 and D2) (Figure 1). Based on the hydrological information, we hypothesized that contamination levels at the North–East side of the polder

Inlet
Direction of the water flow
Emergency outlet
Direction of the elevation gradient
Regular outlet
Inlet to the other polder
* Location pesticide measurements
..... High water area
_ _ Low water area
Line separating polders

1 = Polder Het Langeveld (polder 1), 2 = Noordzijderpolder-Noord (polder 2 North), 3 = Noordzijderpolder-Zuid (polder 2 South), D = sites in watersheds in the nature reserve, F = sites in ditches next to flower fields, P = sites in ditches next to the pastures

Figure 1. Map of the research area

area next to sandy dunes (located above sea level) would be lower than at the South–West side of the area (located below sea level) (Supplemental Data, Figure S1). Therefore, we expected a gradient of pesticide residue concentrations in the area with the sites ordered in the following way: D1-D2>F2>P1>F7>P2>F3>F6–F5>F4 (Figure 1).

In order to verify the hypothesis, the distance between each experimental site and the nature reserve was calculated. Toxic units (TU) calculated for each site were then plotted versus the distance from the nature reserve (most North–West point at the polder 1). Additionally, toxic units were plotted versus the elevation for each experimental site. Data were analyzed with linear regression.

Pesticide measurements

The selection of pesticides for analytical measurements was based on the analysis of authorized pesticides as used in flower growing. Additionally, a historical database of physico-chemical water properties of Waterboard Rijnland (province Southern Holland, year 2010) was analyzed (Van Rooden et al., 2011). Major pesticides applied in the study area were identified and 10 pesticides were selected for measurements (chlorprophm, pirimiphos-methyl, tolclophos-methyl, carbendazim, ethiofencarb, imidacloprid, isoproturon, imazalil, methiocarb, prochloraz) (Supplemental Data, Table S1). Concentrations of these pesticides were measured by Omegam laboratoria BV (Amsterdam, Netherlands) using GC–MS and LC-MS/MS.

Physico-chemical water parameters

Temperature (°C), dissolved oxygen (DO, mg/L), and oxygen saturation (%) at experimental sites were measured with an Oxygen meter Z521 Consort. pH was measured with a Greisinger electronic pH-meter. Conductivity was measured with a conductivity-meter Eijkelkamp Agriresearch Equipment. Conductivity, T, DO and pH were measured at the start and at the end of each experiment. The average value of each parameter was used in the statistical analysis. Dissolved Organic Carbon (DOC) concentrations were quantified using non-dispersive infrared analysis (NDIR). Phosphate, nitrate, and nitrite were measured according to NEN 6663 and NEN-EN-ISO 13395 respectively (OMEGAM laboratory, Amsterdam, The Netherlands). DOC, phosphate, nitrate, and nitrite levels were measured at the end of the experiment.

Test species

Three species (*Daphnia magna*, *Chydorus shpaericus* and *Asellus aquaticus*) were selected as they are important components of food web in aquatic ecosystems. The selected species *D. magna* and *C. sphaericus* belong to the order Cladocera. They have similar modes of ingesting food (filter-feeding) and respiration (integumentary). *D. magna* (adult size 2 - 4 mm) is a planktonic species that has a high ecological importance serving as a food source for larger crustaceans and fish. The smaller-sized *C. sphaericus* (adult size

0.3 - 0.5 mm) is a meiobenthic cladoceran. It plays an important role in the food web by transferring organic matter into biomass that is subsequently consumed by invertebrates and fish (Pieters et al., 2008 and Dekker et al., 2006). The aquatic isopod *A. aquaticus* lives at the river/pond bottom. It is mainly detritivore feeding on particulate organic matter (Graça et al, 1994).

Juveniles of *D. magna* and *C. sphaericus* were obtained from a laboratory culture (National Institute of Public Health and Environment, RIVM, The Netherlands). *A. aquaticus* adult males and females were collected in ditches around nature reserve areas and maintained in the laboratory during 1 month with a 16/8 h light photoperiod and 20° C. *A. aquaticus* juveniles and adults were fed with a diet consisting of dry leaves and fish food. Air was constantly supplied to each aquarium. To provide shelter, black plastic tubes and/or stones were placed at the bottom of aquaria. Once per week ditch water was filtered and 50% was refreshed with Dutch Standard Water (DSW). The variation in temperature, water hardness, nitrate, and nitrite concentrations were recorded weekly with indicator stripes TetraSet (Tetra 6 in 1 Test Kit, Tetra®).

Experimental design

The enclosures for *D. magna* were composed of glass cylinders of 500 ml volume with a 6 cm diameter opening on one side. The opening was covered with fine mesh (mesh size 150 µm) allowing water to exchange with the outside environment, at the same time keeping animals inside the cage. The enclosures for *C. shpaericus and A. aquaticus* were constructed from polyethylene cylinders of 100 ml volume with a 3.5 cm diameter opening on one side closed with mesh (mesh size 150 µm). At each site, 10 juveniles of *D. magna* (36 - 48 h old), *C. sphaericus* (36 - 48 h old) and *A. aquaticus* (2 - 3 weeks old) were placed in each cage and three replicate cages were fixed at each site. As a food source, in each cage with *D. magna*, five drops of algae *Pseudokirchneriella subcapitata* were added, in each cage with *C. shpaericus* – three drops of algae *Nitzschia perminuta* were added, and in cages with *A. aquaticus* – one dry leaf and two pellets of fish food were added. The cages were fixed in the ditch horizontally. Enclosures were retrieved after 21 days.

Response measurements

Initial size measurement of *D. magna* and *C. sphaericus* juveniles (36 - 48 h old) subsampled from the permanent laboratory cultures at RIVM was done prior to field experiments. Initial size measurements of *A. aquaticus* juveniles (2 - 3 weeks old) were performed one day before field deployment. *D. magna* body length was defined as the distance from the most posterior point on the head to the base of the junction of the tail spine with the carapace (Barry, 1998). *C. sphaericus* body length was defined as the distance from the posterior point on the head to the end of the carapace. *A. aquaticus* body length was defined as the distance between the base of the antennae until the top of the pleotelson.

After 21 days, cages were retrieved. Survival of *A. aquaticus* was estimated as the percentage of surviving animals relative to the initial number of animals placed in the cage. *A. aquaticus* reaches maturity in 20 weeks and was not expected to produce juveniles within 21 days. Therefore, reproduction was not estimated for *A. aquaticus*. Juveniles of *D. magna* and *C. sphaericus* were counted. *C. sphaericus* adult females size varies in the range 0.3 mm – 0.5 mm according to Balcer et al. (1984). For *C. sphaericus* all individuals smaller than 0.3 mm were classified as juveniles and all individuals larger than 0.3 mm were considered adults. *D. magna* body length at maturity varies in the range 1.4 mm – 2.0 mm according to Kee & Ebert (1996) and 2.1 mm – 4.0 mm according to Ebert (1994). Therefore, in our study *D. magna* individuals smaller than 2.5 mm were considered juveniles and all individuals larger than 2.5 mm were counted as adults.

Body morphometric parameters of adults were measured. For each species, the mean body length of adults at day 21 was calculated and was used to estimate the somatic growth rate (SGR) according to:

$$SGR = \frac{\mathrm{Ln(Li)} - \mathrm{Ln(L0)}}{\mathrm{d}},$$

where L *i* = mean body length at day 21, L0 = mean body length at day 1, d = total number of days (21 day). The mean values for the survival, growth, and reproduction were calculated from the three replicates and used in statistical analysis.

Data treatment

To analyze experimental data, we used Principal Component Analysis (PCA) and General Linear Model (GLM). PCA was applied to the dataset containing environmental and pesticide data to identify the most variable parameters. Environmental data for PCA analysis were log-transformed. PCA was constructed based on environmental data derived in each of the four experiments. Additionally, all environmental data obtained in four experiments were combined in one dataset and analyzed with PCA. The toxic unit approach is commonly used in evaluation of pesticide mixture toxicity (SCHER, SCCS, SCENIHR, 2012). Concentrations of pesticides at each sampling site were therefore expressed as Toxic Units (TU):

$$\sum_{i=1}^{n} TUi = \frac{\mathrm{Ci}}{NOEC21d, D.magna},$$

where, TU_i is the toxic unit of the pesticide *i, Ci*- is the concentration (mg/L) of the pesticide *i*; and NOEC- the corresponding NOEC (21d) of *D. magna* exposed to substance *i* (mg/L). Toxic units based on $NOEC_{21d,D.\ magna}$ for *D. magna* were also relevant for *C. sphaericus* because these species belong to the same taxonomic group (order Cladocera) and have

similar physiological traits. To investigate the effects of pesticides on the performance of *A. aquaticus* we used the TU based on NOEC for *D. magna*.

To quantify the impact of pesticides and other field relevant factors on the performance of *D. magna, C. sphaericus* and *A. aquaticus* under field conditions (e.g. to link environmental and biota datasets), a GLM was applied, following the general equation:

$$N_i = \alpha + \beta_1 * Abiotic\ Factor1 + \beta_2 * Abiotic\ Factor2 + \ldots + \beta_N * Abiotic\ Factor\ N$$

where α = intercept; *Abiotic Factor1…Abiotic Factor N* = explanatory variables (environmental parameters T, TU, P, Nitrate, Nitrite, DOC, DO); *Ni* = response variable (estimated endpoints).

One of the assumptions of GLM is that explanatory variables are independent. Electric conductivity reflects the amount of inorganic dissolved material in water and its ability to pass electric current. Nitrate and phosphate anions raise the water conductivity (APHA, 1992). Because conductivity is indirectly correlated to phosphate and nitrate concentrations, it was not included in the model. According to the results of PCA, pH did not vary significantly between locations. Additionally, the pH range did not exceed tolerance ranges for the species (pH = 7.2 –8.6). Therefore, pH was also not included in the model. Because toxic units varied a factor of 800 (the highest TU = 8.2 and the lowest TU = 0.01) between locations, values for toxic units were log transformed. For site F1 (year 2011–2012), P1 (year 2012) and F6 (year 2011) where pesticide concentrations were not measured, environmental parameters except toxic units were included in the GLM.

Mean squares of all explanatory variables were calculated. The variable having the largest mean square was added to the GLM first. Remaining explanatory variables were added to the GLM by the order of decreasing their mean square values. After every explanatory variable was added to the model, the percentage of variance explained by the model was calculated. In addition, relationship between response variables (endpoint) and environmental variables that explained at least 20% of variation in the endpoint, as identified by GLM, was analyzed with linear regression analysis. Statistical analyses were performed in GenStat software Version 13.1.0.4470 (VSN International Ltd).

Results and discussion

Variation in the water chemistry data

The best fit between the toxic units and distance from the nature reserve area was observed for the data collected in autumn 2012 ($R^2 = 0.605$ and $R^2 = 0.597$ respectively) (Supplemental Data, Figure S2). In other seasons, there was no gradient in pesticide concentrations depending on the distance or on the altitude. In Autumn 2011, the differences in contamination levels between the sites were the largest (Supplemental Data, Table S1). The highest toxic unit were observed for the site F4 (TU = 8.172) which was likely ascribed to the concentration

of the insecticide pirimiphos-methyl exceeding the NOEC (Supplemental Data, Table S1). The highest average concentration of phosphate was also found at the F4 (Figure 2). Site F4 is distant from the nature reserve and is the lowest site in the area located below sea level (Supplemental Data Figure S1). Water to the ditch where F4 site was deployed is supplied from the rural area next to the polder. This water may already carry substantial amounts of nutrients and pesticides resulting in higher contamination levels at site F4.

Our results thus showed distinct contamination levels at the experimental sites in the four experiments, likely determined by a combination of at least three factors: a) natural flow of water from the dunes to both polders, b) pumping activities by the Water Board and c) agricultural activities in the area around the ditches where the in situ experiments were deployed.

Table 1 represents the PCA on environmental variables obtained in four experiments combined in one dataset. The 1st Principal Component explained 64.9% of the variance in environmental data. The component loading for phosphate was highest in the PC 1 (0.612). The second PC explained 21.4% of variance and was mainly represented by pesticides (−0.779) (Table 1).

A similar trend was observed over time: in all four experiments, phosphate constituted the first principal component and accounted for the largest percentage of variance in the dataset (Supplemental Data, Table S2).

Reproduction and growth of C. sphaericus

The highest mean reproductive output of *C. sphaericus* was recorded at the nature reserve site (D2) (Figure 3). Sites D1 and D2 were characterized by the lowest levels of phosphate and highest concentrations of dissolved oxygen typical for the high water quality of the nature reserve. This observation was consistent with the results of the GLM (Table 2).

Table 1. Results of PCA of water chemistry data collected in four experiments

PC Axis	1	2
% variance	64.9	21.4
Conductivity	0.154	0.267
Dissolved Oxygen	-0.119	0.055
Dissolved Organic Carbon	0.126	0.090
Nitrate	0.418	0.025
Nitrite	0.335	0.058
Phosphate	0.612	0.554
Temperature	-0.015	-0.014
TU	0.533	-0.779
pH	-0.023	0.010

Variance in *C. sphaericus* reproduction was best explained by dissolved oxygen (that constituted 30.5% of total variance, positive correlation) followed by DOC (9.7%, positive correlation) and TU (6.1%, negative correlation) (Table 2, Figure 4). A positive relationship between *C. sphaericus* reproduction and dissolved oxygen concentration was confirmed by linear regression analysis (Supplemental Data, Figure S4). In addition, the sum of nitrate and nitrite concentrations was positively correlated to *C. sphaericus* growth and explained 6.7% of variance in the endpoint (Table 2; Supplemental Data Figure S3).

Table 2. Summary statistics for General Linear Regression (GLM) between the endpoints estimated for three species, TU and abiotic factors

Response variable	Explanatory variable	β-coefficient	s.e.	F stat	p-value	% explanatory variable	% cumulative
Reproduction	Constant	-59	25.6	-2.3	0.031*		
C. sphaericus	DO	1641	0.848	1.94	0.067	30.5	46.5
	DOC	0.048	0.017	2.81	0.010*	9.7	
	TU	-9.03	4.8	-1.88	0.074*	6.1	
	T	2.2	1.2	1.84	0.080**	0.2	0.2
Reproduction	Constant	48.9	19.5	2.51	0.020*		
D. magna	Nitrite_Nitrate	132.8	65.7	2.02	0.055	25.9	31.2
	P	14.75	8.91	1.66	0.112	5.3	
Survival	Constant	218	37.2	5.86	<.001*		
A. aquaticus	T	-10.5	1.8	-5.82	<.001*	45	66.6
	DO	4.06	1.16	3.5	0.002*	17.7	
	TU	5.55	5.87	0.94	0.356	2.6	
	Nitrite_Nitrate	18.6	13.5	1.38	0.181**	1.3	
Growth	Constant	0.389	0.032	12.15	<.001*		
D. magna	DO	0.021	0.005	4.66	<.001*	27.3	64.5
	P	0.021	0.005	4.39	<.001*	31.7	
	DOC	0.000	8.45E-05	-2.14	0.048*	4.6	
	TU	0.024	0.020	1.2	0.247	0.9	0.9
Growth	Constant	0.144	0.016	9.11	<.001*		
C. sphaericus	Nitrite_Nitrate	0.081	0.050	1.63	0.118	6.7	6.7
Growth	Constant	0.5	0.163	3.07	0.006*		
A. aquaticus	DOC	0.0004	0.0001	3.34	0.003*	43.6	46.5
	Nitrite_Nitrate	0.104	0.071	1.47	0.159	1.1	
	T	-0.012	0.009	-1.31	0.207	1.8	

s.e. = standard error, F stat = F statistic, % explanatory variable = percentage of variance explained by the variable, % cumulative = cumulative percentage of explained variance, Nitrite_Nitrate = sum of nitrite and nitrate concentrations
*p<0.05, **p<0.1

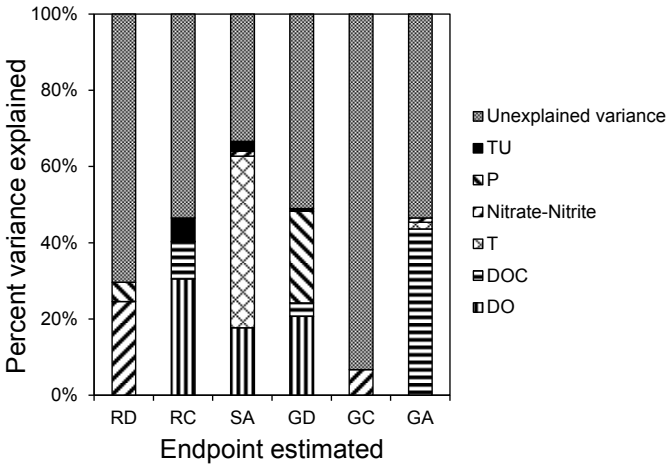

Figure 2. Histogram showing the contribution of each explanatory variable to the total variance explained by the general linear model (GLM). The figure is based on the results presented in the Table 2. RD = reproduction of *D. magna,* RC = reproduction of *C. sphaericus,* SA = survival of *A. aquaticus,* GD = growth of *D. magna,* GC = growth of *C. sphaericus,* GA = growth of *A. aquaticus*

Reproduction and growth of D. magna

D. magna reproductive output was the highest at site F4, located next to the flower field characterized by the highest phosphate levels and toxic units (Figure 3). According to the results of the GLM, variance in *D. magna* reproduction was largely determined by sum of nitrate and nitrite, and phosphate (that explained 31.2% of variation) (Table 2, Figure 4). A similar result was observed for *D. magna* growth: the highest percent of variance in growth (31.7%) was explained by phosphate followed by dissolved oxygen (27.3%) (Table 2, Figure 4). A positive effect of nutrients on the performance of *D. magna* was also confirmed by results of linear regression between *D. magna* reproduction and growth versus nutrients: positive regression coefficients were found (Supplemental Data, Figure S4). According to the PCA results, phosphate was the most variable parameter (Table 1). A previous study of Janse & Van Puijenbroek (1998) also indicated that because of nutrient leakage from agricultural fields, ditches in the Netherlands receive continuous nutrient inputs. Nutrients represent an important factor affecting performance of cladocerans. Nutrients limit growth of unicellular algae that are further consumed by filter-feeding invertebrates (Cotner & Wetzel, 1992). In the semi-field study of Alexander et al. (2013), the effects of insecticides (applied at low concentrations) on aquatic insects reduced at the mesotrophic conditions. In a few instances, the abundances of insects exposed to low concentrations of insecticides increased at the mesotrophic conditions (Alexander et al., 2013). Similarly, better performance of *D. magna* in our study was observed at high nutrient levels in ditches within the agricultural area where

pesticides were found at ambient concentrations. Nutrients appeared to be more important in controlling *D. magna* reproduction and growth than pesticides. Reduction in survival at the relatively short scale (2 - 21 days) is commonly a result of acute effects of chemicals applied at high concentrations that cause mortality. In the study of Baas et al. (2009), survival of *D. magna* in the field experiments conducted in the same region of the Netherlands was compared with model predictions. Mortality of *D. magna* in seven days in situ experiments was precisely predicted and could be related to pesticides, and in many cases to the insecticide pirimiphos-methyl in particular (Baas et al., 2009). In our study, such acute effects were not observed. Pesticide concentrations at ambient conditions were possibly too low to cause direct reduction in survival and reproduction of animals. However, similarly to the study of Baas et al. (2009), dissolved oxygen concentration affected significantly the performance of *D. magna*.

Survival and growth of A. aquaticus

Variation in survival of *A. aquaticus* was determined mainly by temperature and dissolved oxygen (in total explaining 62.7% variation in survival) (Table 2). The correlation coefficient between both survival and growth of *A. aquaticus* and temperature was negative and explained 45% and 1.8% of variance respectively (Table 2). This negative trend was confirmed by linear regression analysis: lower survival of animals was observed at higher temperatures (Supplemental Data, Figure 4). According to the study of Roshchin & Mazelev (1979), the most favorable temperature range for *A. aquaticus* growth at which energy use is optimized is 14.5–18.8° C. The authors suggest that at the higher temperatures, oxygen consumption by the animals is larger, which may result in a lower proportion of energy available to growth, leading to reduced growth rates. The water temperature in our study varied in the range of 15° C – 21° C. When the water temperature was high, which was also associated with reduced dissolved oxygen levels, the growth of animals was possibly impaired because more energy was spent for respiration than for growth. This finding was confirmed by GLM results showing that dissolved oxygen was the second important factor controlling *A. aquaticus* survival (explained 17.7% of variation, positive regression coefficient) (Figure 4). In our study, survival of *A. aquaticus* was reduced at higher temperatures exceeding the optimal range and at low dissolved oxygen levels.

The growth rate of *A. aquaticus* was positively correlated to DOC (explaining 43.6% of variation, positive regression coefficient) (Table 2, Figure 4, Supplemental Data, Figure S4). Elevated DOC levels in the ditches studied possibly stimulated algae growth, which was another food source for *A. aquaticus* in addition to the leaves added to cages at the beginning of experiment (Elvins, 2004).

Pesticides expressed as toxic units and nutrients did not cause an effect on the survival and growth of *A. aquaticus*. The aquatic isopod *A. aquaticus* is described to be a relatively tolerant species to pesticides (Hynes, 1960). This finding was confirmed by our results: DOC, dissolved oxygen and temperature, but not pesticides controlled survival and growth of *A. aquaticus*.

In our study, abiotic factors likely related to food availability for invertebrates (like nutrients, DOC) as well as abiotic factors fluctuating beyond the species tolerance limits (like temperature) explained the largest percentage of variation in survival, growth, and reproduction of animals.

Conclusions

In the current research *D. magna, C. sphaericus* and *A. aquaticus* were exposed in the field where ambient concentrations of pesticides are found. Pesticides present in water at low concentrations (expressed as TU) negatively affected reproduction of *C. sphaericus*. However no significant correlation between reproduction and growth of *D. magna* and pesticides was identified that was in turn positively affected by nutrients. Growth of *A. aquaticus* was positively correlated to DOC. Considering the fact that in agricultural fields, pesticides are often applied in combination with fertilizers, and that pesticides and nutrients interact strongly, it is important to include nutrients in the interpretation of field toxicity data. Our findings suggest a high importance of abiotic factors in structuring aquatic communities in realistic environments, underlining the importance of a multiple stressor approach in describing the field effects of pesticides.

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Supplemental information

Table S1. Concentrations of pesticides measured at the experimental locations (in µg/L)

	PirM	**Meth**	**Imzl**	**Pr**	**Carb**	**Ethfc**	**TolM**	**Ispr**	**Chlor**	**Imdc**	
Group	insecticide	insecticide	fungicide	fungicide	fungicide	insecticide	fungicide	herbicide	herbicide	insecticide	
DT50. days	117	28	stable	stable	stable	n.a.	9	1560	stable	stable	TU
NOEC. µg/L	0.08	0.1	10	12.5	16	16	100	120	450	1800	
LOD. µg/L	0.01	0.02	0.01	0.2	0.02**	0.05	0.01	0.01	0.02	0.05**	
D1-S-11	0.005	0.01	0.005	0.1	0.01	0.025	0.005	0.005	0.01	0.025	0.072
D2-S-11	0.005	0.01	0.005	0.1	0.01	0.025	0.005	0.005	0.01	0.025	0.072
P1-S-11	0.005	0.01	0.005	0.1	0.16	0.025	0.005	0.005	0.11	0.025	0.081
P2-S-11	0.005	0.01	0.005	0.1	0.18	0.025	0.005	0.005	0.06	0.025	0.082
F2-S-11	0.005	0.01	0.005	0.1	0.14	0.025	0.005	0.005	0.04	0.025	0.080
F7-S-11′	0.005	0.01	0.005	0.1	0.20	0.025	0.005	0.005	0.09	0.025	0.084
F3-S-11	0.005	0.01	0.005	0.1	0.12	0.025	0.03	0.005	0.06	0.025	0.079
F4-S-11	0.005	0.01	0.005	0.1	0.01	0.025	0.005	0.01	0.07	0.025	0.072
F5-S-11	0.005	0.01	0.005	0.1	0.01	0.025	0.005	0.005	0.07	0.025	0.072
F6-S-11	0.005	0.01	0.005	0.1	0.01	0.025	0.005	0.005	0.06	0.025	0.072
D1-A-11	0.005	0.01	0.005	0.1	0.01	0.025	0.005	0.005	0.01	0.025	0.072
D2-A-11	0.005	0.01	0.005	0.1	0.01	0.025	0.005	0.005	0.01	0.025	0.072
P1-A-11	0.15	0.01	0.005	0.1	2.9	0.025	0.005	0.005	0.01	0.13	2.065
P2-A-11	0.005	0.01	0.005	0.1	0.1	0.025	0.005	0.005	0.01	0.025	0.151
F7-A-11′	0.01	0.01	0.005	0.1	0.28	0.025	0.005	0.005	0.02	0.025	0.077
F3-A-11	0.005	0.01	0.02	0.1	0.04	0.025	0.005	0.005	0.01	0.025	0.075
F4-A-11	0.65	0.01	0.005	0.1	0.62	0.025	0.005	0.005	0.01	0.025	8.172

Table S1. Concentrations of pesticides measured at the experimental locations (in µg/L) (*Continued*)

	PirM	Meth	Imzl	Pr	Carb	Ethfc	TolM	Ispr	Chlor	Imdc	
Group	insecticide	insecticide	fungicide	fungicide	fungicide	insecticide	fungicide	herbicide	herbicide	insecticide	
F5-A-11	0.005	0.01	0.005	0.1	0.03	0.025	0.005	0.005	0.01	0.025	0.073
D1-S-12	0.005	0.01	0.005	0.1	0.01	0.025	0.005	0.005	0.01	0.025	0.072
D2-S-12	0.005	0.01	0.005	0.1	0.01	0.025	0.005	0.005	0.01	0.025	0.072
P2-S-12	0.005	0.01	0.005	0.1	0.03	0.025	0.005	0.005	0.01	0.025	0.073
F2-S-12	0.005	0.01	0.005	0.1	0.55	0.025	0.005	0.005	0.01	0.025	0.106
F3-S-12	0.005	0.01	0.005	0.1	0.21	0.025	0.005	0.005	0.01	0.025	0.084
F4-S-12	0.005	0.01	0.005	0.1	0.18	0.025	0.005	0.005	0.01	0.025	0.082
F5-S-12	0.005	0.01	0.005	0.1	2	0.025	0.005	0.005	0.01	0.025	0.196
F6-S-12	0.005	0.01	0.005	0.1	2	0.025	0.005	0.005	0.01	0.025	0.196
D1-A-12	n.d.	0.01*	0.005*	0.1*	n.d.	0.025*	0.005*	0.005	0.01*	0.02	0.009
D2-A-12	n.d.	0.01*	0.005*	0.1*	0.01	0.025*	0.005*	0.005	0.01*	0.02	0.009
P2-A-12	n.d.	0.01*	0.005*	0.1*	0.02	0.025*	0.005*	0.005	0.01*	0.03	0.010
F2-A-12	n.d.	0.01*	0.005*	0.1*	0.01	0.025*	0.005*	0.005	0.01*	0.04	0.009
F3-A-12	n.d.	0.01*	0.005*	0.1*	0.04	0.025*	0.005*	0.005	0.01*	0.36	0.011
F4-A-12	n.d.	0.01*	0.005*	0.1*	0.07	0.025*	0.005*	0.005	0.01*	0.24	0.013
F5-A-12	n.d.	0.01*	0.005*	0.1*	0.05	0.025*	0.005*	0.005	0.01*	1.04	0.012
F6-A-12	n.d.	0.01*	0.005*	0.1*	0.13	0.025*	0.005*	0.005	0.01*	0.05	0.017

Chlor = chlorprofam, Pir-meth = pirimiphos-methyl, Tolc-meth = tolclophos-methyl, Carb = carbendazim, Ethiofen = ethiofencarb, Imidacl = imidacloprid, Ispr = isoproturon, Imaz = imazalil Meth = methiocarb, Pr = prochloraz, DT50 = degradation time for 50% of a compound (hydrolysis, pH = 7, T = 20ºC), NOEC = No Observed Effect Concentration for *D. magna* (21 day test, endpoint reproduction), LOD = limit of detection, TU = sum of toxic units, n.d. = not detected, S = spring, A = Aautumn, n.a .= data not available

** LOD of carbendazim and imidacloprid for samples collected in autumn 2012 was 0.01 µg/L

′ at the site F7 pesticide concentrations were measured in 2011, but in situ experiments were not deployed, and data from the site F7 was used in the analysis of the trends in water chemistry data within the area

* in autumn 2012 concentrations of prochloraz, ethiofencarb, imazalil, tolclophos-methyl, chlorprofam and methiocarb were not measured, and were assumed to be below the limit of detection (additionally, given their high NOEC and low concentrations measured in previous seasons, these pesticides contributed negligibly to toxic units)

Table S2. Results of PCA of water chemistry data collected in each of the four experiments

	PC	1	2
	% variance	63.1	35.9
Spring 2011	Dissolved Oxygen	0.066	-0.033
	DOC	0.069	0.001
	Nitrite	-0.460	0.838
	Phosphate	0.880	0.433
	Temperature	0.008	0.312
	TU	0.033	0.104
	pH	-0.056	-0.002
	PC	1	2
Autumn 2011	% variance	70.7	21.7
	Conductivity	0.320	0.338
	Dissolved Oxygen	-0.072	-0.021
	DOC	0.029	0.036
	Nitrate	0.426	0.135
	Nitrite	0.284	0.180
	Phosphate	0.618	0.331
	Temperature	0.001	-0.006
	TU	0.498	-0.851
	pH	-0.007	-0.006
Spring 2012	PC	1	2
	% variance	91.8	6.3
	pH	0.069	0.038
	TU	0.172	0.564
	Temperature	0.000	-0.021
	Phosphate	0.953	-0.258
	Nitrite	-0.105	-0.474
	DOC	-0.032	-0.489
	Dissolved Oxygen	0.149	0.211
	Conductivity	0.151	0.325
Autumn 2012	PC	1	2
	% variance	84.8	10.3
	Conductivity	0.129	-0.084
	Dissolved Oxygen	-0.066	0.145
	DOC	0.262	-0.589
	Nitrate	0.191	-0.636
	Nitrite	0.241	0.415
	Phosphate	0.899	0.216
	Temperature	0.011	0.003
	TU	0.066	0.040
	pH	-0.052	0.020

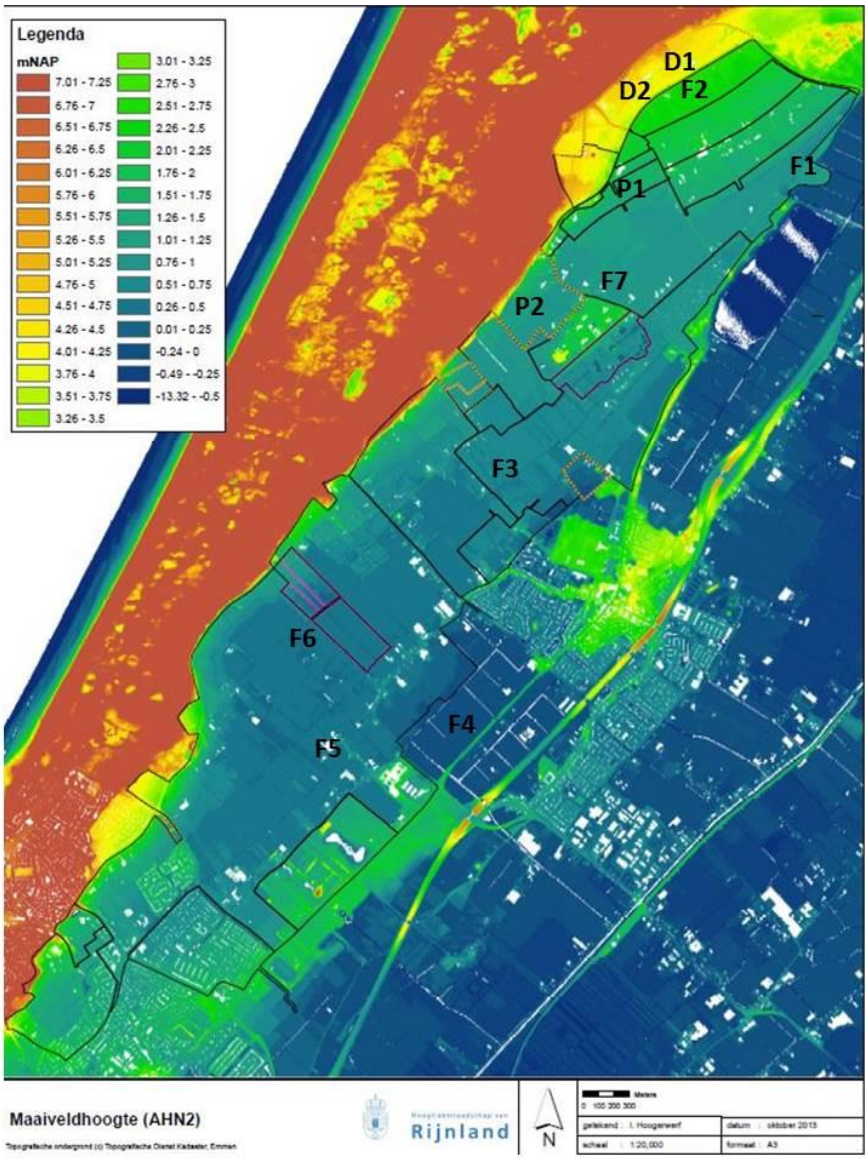

Figure S1. Topographic surface of the research area (source: Water Board Rijnland, October 2013)

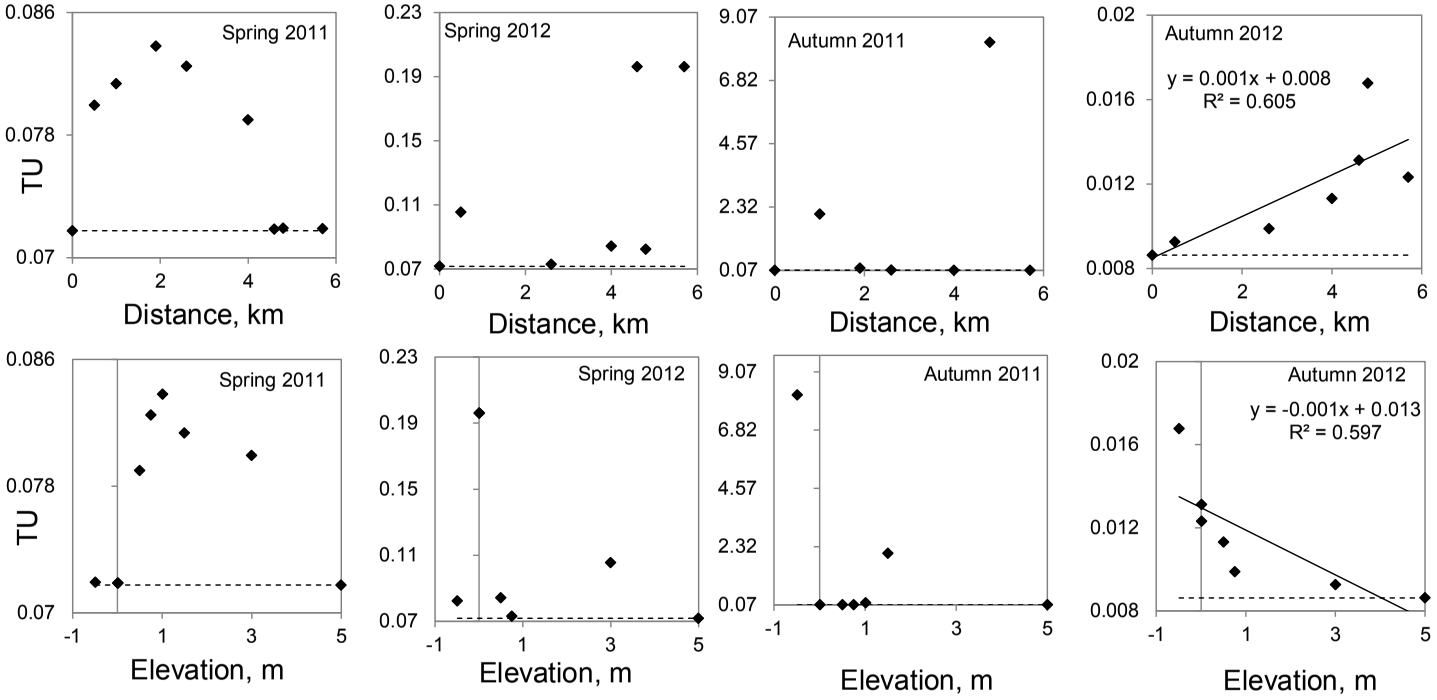

Figure S2. Toxic Units (TU) plotted versus the distance of the experimental location from the nature reserve and the elevation. Dotted line shows the limit of detection. When relationship between TU and distance/elevation was statistically significant ($p<0.05$), regression line and equation are shown

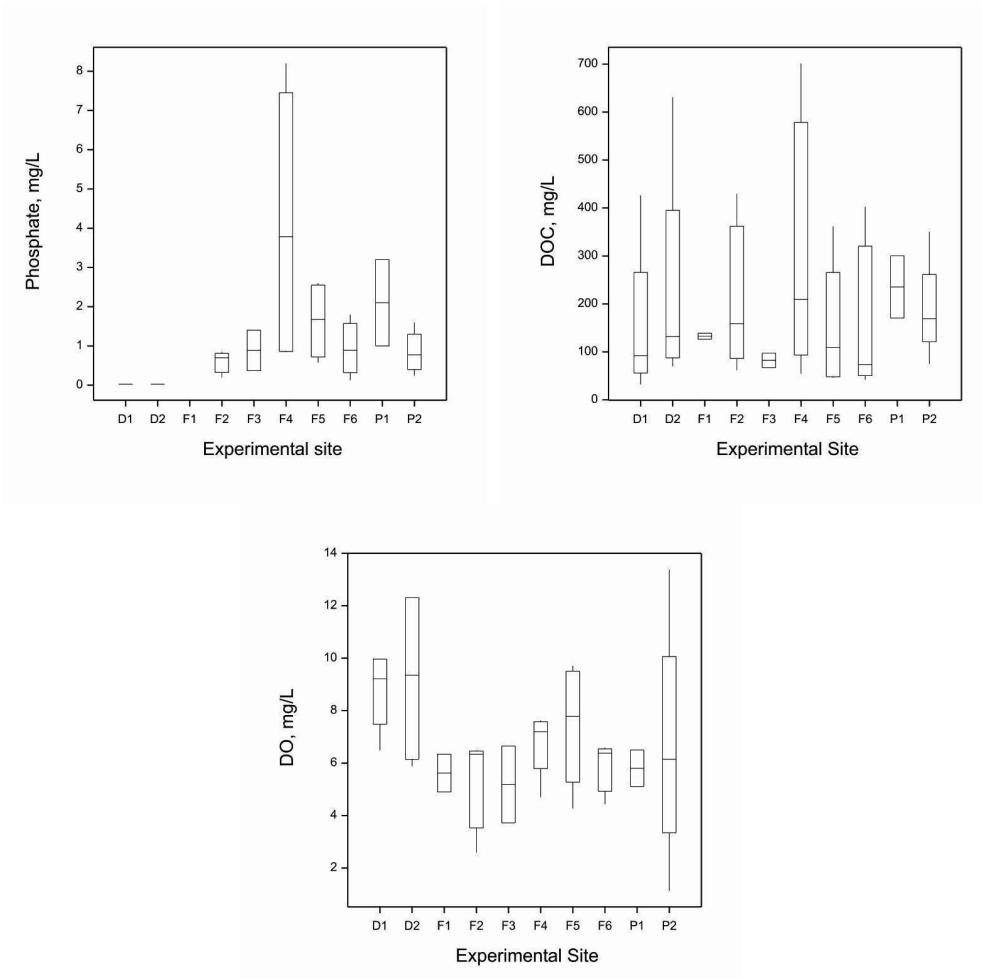

Figure S3. Boxplot of the phosphate (mg/L), Dissolved Organic Carbon (DOC, mg/L), Dissolved Oxygen (DO, mg/L) concentrations at experimental locations based on the data combined from the four experiments. D1 and D2 – experimental sites in nature reserve. F1, F2, F3, F4, F5, F6, P1 and P2 – sites in agricultural area. The graph shows the median (horizontal line), minimum and maximum values (vertical bars), and distribution of the 50% data (the box)

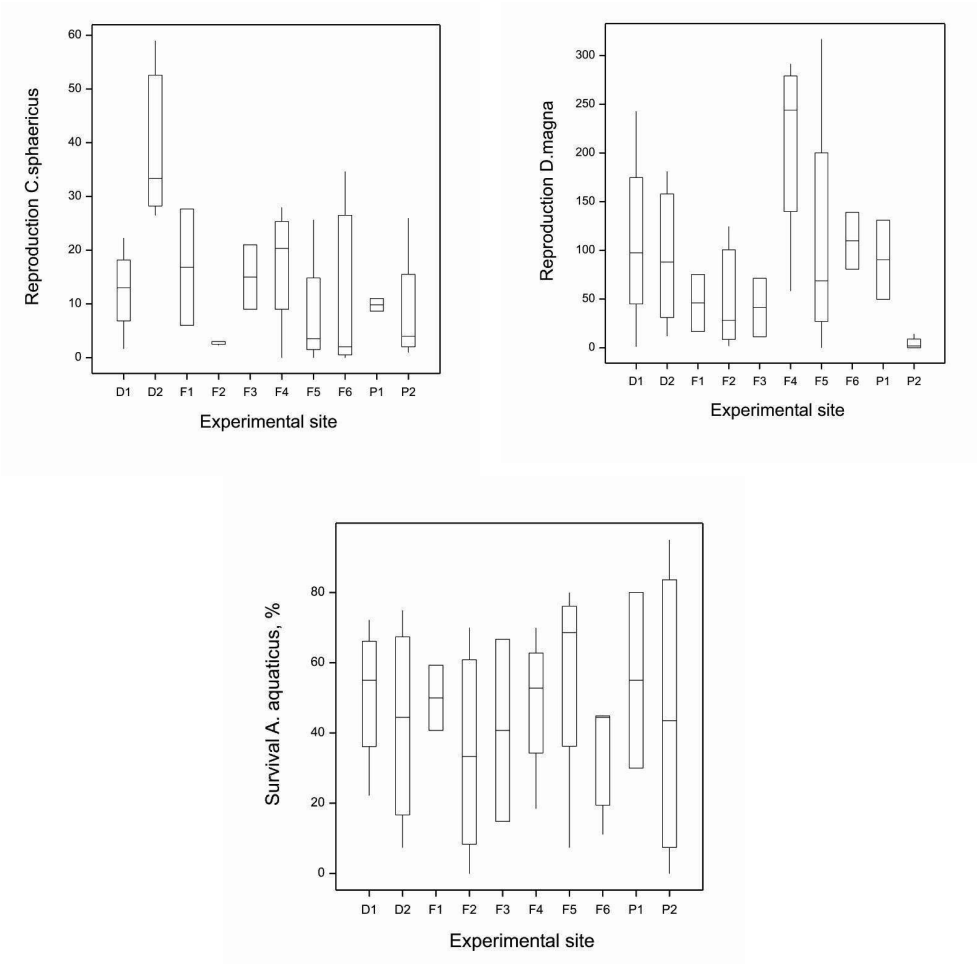

Figure S4. Boxplot of the reproduction output (number of juveniles at 21 day) for *D. magna* and *C.sphaericus* and survival of *A. aquaticus* (%) at experimental locations based on the data combined from the four experiments. D1 and D2 – experimental sites in nature reserve. F1, F2, F3, F4, F5, F6, P1 and P2 – sites in agricultural area. The graph shows the median (horizontal line), minimum and maximum values (vertical bars), and distribution of the 50% data (the box)

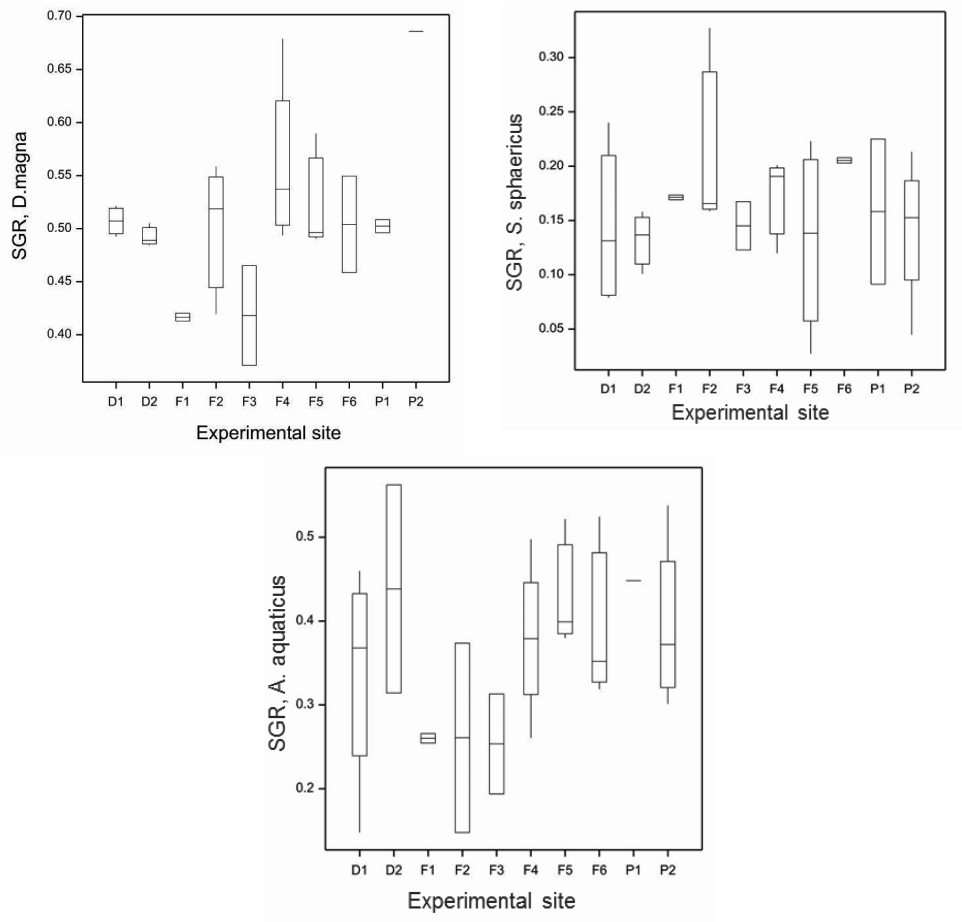

Figure S5. Boxplot of the Somatic Growth Rate (SGR, µm/day) for *D. magna* and *C.sphaericus* and *A. aquaticus* at experimental locations based on the data combined from the four experiments. D1 and D2 – experimental sites in nature reserve. F1, F2, F3, F4, F5, F6, P1 and P2 – sites in agricultural area. The graph shows the median (horizontal line), minimum and maximum values (vertical bars), and distribution of the 50% data (the box)

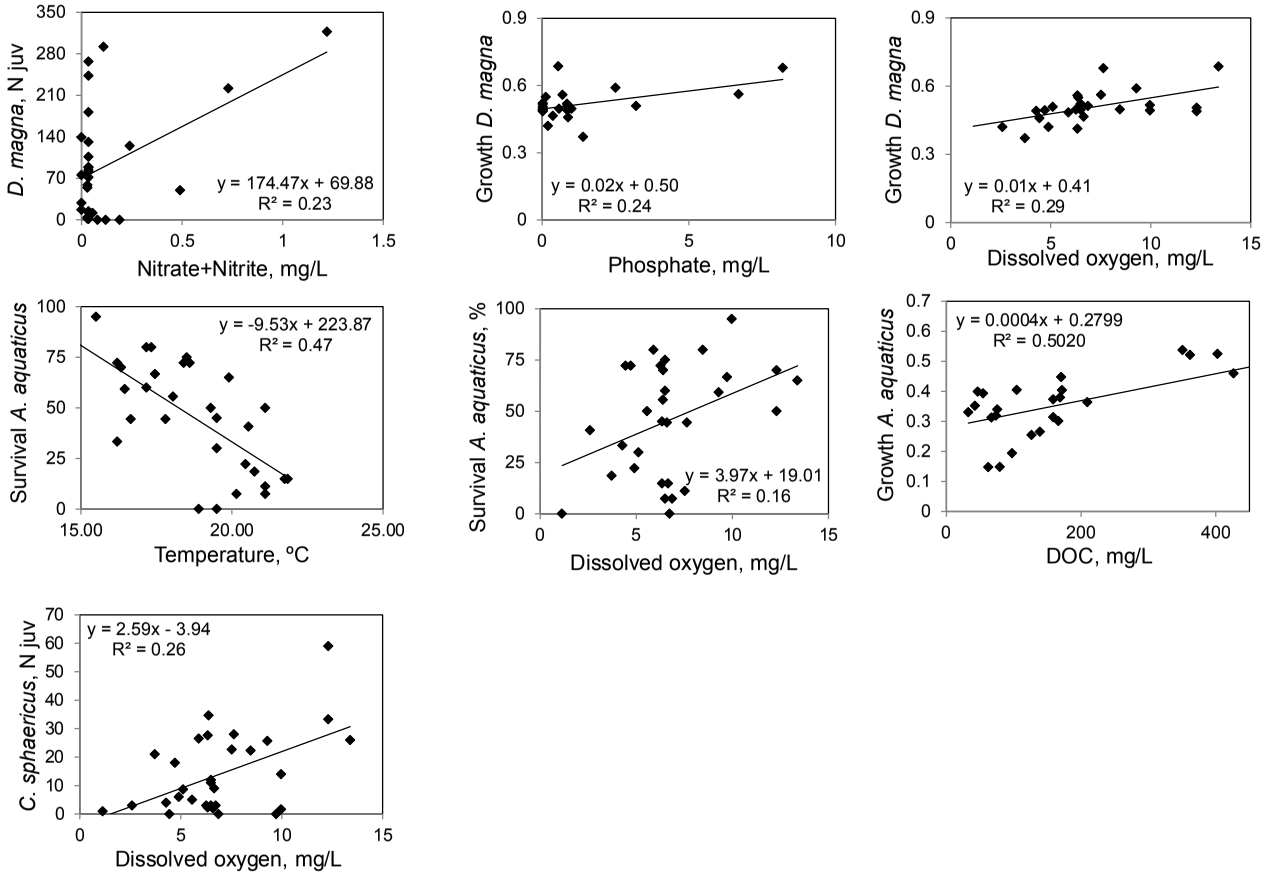

Figure S6. Linear regression analysis between the endpoints estimated for *D. magna, C. sphaericus* and *A. aquaticus* and environmental variables identified to be statistically significant in GLM model and explaining more than 20% variation in the endpoint

CHAPTER 6

IMPACT OF IMIDACLOPRID ON *DAPHNIA MAGNA* UNDER DIFFERENT FOOD QUALITY REGIMES

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Abstract

Aquatic ecosystems are characterized by fluctuating conditions that have direct effects on aquatic communities but also indirect influences such as changing the toxicity of chemicals. Because the effect of food quality on pesticide toxicity has rarely been studied, in the present study *Daphnia magna* juveniles supplied with 4 different food quality levels were exposed to a range of imidacloprid concentrations for 21 d. Food quality was expressed as carbon:phosphorus ratios of algae *Pseudokirchneriella subcapitata* (C:P 35, C:P 240, C:P 400, and C:P 1300). Survival, growth rates, and reproduction of *D. magna* were monitored, and the combined effects of imidacloprid exposure and the phosphorus content of algae were analyzed. A stronger effect on survival was observed at the P-deficient diet (C:P 1300), confirmed by lower 10% effect concentration (EC10) values at days 7, 9, 15, and 21 compared to diets with higher phosphorus contents. Similarly, the growth rate was reduced when *D. magna* were supplied with algae of low phosphorus content at imidacloprid exposure conditions. The highest reproductive output was observed for *D. magna* fed the optimal phosphorus diet (C:P 240), both at control and exposed conditions. Poor food quality increased the sensitivity of nontarget species to pesticide exposure, potentially leading to an underestimation of adverse effects on aquatic communities in the field

Key words: *Daphnia magna*, imidacloprid, algae, food quality, toxicity

Introduction

The toxicity of pesticides to aquatic invertebrate species is commonly assessed based on laboratory tests under controlled conditions, such as temperature, photoperiod, and standardized feeding regime (OECD, 2012). Contrary to the laboratory setting, however, nature is characterized by fluctuating environmental conditions. Apart from physical conditions such as temperature, pH, and salinity, the ecological conditions for aquatic species, such as quantity and quality of food, also vary. The availability of phosphorus is an important factor controlling productivity of phytoplankton algae, which are primary producers in aquatic ecosystems. Aquatic algae in turn serve as a food source for primary consumers represented by zooplankton (Lampert & Sommer, 2007). Aquatic invertebrates of the subphylum Cladocera constitute a dominant group of zooplankton mainly in freshwater ecosystems. The most well-known group is the daphnids, among which *Daphnia magna* Straus is a common species used in standard toxicity testing (OECD, 2012, OECD, 2004).

Literature mostly focuses on either the sensitivity of aquatic invertebrates to algal nutritional levels or on chemically induced effects. To date, the toxicity of only a few chemicals, including 3,4-dichloroaniline, fenoxycarb, and chlorpyrifos (Rose et al., 2002), endosulfan (Barry et al., 1995, Barry, 1996) and esfenvalerate (Barry et al., 1995) to aquatic cladoceran species supplied with different algae cell concentrations (estimated as number of cells in 1 mL) has been studied. Organisms are sensitive not only to food quantity, however, but also to food quality. The elemental food composition (estimated as C:P ratio) is an important factor influencing the performance of cladocerans (Plath & Boersma, 2001; Sterner & Schulz, 1998). Sensitivity of daphnids to nutritional levels expressed as algae phosphorus content was described at the physiological level (growth rate: Plath & Boersma, 2001; Becker & Boersma, 2003; Seidendorf et al., 2010; DeMott & Van Donk, 2003, reproduction: Becker & Boersma, 2003; DeMott & Van Donk, 2003) and at the biochemical level (calcium balance (He & Wang, 2009)). Yet, the effect of the algal phosphorus concentration on the toxicity of chemicals to daphnids has been studied for only a few compounds: herbicide WeatherMAX Roundup (referred as concentration of glyphosate (Lessard & Frost, 2012)) and antibiotic fluoxetine (Hansen et al., 2008). However, no study focused on the combined effects of nutritional quality of algae and neonicotinoid insecticides on *D. magna*. In the present study, we focus on the combined effects of insecticide imidacloprid and algae nutritional levels to *D. magna*.

Imidacloprid belongs to the group of neonicotinoid insecticides that block the nicotinergic neuronal pathway in invertebrates. This blockage of the nicotinic receptor in the neurons leads to the accumulation of the neurotransmitter acetylcholine (Matsuda et al., 2001), resulting in paralysis of the insect, and consequently death. The biochemical activity of imidacloprid in insects and other arthropods appears to be mainly agonistic (Matsuda et al., 2001). Roessink et al. (2003) reported a higher acute toxicity of imidacloprid to mayfly (Ephemeroptera) and caddisfly (Trichoptera) species compared with macrocrustaceans and

insect species belonging to the orders Hemiptera, Megaloptera, and Diptera. The median effect concentration (EC50) of imidacloprid for microcrustacean *D. magna* is 85 mg/L (48-h test, immobility endpoint (Posthuma-Doodeman, 2008)), which is considerably higher than median lethal concentration for mayfly species of 26.3 µg/L (*Cloeon dipterum*, 96-h test, Roessink et al., 2003). Despite its low acute toxicity to daphnids, in the semi-field conditions imidacloprid caused significant reduction in abundances of aquatic faunal assemblages (Hayasaka et al., 2011; Sánchez-Bayo & Goka, 2006). This findings suggest a high potential for imidacloprid to cause adverse effects on nontarget species in the realistic environment.

The aim of the present study was to investigate the effect of the insecticide imidacloprid at a range of nutritional levels (defined as algae C:P ratios) on *D. magna* (subphylum Crustacea, suborder Cladocera). In the present study, toxicological endpoints used were survival, growth, and reproduction, all relevant for population growth. To mimic the differences in food quality, 4 algal phosphorus levels were tested. We hypothesized that exposure to a range of imidacloprid concentrations at P-deficient conditions results in severe effects, such as reduced reproductive output, survival, and growth rate of *D. magna* compared with P-high conditions.

Materials and methods

Test species and culture conditions

Juveniles of *D. magna* were obtained from the laboratory culture of the National Institute of Public Health and the Environment (RIVM, Bilthoven, The Netherlands). Parent animals were cultured under standard laboratory conditions at 20 °C, and a 16:8-h light:dark photoperiod. Adult *D. magna* were raised in 1-L plastic jars in M4 medium described in Elendt (1990). The culture medium was renewed twice per week. Daphnids were fed with the algal cells *Pseudokirchneriella subcapitata*, which were cultured in 2-L bottles in a Woods-Hole medium. The culture medium was replaced once per week. Algae were centrifuged at 7500 RCF in 50-mL falcon tubes, suspended in M4 medium, and fed to *D. magna*.

Preparing different phosphorus levels

The 4 C:P levels of algae were selected for the experiment based on the analysis of literature reporting C:P levels limiting performance of daphnids (Seidendorf et al., 2010; DeMott & Van Donk, 2003; Lessard & Frost, 2012; Hansen et al., 2008; Urabe et al., 1997). To study the effect of the algal phosphorus content on *D. magna* responses, P-free Woods-Hole medium was prepared and divided between 4 2-L bottles. Four different concentrations of K_2HPO_4 and algae (*P. subcapitata*) were subsequently added to the different P levels. Phosphorus concentration in the P-optimal treatment (C:P 240) is the same as in the Woods-Hole medium used in the standard laboratory procedure for *P. subcapitata*.

The algae were adapted to these 4 different phosphorus conditions during 7 d to obtain algae cultures of different nutritional levels at the stationary growth phase. This procedure allowed to obtain sufficient algal biomass to initiate the nutritional experiment. Algae cultures containing the 4 different phosphorus concentrations were kept in individual 2-L bottles with constant aeration and a 24-h light period. Measurements of carbon and phosphorus in algae cultures were made after 7 d adaptation. Table 1 depicts the nutritional levels tested during the experiment (C:P 35, C:P 240, C:P 400, C:P 1300).

Table 1. Algae culture conditions and C:P levels used in the nutritional experiments with *D. magna*.

Reference	K_2HPO_4 addition, mg/L	Dissolved P, mg/L	Total P, mg/L	Particulate P, mg/L	DOC, mg/L	TOC, mg/L	Molar C:P ratio
P-high	16.80	<0.05	3.80	3.78	44.21	96.70	35
P-optimum	8.40	<0.05	1.00	0.98	32.79	87.50	240
P-low	2.80	<0.05	0.38	0.35	37.76	51.70	400
P-very low	0.28	<0.05	0.09	0.07	31.13	19.90	1300

Dissolved P = concentration of dissolved phosphorus, Total P = concentration of total phosphorus, Particulate P = concentration of particulate phosphorus (bound to algae), DOC = dissolved organic carbon concentration, TOC = total organic carbon concentration

For the determination of the organic carbon content, algae culture was filtered through glass-fiber 45-µm pore size filters (Whatman GF/C). Dissolved organic carbon concentrations were determined using non-dispersive infrared analysis. Total organic carbon concentrations were quantified by high temperature combustion/direct injection. The concentration of dissolved and total phosphorus in the algae culture was determined according to the OMEGAM laboratory NEN 6663 (Amsterdam, the Netherlands). The concentration of particulate phosphorus was determined as the difference between total and dissolved phosphorus concentrations. Because the concentration of dissolved phosphorus was below the limit of detection at 4 treatments, half of the detection limit was used to calculate the concentration of particulate phosphorus.

Phosphate is taken up by algae quickly, leading to the depletion in extracellular phosphorus concentration (Yao et al., 2011). At the same time, algal cell density and internal phosphorus concentration increase (Yao et al., 2011). For this reason, after 7 d, we found similar concentrations of external dissolved phosphorus in 4 algae cultures (<0.05 mg/L), even if the concentration of total phosphorus differed (Table 1). Total phosphorus in turn includes all forms of phosphorus: dissolved and particulate phosphorus. In the present study, particulate phosphorus means phosphorus bound to organic matter. Therefore, after 7 d, inorganic phosphorus was taken up by the algae and transformed to particulate

phosphorus. Before being fed to daphnids, algae cultures were centrifuged at 7500 RCF and only the particulate fraction (algae cells dissolved in M4 medium) was used during the experiment.

Test set up

The *D. magna* neonates less than 24 h old were exposed for 21 d. The 6 different concentrations of imidacloprid and a blank at 4 algal phosphorus levels were prepared. Each experimental treatment consisted of 3 replicates with 5 neonates in each replicate chamber (this resulted in $7 \times 4 \times 3 = 84$ test chambers). The experiment was performed in 100-mL test chambers, with 50 mL media in each test chamber. The M4 media containing a range of imidacloprid concentrations were transferred to the test chambers. Algae containing 4 different P concentrations and *D. magna* neonates were subsequently added to the test chambers. All experiments were conducted in a 16:8-h light:dark photoperiod at 20 °C.

Test chambers were not aerated during the experiment. The M4 media containing imidacloprid was renewed every 3 d to ensure continuous exposure to imidacloprid and also to suppress bacteria and fungi growth. Feeding with algae cultured at 4 phosphorus levels was done on the same day as the medium renewal. Feeding with 4 different diets was normalized based on the amount of total organic carbon (0.05 mg C/Daphnia) for each of the 4 diets. Temperature, pH, oxygen saturation, and water hardness were recorded 3 times during the experiment at the time of medium renewal and in freshly prepared medium.

Preparing different imidacloprid concentrations

The concentration range was chosen based on the reported acute and chronic toxicity data for imidacloprid: chronic 21-d no-observed-effect concentration (NOEC) for *D. magna* with endpoint of reproduction, 1.8 mg/L; acute 48-h EC50 for *D. magna* with an endpoint of immobility, 85 mg/L; EC50 for *P. subcapitata* algae, above 100 mg/L (Posthuma-Doodeman, 2008). Nominal concentrations were 1.8 mg/L, 25 mg/L, 45 mg/L, 60 mg/L, 85 mg/L, and 130 mg/L. The 6 different imidacloprid concentrations were prepared by diluting an imidacloprid stock solution in M4 media. The concentration of the stock solution was 400 mg/L, which is lower than the water solubility limit of imidacloprid (610 mg/L), so no solvent was added (US EPA, 1996). The purity of the test substance as reported by the provider Sigma Aldrich Chemie BV was 99.7%.

Analytical measurements

Chemical analysis was performed for 3 imidacloprid concentrations (45 mg/L, 85 mg/L, and 130 mg/L; 1 replicate for each treatment) in freshly prepared medium and old medium (after 3 d exposure) in samples selected randomly in time. At least 2 measurements in fresh and old medium at 3 concentrations were made. Chemical analysis was performed using a 3200 Q Trap liquid chromatography–tandem mass spectrometer (LC/MS/MS; Applied Biosystems). External standard calibration was done using 6 calibration points (1 µg/L,

10 µg/L, 20 µg/L, 50 µg/L, 70 µg/L, and 120 µg/L) plus a blank. The limit of quantification was 0.01 µg/L. Samples were diluted before the analysis in the proportion 1:1000. Measured concentrations were 44.6 ± 3.1 mg/L; 94 ± 2.5 mg/L; and 158.0 ± 6.5 mg/L, respectively. Actual time-weighted mean concentrations of 2.0 mg/L, 27.6 mg/L, and 66.3 mg/L were estimated assuming similar deviation from the nominal concentrations (average 10.5%).

Because the concentration of imidacloprid was expected to decline slightly over the period of 3 d between medium renewals (half life time DT50 in microcosm = 14.8 d (Posthuma-Doodeman, 2008)), the time-weighted mean concentration was calculated as follows:

$$TWConc = \frac{Conc\ 0 - Conc\ 1}{Ln(Conc\ 0) - Ln(Conc1)} * time\ ,$$

where *TWCon*c is the time-weighed concentration for the renewal period; time is the number of days in the renewal period; *Conc 0* is the measured concentration of imidacloprid at the start of the renewal period; and *Conc 1* is the measured concentration of imidacloprid at the end of the renewal period (OECD, 2012). The average concentration per treatment was used in the statistical analysis (US EPA, 1996).

Estimated endpoints

Survival and reproduction of parent animals was estimated daily during the 21-d experiment. Survival was calculated as the proportion of live animals. Animals were considered dead when no movement of antennae/appendages and no swimming behavior were observed. Offspring produced each day were counted daily and transferred to a new series of test chambers containing varying imidacloprid/phosphorus concentrations. Survival of juveniles was also recorded. The number of juveniles produced daily was divided by the number of live adults present. The net reproductive rate (R0) was determined as the cumulative number of juveniles per adult produced in 21 d. Average reproduction per day was determined as average number of juveniles produced per adult per day. Average values for R0 and average reproduction per day between the 3 replicates and standard deviation were calculated.

Body length of the parent animals was measured every 2 d under a microscope STEM SR Zeiss fitted with a micrometer eyepiece. At least 2 randomly selected live parent animals were measured from each test replicate (resulting in 6 size measurements per treatment, every 2 d). Live animals were placed in a petri dish, and the volume of water around the animals was reduced with a pipette to immobilize the animal, and then the animal was measured. *D. magna* body length was defined as the distance from the most posterior point on the head to the junction of the carapace with the tail spine (Barry, 1998).

Growth rate was estimated using 2 different methods. The somatic growth rate (SGR) provided information on body length increment per day. Additionally, the Von Bertalanffy

growth model was fitted that is widely applied to study effects of various stressors on growth of animals.

The somatic growth rate (SGR) was calculated based on the formula

$$SGR = \frac{\ln(L2) - \ln(L1)}{time},$$

where L_1 = the average measured length of neonates at the day of the initiation of the experiment and L_2 = the average measured length after 21 days, time = duration of experiment (21 day). The average SGR per treatment and the standard error of the mean was used for statistical analysis. Additionally, the Von Bertalanffy growth model was applied to estimate growth rates for *D. magna* using mean length at time data

$$L_t = L_{max}\,(1 + e^{-K(t-t0)}),$$

where L_t = body length of *D. magna* at time t; L_{max} = length that can be reached at an infinite time, or a maximum potential length that can be reached at given conditions; K = growth rate; t = time (days); t_0 = theoretical age at $L_t = 0$. The parameters of the Von Bertalanffy growth model were obtained by constructing a Ford-Walford plot introduced by Ford (1993) and Walford (1946). A Von Bertalanffy growth model was constructed for the control and the imidacloprid concentrations 2.0 mg/L and 27.6 mg/L, because animals at these treatments survived for 21 days, allowing comparison between food regimes. Mean length at time t (L_t) was then plotted versus L_t predicted by the Von Bertalanffy growth model and the R^2 coefficient was estimated.

Data treatment

Two-way analysis of variance (ANOVA; 95% confidence interval) with replicates was performed to test the effect of 2 independent factors (imidacloprid and phosphorus concentrations) and the interaction between them on *D. magna* body length at days 3, 9, 15, and 21, as well as on the net reproductive rate (R0). For the two-way ANOVA, analysis of body size measurements at days 3, 9, 15, and 21 at control conditions (C0), imidacloprid concentrations of 2.0 mg/L (C1), 27.6 mg/L (C2), and 44.6 ± 3.1 mg/L (C3) were used. Relationships between *D. magna* somatic growth rate and C:P ratio at different imidacloprid exposure conditions were analyzed with simple linear regression. A slope, intercept, and R^2 were derived for each imidacloprid concentration.

Dose–response relationships between *D. magna* survival and imidacloprid concentration were analyzed by plotting *D. magna* survival at days 5, 7, 9, 15, and 21 (for C:P 35, C:P 240, C:P 400, and C:P 1300) versus the corresponding imidacloprid concentration (log transformed). GraphPad Software was used to obtain a logistic model following the equation

$$Y = \frac{(max + min)}{1 + \left(\frac{x}{EC_{50}}\right)^{-H}} + min,$$

where *min* = minimum response, *max* = maximum response, *x* = concentration of imidacloprid, *EC50* = concentration of imidacloprid that causes 50% of *D. magna* mortality, *H* = Hill slope. EC10 values were calculated using the following equation

$$EC_F = \left(\frac{F}{100 - F}\right)^{1/H} * EC_{50}$$

where EC_F = EC10, *H* = the Hill Slope value and *F* is 10 or 20.

EC10 values were derived for 5, 7, 9, 15 and 21 days of exposure in order to compare effects of imidacloprid on *D. magna* fed with four diets at different ages. EC50 values between four food regimes were compared using an extra sum-of-squares F-test.

Time-to-event analysis was applied to evaluate the median effective time that causes 50% mortality of *D. magna* (ET50) for six imidacloprid concentrations used in the experiment using empirical model described in Sánchez-Bayo (2009). Calculations were made for each food quality regime. ET50 (y) was calculated using the hyperbolic model

$$y = a \times x^{-b},$$

where *y* = ET50 value, *x* = concentration of imidacloprid.

In order to obtain coefficients *a* and *b,* time to 50% mortality of *D. magna* obtained in the experiment for days 5, 7, 9, 15 and 21 was plotted versus imidacloprid concentrations and fitted with linear regression

$$\mathrm{Ln(ET50)} = a' - b \times \ln(C), a' = \ln(a)$$ (Sánchez-Bayo, 2009)

Because reliable confidence intervals could not be derived for EC50 at C:P 1300 (days 15 and 21), it was excluded from the analysis. In order to validate the model, EC50 values for days 5, 7, 9, 15 and 21 were extrapolated using the hyperbolic model for days 5, 7, 9, 15 and 21. Estimated versus predicted EC50 values were analyzed with linear regression.

Results

Effects of imidacloprid and phosphorus on the survival of Daphnia magna

Mortality increased with increasing imidacloprid concentrations in the water. Adverse effects on the survival of daphnids were shown to increase with decreasing food quality. Survival of *D. magna* fed with the low-phosphorus diet, C:P 1300, at an imidacloprid concentration of 44.6 ± 3.1 mg/L reached 0% at day 14, whereas at other diets it remained at 5% to 15% during the 21-d experiment (Figure 1).

Survival of *D. magna* at days 5, 7, 9, 15, and 21 can be found in the Supplemental Data, Tables S1 and S2, along with comparisons between all pairs of EC50 values at 4 food quality regimes. No trend was seen in EC50 values between food regimes derived for days 5, 7, and 9, whereas EC10 values were lower at C:P 1300 compared with other diets starting from day 7 (Table 2). Respective Hill slope values were also lower at C:P 1300 at days 7 through 21 than with other diets (Table 2). A more negative slope indicates a steeper curve and faster response to changing exposure conditions. At days 15 and 21, both EC50 and EC10 values were lower with a P-deficient diet, C:P 1300 (Table 2). However, a comparison between EC10 and EC50 between C:P 1300 and other diets for 15 and 21 d was not possible because the 95% confidence intervals for these parameters at C:P 1300 could not be fitted.

Highest absolute slope value (b) and intercept (a) between the time to 50% mortality and imidacloprid concentration was found for P-optimal conditions (C:P 240) and lowest for P-deficient conditions (C:P 400; Figure 2 and Table 3).

For an imidacloprid concentration of 2 mg/L, the highest predicted ET50 was found at C:P 240 (Figure 3; Supplemental Data, Table S3). At the imidacloprid concentrations 27.6 mg/L to 158 mg/L, the highest ET50 was derived at C:P 400 and the lowest at C:P 240 (Figure 3; Supplemental Data, Table S3). A relatively good fit was obtained between the estimated and predicted in the hyperbolic model EC50 values ($R^2 = 0.62$; Supplemental Data, Table S4 and Figure S1).

Effects on the growth rate

The Von Bertalanffy growth model fitted with the experimental mean length at time data for *D. magna* showed that lowest values for maximum hypothetical length (Lmax) were reached at P-deficient diet C:P 1300, at control and imidacloprid exposure conditions (Table 4). Body lengths of *D. magna* over the 21-d experiment at 4 diets can be found in the Supplementary Data, Figure S2. At control conditions, the highest K was observed at C:P 35; however, larger Lmax was attained at C:P 240. At imidacloprid conditions, the highest Lmax was observed at C:P 35 (Table 4 and Figure 4).

At all diets, imidacloprid induced a negative effect on the *D. magna* SGR (Figure 5). However, differences in SGR between the control and the lowest imidacloprid concentration of 2 mg/L were negligible. A negative regression slope between SGR and log C:P was found for the control and imidacloprid exposure conditions (Table 5). With increasing C:P level (lowering P content of algae), SGR decreased. The absolute slope value (b) was larger at higher imidacloprid concentrations of 27.6 mg/L to 44.6 mg/L (Table 5).

Results of the 2-way ANOVA showed significant effects of phosphorus, imidacloprid, and their interaction on the body length of *D. magna* at ages 3 d and 21 d (Table 6).

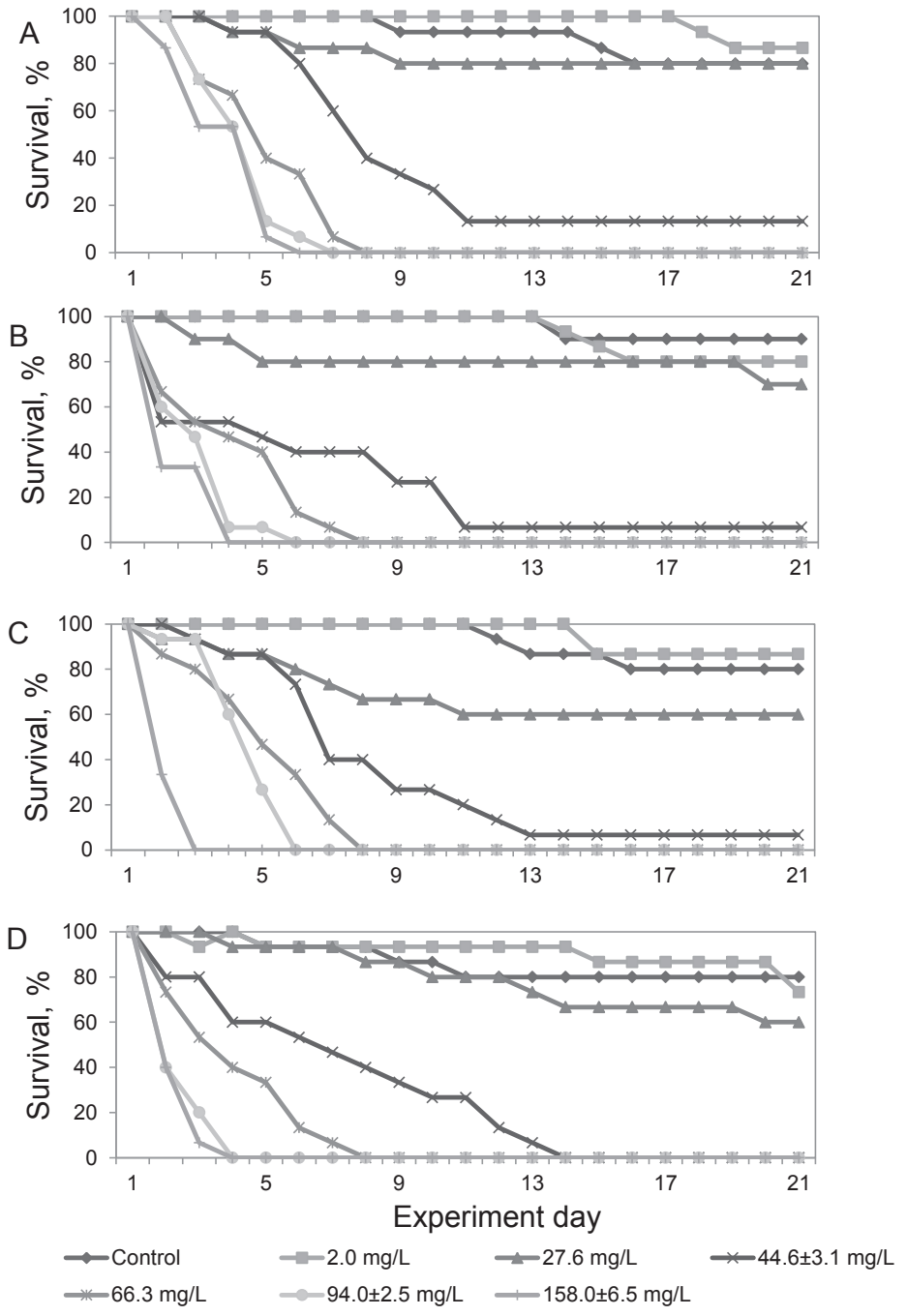

Figure 1. Effect of imidacloprid on the survival of *D. magna* supplied with different food regimes (Mean survival at C:P 35 (A), C:P 240 (B), C:P 400 (C) and C:P 1300 (D))

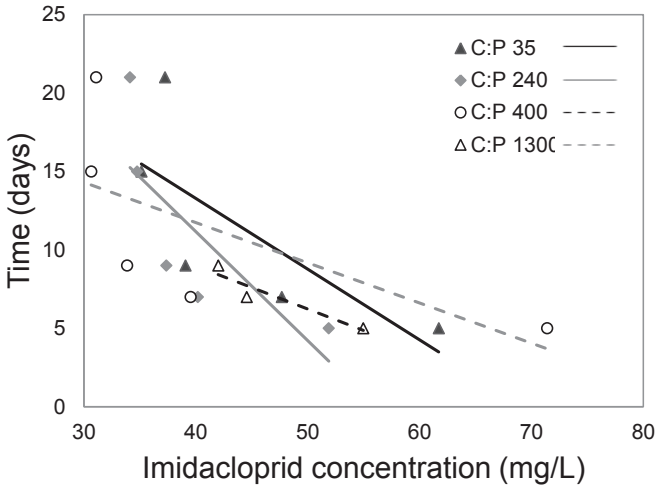

Figure 2. Time to 50% mortality of *D. magna* plotted versus imidalcoprid concentration

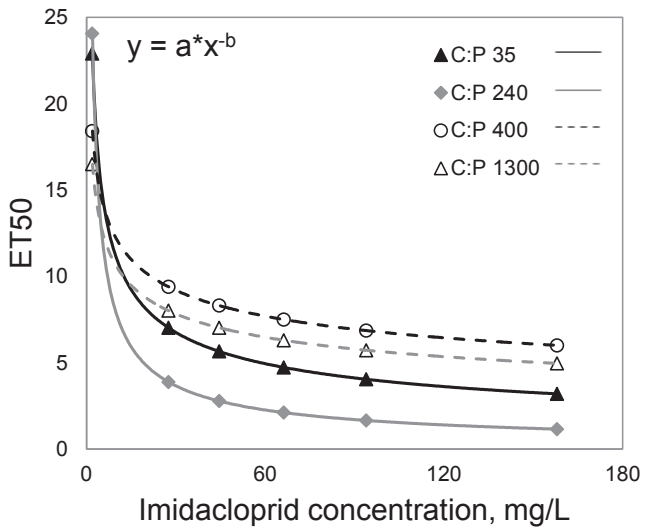

Figure 3. Relationship between median time to 50% effect (ET50) and imidacloprid concentration fitted with hyperbolic curve

Table 2. EC50 and EC10 values for 5, 7, 9, 15 and 21 days for *D. magna* exposed to imidacloprid at four food regimes (endpoint survival).

		C:P35	C:P 240	C:P 400	C:P 1300
Day 5	EC50	61.72 (56.05 to 67.96)	51.88 (37.63 to 71.53)	71.41 (54.14 to 94.19)	54.97 (44.43 to 68.01)
	EC10	80.83 (73.03 to 88.63)	144.64 (97.38 to 191.89)	141.92 (102.12 to 181.72)	95.11 (74.71 to 115.51)
	H	-8.15	-2.14	-3.20	-4.01
	d.f.	17	17	17	17
	R^2	0.94	0.94	0.90	0.89
Day 7	EC50	47.69 (44.74 to 50.84)	40.17 (35.00 to 46.11)	39.53 (34.10 to 45.81)	44.55 (40.13 to 49.46)
	EC10	67.66 (63.33 to 71.99)	68.65 (59.16 to 78.14)	79.69 (67.89 to 91.49)	60.10 (53.81 to 66.40)
	H	-6.28	-4.10	-3.13	-7.34
	d.f.	17	17	17	17
	R^2	0.98	0.95	0.96	0.93
Day 9	EC50	39.07 (35.61 to 44.77)	37.36 (32.70 to 42.70)	33.87 (29.88 to 38.40)	42 (36.71 to 48.04)
	EC10	59.85 (52.98 to 66.71)	55.96 (48.47 to 63.45)	60.06 (52.50 to 67.61)	54.16 (46.86 to 61.47)
	H	-5.43	-5.44	-3.84	-8.64
	d.f.	17	17	17	17
	R^2	0.96	0.95	0.96	0.93
Day 15	EC50	35.14 (31.26 to 39.51)	34.76 (28.78 to 41.98)	30.65 (26.67 to 35.22)	28.35 (no CI)
	EC10	47.16 (52.69 to 41.62)	43.28 (35.06 to 51.50)	42.56 (36.62 to 48.50)	29.63 (no CI)
	H	-7.47	-10.02	-6.69	-49.61
	d.f.	17	16	17	17
	R^2	0.97	0.94	0.93	0.98
Day 21	EC50	37.24 (31.83 to 43.58)	34.12 (29.26 to 39.78)	31.1 (26.89 to 35.98)	28.38 (no CI)
	EC10	47.16 (39.72 to 54.60)	43.40 (36.71 to 50.09)	42.85 (36.59 to 49.11)	29.62 (no CI)
	H	-9.30	-9.13	-6.86	-51.36
	d.f.	17	17	17	17
	R^2	0.96	0.95	0.93	0.96

H = hillslope value, d.f. = degrees of freedom, 95% CI = 95% confidence interval, * no CI = confidence intervals could not be fitted

Table 3. Parameters of the regression equation ln (*ET50*) = $a + b \times \ln(C)$ fitted to the data shown at the Figure 2

C:P ratio	Intercept (*a*)	Slope (*b*)	R^2	n
35	31.316	-0.451	0.57	5
240	38.981	-0.696	0.59	5
400	21.998	-0.257	0.46	5
1300	19.958	-0.275	0.89	3

Table 4. Summary of parameters estimated in Von Bertalanffy growth model for *D. magna* supplied with four food regimes at control conditions (C0) and exposed to imidacloprid concentrations 2.0 mg/L (C1) and 27.6 mg/L (C2).

C: P ratio	Estimated parameters	C0 (0 mg/L)	C1 (2.0 mg/L)	C2 (27.6 mg/L)
C:P 35	*K*	0.43	0.39	0.30
	L_{max}	2400.9	2660.4	1987.8
	t_0	-1.76	-0.49	-0.74
	R^2	0.89	0.89	0.88
C:P 240	*K*	0.40	0.40	0.27
	L_{max}	2751.1	2639.6	1958.4
	t_0	-0.72	-0.44	-1.59
	R^2	0.90	0.86	0.87
C:P 400	*K*	0.38	0.33	0.28
	L_{max}	2546.4	2481.6	1707.0
	t_0	-0.99	-1.15	-1.21
	R^2	0.87	0.90	0.71
C:P 1300	*K*	0.36	0.29	0.30
	L_{max}	2209.4	2444.5	1622.5
	t_0	-0.37	-0.36	-2.53
	R^2	0.90	0.88	0.86

L_{max} = hypothetical maximum length of *D. magna*, *K* = growth rate, t_0 = constant at which an organism has a length $L_t = 0$, R^2 = correlation coefficient between observed and predicted in the model data

Table 5. Parameters fitting regression equation $SGR = a + b \times \log(SGR)$ describing relationship between Somatic Growth Rate (SGR) of *D. magna* and C:P ratio at different imidacloprid exposure conditions and control

Imidacloprid concentration	Slope (*b*)	Intercept (*a*)	R^2	N
0 mg/L	-0.003	0.056	0.58	4
2.0 mg/L	-0.002	0.052	0.11	4
27.6 mg/L	-0.006	0.047	0.88	4
44.6±3.1 mg/L	-0.006	0.035	0.76	3

Table 6. Summary statistics for the two-way analysis of variance explaining *D. magna* body length at days 3, 9, 15 and 21; net reproductive rate (R0) and age at maturity at different exposure conditions.

Parameter	Source of variation	*f stat*	*p-value*	*f crit*
R0	I	3.34	0.09	4.49
	P	4.72	0.02*	3.24
	I x P	0.76	0.53	3.24
Age at maturity	I	0	1	4.49
	P	56.33	1.0E-08*	3.24
	I x P	2.00	0.15	3.24
Body length day 3	I	25.31	*2.62E-12**	2.53
	P	3.71	*0.016**	2.76
	I x P	2.43	*0.012**	1.92
Body length day 9	I	193.50	*8.37E-27**	2.80
	P	2.09	*0.114*	2.80
	I x P	3.65	*0.002**	2.08
Body length day 15	I	76.11	*1.17E-13**	3.26
	P	10.29	*4.93E-05**	2.87
	I x P	1.51	*0.204*	2.36
Body length day 21	I	80.58	*5.09E-14**	3.26
	P	17.63	*3.29E-07**	2.87
	I x P	5.02	*0.0008**	2.36

I = imidacloprid, P = phosphorus content of algae, I x P = interaction of imidacloprid and phosphorus, *f stat* = F-statistic, *f-crit* = F-critical, * variable significance at $p<0.05$

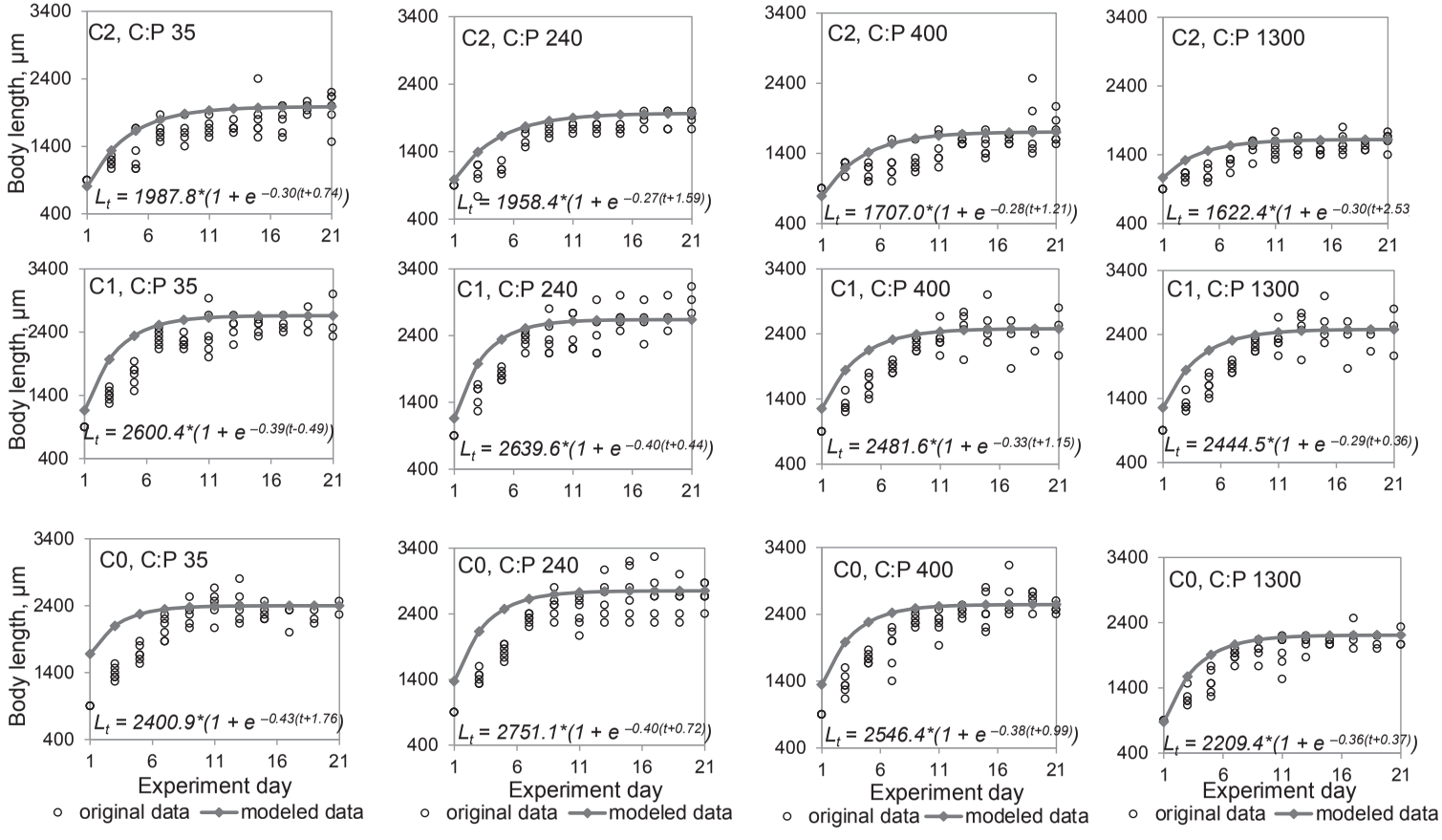

Figure 4. Body length of *D. magna* supplied with diets C:P 35, C:P 240, C:P 400 and C:P 1300 at control conditions (C0) and exposed to imidacloprid concentrations 2.0 mg/L (C1) and 27.6 mg/L (C2) fitted with Von Bertalanffy growth model

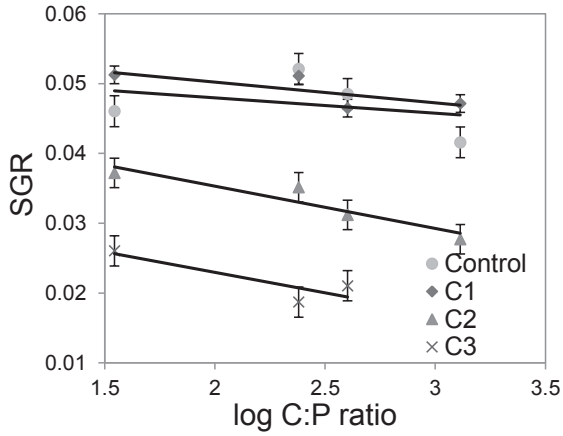

Figure 5. Somatic growth rate (µm/day) of *D. magna* exposed to a range of imidacloprid concentrations plotted versus log C:P ratios (shown on the graph are mean somatic growth rate and standard error). C1= 2.0 mg/L; C2= 27.6 mg/L; C3= 44.6±3.1 mg/L

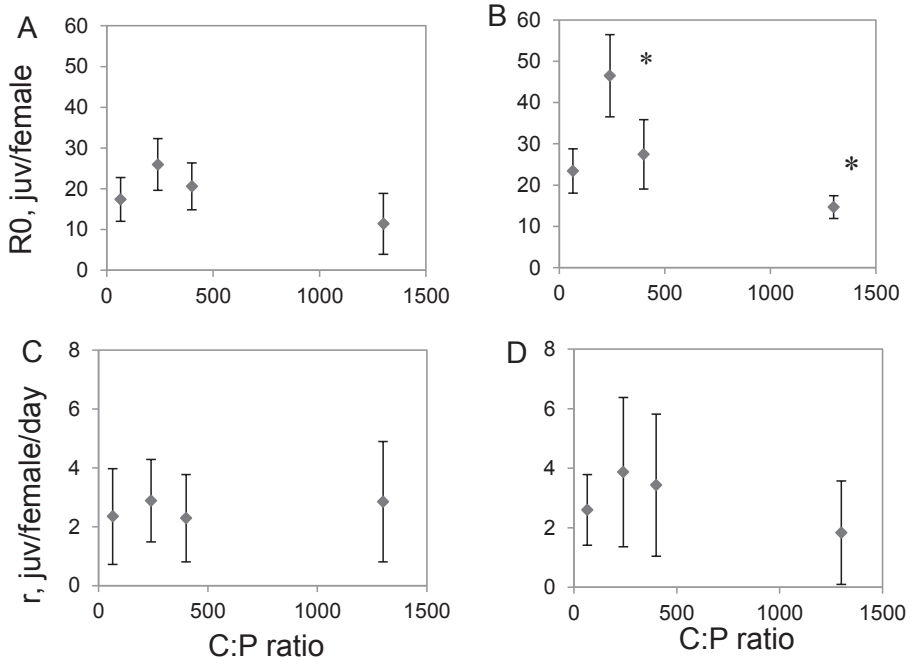

Figure 6. Net reproductive rate (R0, juv/female), average reproduction per day (juv/female/day) of *D. magna* at the control conditions (A, C) and imidacloprid concentration 2.0 mg/L (B, D) and age at maturity (E), * significantly different from other C:P levels at $p<0.1$

Effects on reproduction

Production of juveniles was observed at control exposure conditions and at imidacloprid concentration of 2.0 mg/L. No reproduction was observed at the higher imidacloprid concentrations. Two-way ANOVA revealed a significant effect of phosphorus and imidacloprid on the reproductive output R0 (Table 6). The effect of imidacloprid–phosphorus interaction was not significant ($p > 0.05$; Table 6). However, the mean net reproductive rate (R0) was the highest at C:P 240 (optimal conditions) compared with other diets at control and imidacloprid concentrations of 2 mg/L (Figure 6A, B). The lowest mean reproductive output, R0, was observed for the P-deficient diet both at control and imidacloprid exposure conditions (C:P 1300) (Figure 6A, B). Average reproduction per day did not differ significantly for *D. magna* fed with different diets at control conditions and imidacloprid concentrations ($p > 0.05$) (Figure 6C, D).

Discussion

Varying environmental conditions, including nutrient concentrations, are unavoidable characteristics of natural aquatic ecosystems. Within agricultural areas, concentrations of nutrients in surface waters vary significantly depending on local farming activities, fertilizer application, and the amount of precipitation. However, in ecological effect predictions the variable environmental conditions are hardly considered. Earlier research demonstrated that differences in toxicity between laboratory and field exposures range as a factor of 1.2 to 10 for the nutritional state (Heugens et al., 2001) When subjected to multiple stressors in a natural aquatic environment, organisms are more prone to diet change or food deficiency (Kooijman & Metz, 1984). Hence, extrapolation of results obtained in the laboratory to the field deals with high uncertainty (Selck et al., 2002).

Effects on survival

Lower EC10 values at days 7 through 21 were found at the P-deficient diet, C:P 1300, suggesting a greater effect of imidacloprid on *D. magna* survival at poor nutrient diet.

Results of time-to-event analysis indicated that *D. magna* supplied with P-optimal food had the highest absolute value of regression slope (b) between time to 50% mortality and imidacloprid concentration (Table 3, Figure 3). Therefore, the gradient of response to imidacloprid at C:P 240 was larger compared with other diets (Table 3 and Figure 3). As a result, at C:P 240 *D. magna* ET50 estimated in a hyperbolic model was lower compared with other food regimes. On the contrary, at lower phosphorus conditions of C:P 400, higher ET50 values were derived compared with other diets. This result was found for high imidacloprid concentrations of 27.6 to 158 mg/L. Reduced growth and reproduction at P-low conditions was possibly compensated for by larger time to mortality when exposed to high imidacloprid concentrations.

However, at the lowest imidacloprid concentration of 2 mg/L, the longest time-to-mortality was found for an optimal diet to be C:P 240. At the P-optimal treatment, the highest reproductive output was obtained at the control and the imidacloprid exposure of 2 mg/L (Figure 6). Therefore, when exposed to a low imidacloprid concentration, close to the NOEC (1.8 mg/L, Posthuma-Doodeman, 2008), the optimal feeding regime C:P 240 was found to be the most favorable for reproduction and life duration of *D. magna*.

Effects on the growth rate

A negative effect of low phosphorus content on the growth rate of *D. magna* was found at P-deficient conditions based on the results of the Von Bertalanffy growth model and the somatic growth rate (Figures 4 and 5). Phosphorus is stored in algae cells as polyphosphate (Powell et al., 2008; Miyachi et al., 1964). Addition of K_2HPO_4 to the phosphorus-sufficient algae results in the increase of total cellular phosphorus and polyphosphate (Eixler et al., 2006). On the contrary, at the conditions of starvation, the total cellular phosphorus content of algae decreases (Aitchinson & Butt, 1973). In the present study, the total phosphorus concentration of algae *Pseudokirchneriella subcapitata* was the lowest at C:P 1300, which explains its poor nutritional quality for *D. magna* (Table 1). In previous studies, algae grown at conditions of P-deficiency increased the thickness of the cell wall, which resulted in lower digestion rates for *D. magna* and consequently reduced growth (DeMott WR & Van Donk, 2013). This was proposed to be a defensive mechanism of algae against grazing by *D. magna* at poor nutrient conditions (Van Donk et al., 1997). DeMott & Van Donk (2013) suggested that in the algae resistant to digestion, the cell wall remains undamaged when passing through the gut of daphnids. Therefore, in conditions of phosphorus deficiency, carbon and phosphorus of algae cannot be fully assimilated by daphnids (DeMott WR & Van Donk, 2013). Also, in the study by Frost et al (2008), when the algae C:P ratio increased (meaning lowered P content), the percentage of P in the body mass of *D. magna* decreased at the control treatment. The growth rate of daphnids in turn depends on the amount of carbon assimilated (DeMott WR & Van Donk, 2013). Results of daphnids' growth rates, as determined in our experiment, especially at the high C:P levels, could therefore be a possible result of reduced carbon and phosphorus incorporation by *D. magna* fed with P-deficient algae. In poor nutrient conditions, values for both growth rate K and maximum hypothetical length Lmax derived in the Von Bertalanffy model were lower compared to P-sufficient diets. At the same time, *D. magna* provided with algae of low P content could have higher filtering activity, which resulted in more energy spent for filtering and faster passage of algae through the gut (Plath & Boersma, 2001). As a result, higher energy costs for filtering activity may lead to a reduced growth rate and lower reproduction at a P-deficient diet. Therefore, the energy demand of *D. magna* supplied with algae of low phosphorus level (C:P 1300) may not be fulfilled. Similar results of the negative effects of low algal phosphorus content on the growth of daphnids were found in a number of previous studies (Plath & Boersma, 2001; Urabe et al., 1997; Van Donk et al., 1997). Conversely, when supplied

with P-sufficient algae, the feeding rate of *D. magna* is lower compared with P-deficient conditions (Plath & Boersma, 2001). Consequently, a lower amount of energy is allocated to filtering, that results in higher growth rates at P-sufficient conditions.

Urabe et al. (1997) confirmed that phosphorus determined food quality for *D. magna* and estimated the C:P ratio threshold for algae growth (C:P ≤ 300). *Daphnia magna* fed with algae of C:P lower than 300 are not limited by the phosphorus in food. This observation agrees with our results: lower growth and Lmax were found at limited conditions of C:P 1300. Plath and Boersma observed reduced somatic growth rates at low C:P (approximately 30) (Plath & Boersma, 2001). These authors argued that this effect can be explained by a lower incorporation of carbon by *D. magna* as a result of the reduced feeding rate at P-rich conditions. This result could not be confirmed. However, the hypothetical body length Lmax derived from the Von Bertalanffy model was higher at P-optimal conditions (C:P 240) than at P-rich (C:P 35 at control conditions). Additionally, in the study by Plath and Boersma, a significant reduction of somatic growth (approximately 3-fold) was observed at a P-deficient C:P level of approximately 640 (Plath & Boersma, 2001). The duration of their experiments (6 d) differed from the present study, and K_2HPO_4 was added to algae cultures 24 h before the start of the experiment (Plath & Boersma, 2001). In our study, algae were adapted to different nutritional levels during 7 d and likely changed their biochemical composition.

According to the previous studies, the optimal effects of environmental conditions on *D. magna* growth rate were derived from the 21-d experiment. Differences in the modeled Von Bertalanffy growth estimates obtained in the 21-d and 41-d experiments were not significant in the study of Martínez-Jerónimo (2012). Similarly, in the present study, the increase in body size at 11 d to 21 d was generally smaller, likely because of the resource limitation (more energy allocated to reproduction and not to growth irrespectively of the diet). The experiment of 21 d was sufficient to estimate the effects of food limitation on the growth rate of *D. magna*.

Previous studies have emphasized that octanol–water partitioning coefficient (K_{OW}) relates to sorption of chemicals in a positive manner. In the study of Rose et al. (2002) the hydrophobic fenoxycarb caused substantial toxicity to *D. magna* at the highest algae concentration used. This was likely because a larger amount of fenoxycarb was adsorbed to organic matter and harvested by animals supplied with a high food level (Rose et al., 2002). A similar result of larger effect of herbicide glyphosate on *D. magna* growth supplied with P-rich food was found by Lessard & Frost (2008). This result was explained by lower incorporation of toxin by daphnids at P-deficient conditions (Lessard & Frost, 2008). Higher toxicity at a nutrient-rich diet was found for the pharmaceutical fluoxetine (Hansen et al., 2008). On the contrary, Barry et al. (1995) proposed that the metabolic degradation of hydrophobic chemicals by algae can lead to lower effects on *D. magna* exposed at high food conditions (Barry et al., 1995). However, this statement does not apply to the chemicals that also have toxic metabolite products.

Imidacloprid is a hydrophilic insecticide that has a lower tendency to bind to organic matter (water solubility = 610 mg/L, log K_{OW} = 0.57). Therefore, at the conditions of imidacloprid exposure, the quantity and quality of algae supplied to daphnids within the optimal feeding range does not affect toxic response. In our study, only at the conditions of phosphorus deficiency (C:P 1300) was the effect of imidacloprid on survival, growth, and reproduction more pronounced. Food limitation possibly acted as an additional stressor that led to higher toxicity when supplied with algae of low nutritional quality. Following the concept of Van Straalen (2003), under sufficient food conditions invertebrates likely withstand easier additional stresses, and our results clearly show that at phosphorus-sufficient diets, high imidacloprid concentration was easier to battle.

Effects on reproduction

The imidacloprid exposure concentration of 2.0 mg/L used in the experiment is close to the earlier reported NOEC for imidacloprid (1.8 mg/L in 21-d test, endpoint reproduction) (Posthuma-Doodeman, 2008). Because a low imidacloprid concentration was used, average reproduction per day for exposed animals did not differ significantly from the control. At C:P 240 higher reproductive output was found at the exposed treatment (Figure 6A and B). The lowest value of R0 (net reproductive rate) was observed at the P-deficient diet (C:P 1300) at the control conditions and at an imidacloprid concentration of 2.0 mg/L (Figure 6). As a result of lower growth rate at P-deficient conditions, smaller body size was reached. *D. magna* start reproducing when critical body size is achieved. Because of the reduced growth rate at P-deficient conditions, *D. magna* attained critical body length later than with the other diets. This has possibly led to delayed age at maturity and consequently lower reproduction at P-poor conditions. Under conditions of P-deficiency, *D. magna* is likely to allocate higher proportion of energy toward maintaining survival. Consequently, the proportion of energy available for reproduction is reduced (Bradley & Baird, 1991). The energy obtained by the organism is balanced between somatic maintenance (growth) and reproduction: when high growth is reached, less energy is available for reproduction (Kooijman, 2010). This complies with the dynamic energy budget theory, which allows calculating costs that are made by organisms to deal with various natural and anthropogenic stressors (Kooijman, 2010). Thus, in the present study we found a larger time to mortality (ET50) at P-poor conditions characterized by lower reproductive output.

Imidacloprid concentrations used in the experiment were significantly higher than usually found in Dutch surface waters (0.1–1.5 µg/L, Waterboard Rijnland, measurements of 2010 (Van Rooden et al., 2001). Selection of relatively high concentrations is also explained by the fact that cladoceran *D. magna* is more tolerant to imidacloprid compared to insect or other crustacean species (Roessink et al., 2013). This allowed detecting effects on *D. magna* survival and growth on a relatively short time scale of 21 d. In general, surface waters around intensively used arable fields contain phosphorus concentrations that are considerably higher compared to surface waters in areas with less intensive land use and nature-protected areas

(e.g., data waterboard Rijnland period 1993–2007 for the southern part of The Netherlands (Gerrits, 2010), or Gao et al., 2012, period 2005–2006 for Southwestern China). Based on the results of the current study, we can conclude that under oligotrophic conditions (i.e., low P levels), imidacloprid pollution will result in more pronounced effects on crustaceans.

Conclusions

The interactive effect of imidacloprid exposure and the elemental composition of algae (C:P ratio) on the performance of *D. magna* was shown to be ambiguous. Higher impact on survival and growth of daphnids was observed at phosphorus-deficient conditions. Based on the experimental results, one can conclude that toxicity of imidacloprid increased at a P-deficient diet, as seen by the observed effects on survival, growth rate, and reproduction. This was confirmed by lower EC10 values, growth rates, and reproductive output of *D. magna* at the conditions of P-deficiency. Combined effects of toxicants and abiotic factors challenge the estimation of pesticide risks on daphnids populations in freshwater ecosystems. Results can be applied to predict limiting ratios of carbon:nutrients for daphnids at the conditions of toxic stress. In field situations, multiple abiotic factors are present, and, therefore, combined effects of chemicals and natural stressors can be expected. The interactive effects of resource limitation and toxic stress on organisms need to be considered in risk assessment of chemicals.

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Supplemental information

Table S1. Mean survival and standard deviation (in italic) of *D. magna* supplied with four different food regimes at different days of exposure (5, 7, 9, 15 and 21) at a range of imidacloprid concentrations: C1 = 2.0 mg/L, C2 = 27.6 mg/L, C3 = 44.6±3.1 mg/L, C4 = 66.3 mg/L, C5 = 94±2.5 mg/L; C6 = 158.0±6.5 mg/L

Day	**5**				**7**				**9**				**15**				**21**			
C:P	35	240	400	1300	35	240	400	1300	35	240	400	1300	35	240	400	1300	35	240	400	1300
C0	100.0	100.0	100.0	93.3	100.0	100.0	100.0	93.3	93.3	93.3	100.0	86.7	86.7	90.0	93.3	80.0	80.0	90.0	86.7	80.0
	0	*0*	*0*	*11.5*	*0*	*0*	*0*	*11.5*	*11.5*	*11.5*	*0*	*11.5*	*11.5*	*14.1*	*11.5*	*0*	*0*	*14.1*	*11.5*	*0*
C1	100.0	100.0	100.0	93.3	100.0	100.0	100.0	93.3	100.0	100.0	100.0	93.3	100.0	86.7	86.7	86.7	86.7	80.0	86.7	73.3
	0	*0*	*0*	*11.5*	*0*	*0*	*0*	*11.5*	*0*	*0*	*0*	*11.5*	*0.0*	*23.1*	*23.1*	*11.5*	*11.5*	*20.0*	*23.1*	*11.5*
C2	93.3	80.0	86.7	93.3	86.7	80.0	73.3	93.3	80.0	80.0	66.7	86.7	80.0	80.0	60.0	66.7	80.0	73.3	60.0	60.0
	11.5	*20.0*	*11.5*	*11.5*	*11.5*	*20.0*	*23.1*	*11.5*	*20.0*	*20.0*	*11.5*	*11.5*	*20.0*	*20.0*	*20.0*	*11.5*	*20.0*	*11.5*	*20.0*	*20.0*
C3	93.3	46.7	86.7	60.0	60.0	40.0	40.0	46.7	33.3	26.7	26.7	33.3	13.3	6.7	6.7	0	13.3	6.7	6.7	0
	11.5	*11.5*	*11.5*	*34.6*	*0*	*20.0*	*0*	*30.6*	*11.5*	*23.1*	*23.1*	*30.6*	*11.5*	*11.5*	*11.5*		*11.5*	*11.5*	*11.5*	
C4	40.0	40.0	46.7	33.3	6.7	6.7	13.3	6.7	0	0	0	0	0	0	0	0	0	0	0	0
	20.0	*0*	*30.6*	*11.5*	*11.5*	*11.5*	*11.5*	*11.5*												
C5	13.3	6.7	26.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	11.5	*11.5*	*11.5*																	
C6	6.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	11.5																			

Table S2. Summary statistics for comparison of EC50 values between four food quality regimes using extra sum-of-squares F test.

Days of exposure	Parameters estimates	Comparison of EC50 between food regimes					
		35-240	35-400	35-1300	1300-400	1300-240	240-400
5 days	*p-value*	0.383	0.235	0.282	0.110	0.794	0.241
	F(DFn,DFd)	0.78 (1.34)	1.47 (1.34)	1.19 (1.34)	2.69 (1.34)	0.07 (1.34)	1.42 (1.34)
7 days	*p-value*	0.218	0.023*	0.787	0.045*	0.217	0.358
	F(DFn,DFd)	1.57 (1.34)	5.72 (1.34)	0.074 (1.34)	4.32 (1.34)	1.58 (1.34)	0.87 (1.34)
9 days	*p-value*	0.805	0.327	0.307	0.077*	0.419	0.252
	F(DFn,DFd)	0.06 (1.34)	0.99 (1.34)	1.08 (1.34)	3.34 (1.34)	0.67 (1.34)	1.36 (1.34)
15 days	*p-value*	0.915	0.136	*N*	*N*	*N*	0.407
	F(DFn,DFd)	0.012 (1.33)	2.34 (1.34)	*N*	*N*	*N*	0.71 (1.33)
21 days	*p-value*	0.389	0.082*	*N*	*N*	*N*	0.417
	F(DFn,DFd)	0.77 (1.31)	3.23 (1.32)	*N*	*N*	*N*	0.67 (1.33)

DFn = degrees of freedom numenator, DFd = degrees of freedom denominator, *N* = comparison of EC50 values was not possible because confidence intervals for EC50 at days 15 and 21 at C:P 1300 could not be fitted (* $p<0.1$)

Table S3. Estimated median time to 50% of *D. magna* fed with four diets and exposed to a range of imidacloprid concentrations.

Imidaclorprid concentration, mg/L	C:P ratio			
	35	**240**	**400**	**1300**
2	22.91	24.07	18.41	16.49
27.6	7.02	3.88	9.39	8.01
44.6	5.65	2.78	8.30	7.02
66.3	4.73	2.11	7.50	6.30
94	4.04	1.65	6.86	5.72
158	3.19	1.15	6.00	4.96

Table S4. EC50 concentrations estimated in the experiment with 95% confidence intervals (CI) for *D. magna* compared to EC50 concentrations predicted using the hyperbolic model

C:P ratio	Time of exposure (days)	Predicted EC50, mg/L	Measured EC50, mg/L	95% CI
35	5	57.77	61.72	56.05 to 67.96
	7	27.46	47.69	44.74 to 50.84
	9	15.75	39.07	35.61 to 44.77
	15	5.09	35.14	31.26 to 39.51
	21	2.42	37.24	31.83 to 43.58
240	5	18.99	51.88	37.63 to 71.53
	7	11.72	40.17	35.00 to 46.11
	9	8.18	37.36	32.70 to 42.70
	15	3.93	34.76	28.78 to 41.98
	21	2.43	34.12	29.26 to 39.78
400	5	316.80	71.41	54.14 to 94.19
	7	85.67	39.53	34.10 to 45.81
	9	32.26	33.87	29.88 to 38.40
	15	4.43	30.65	26.67 to 35.22
	21	1.20	31.1	26.89 to 35.98
1300	5	151.12	54.97	44.43 to 68.01
	7	44.62	44.55	40.13 to 49.46
	9	17.94	42	36.71 to 48.04
	15	2.82	28.35	no CI
	21	0.83	28.38	no CI

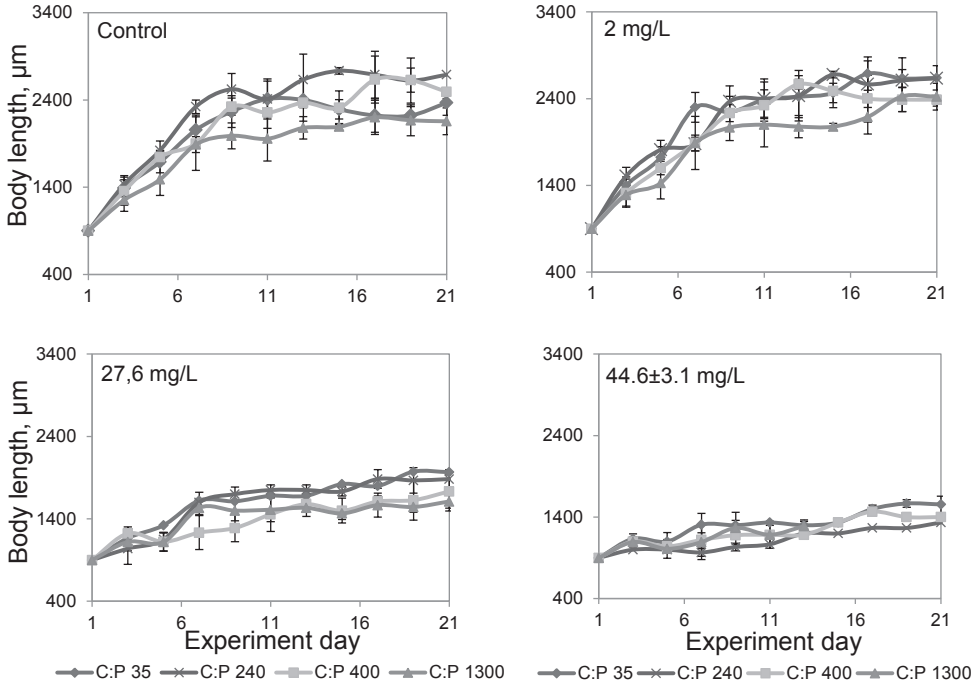

Fig. S2. Body length of *D. magna* at control and imidacloprid exposure concentrations 2.0 mg/L, 27.6 mg/L and 44.6±3.1 mg/L supplied with four food quality regimes

CHAPTER 7

DISCUSSION

Scientific scope and research questions

This PhD thesis provides an insight on the effects of pesticides on aquatic biota in the field. The community composition of aquatic biota inhabiting aquatic ecosystems is determined by different abiotic factors, such as hydrological and environmental factors, intrinsic to aquatic ecosystems. Additional to these abiotic factors, biotic interactions describe a high degree of fluctuation among communities (Clements et al., 2012).

Human activities related to agriculture modify natural aquatic ecosystems. In this respect, pesticide and nutrient pollution of freshwater ecosystems represents one of the central environmental issues worldwide. A substantial amount of research has been dedicated to understanding and characterizing the effects of chemicals on different levels of biological organization: organisms, populations, communities, and ecosystems. The assessment of chemical effects on high organizational levels is challenging due to the high variability in abiotic and biotic factors found in natural ecosystems, which can interfere with chemicals in their effects on aquatic biota. The field relevance of pesticide effects on aquatic biota receives an increasing attention in ecotoxicology.

The research aims of this PhD project were:

A. To determine which role is assigned to pesticides within the complex field setting in shaping the community composition of aquatic macrofauna
B. To determine the magnitude of impact of field-relevant factors on aquatic macrofauna communities in water systems located in close proximity to agricultural fields and to identify the combined effects of field-relevant factors and pesticides on aquatic biota.

To address these aims, the following research questions were formulated:

1. Did pesticide levels and macrofauna diversity in ditches next to flower bulb fields change over the previous decades?
2. What proportion of the total variance in the community composition of aquatic macrofauna can be explained by pesticides, environmental factors (nitrate, nitrite, phosphate, dissolved organic carbon, dissolved oxygen, temperature and macrophyte coverage), the presence of other biota and time (seasonal and annual variation)? What is the predictive power of the taxonomic approach in quantifying pesticide effects on aquatic macrofauna in the field?
3. What are the effects of pesticides combined with environmental factors (nitrate, nitrite, phosphate, dissolved organic carbon, dissolved oxygen and temperature) on aquatic invertebrates exposed in situ in ditches adjacent to flower bulb fields?
4. Does food quality (expressed as the carbon: phosphorus ratio of algae) affect the responses of *Daphnia magna* to the insecticide imidacloprid?

Field research was based in the flower bulb growing area of the Netherlands. Various approaches were used in research, ranging from field monitoring of aquatic macrofauna and

in situ bioassays with aquatic invertebrates, to laboratory toxicity experiments. In addition, a database containing pesticide, water chemistry and macrofauna data collected in the flower growing area of the Netherlands over the previous decades, was analyzed.

Answers to the research questions

RQ1. Pesticide levels in ditches bordering flower bulb fields (expressed as TU normalized by the number of pesticides measured per sample) were stable between 1980 - 1998, followed by a decrease in 1998 - 2010. The lowest TU were observed in the years 2000 - 2004. The concentrations of the most frequently measured individual pesticides (for instance, carbendazim, pirimiphos-methyl, imidacloprid, chloridazon, isoproturon and chlorpropham) decreased over time. The Shannon diversity index expressing macrofauna diversity in ditches of the flower bulb growing area and in the watersheds of nature reserve tended to increase over time. Pesticide and macrofauna data were not collected consistently over time and sampling site. Therefore, causal relationships between pesticide levels in surface waters and macrofauna diversity could not be elucidated. It was concluded that further research based in the field is required to infer causal relationships between pesticide levels in ditches and macrofauna diversity.

RQ2. The largest proportion of variance in both the taxonomic and trait composition of macrofauna community was explained by environmental factors, followed by pesticides and by time, as quantified by a variance partitioning procedure based on the partial redundancy analysis (pRDA). Water chemistry parameters were highly variable in ditches of the study area. Concentrations of nutrients fluctuated in a range significantly exceeding the water quality standards. For example, the concentration of phosphate in ditches ranged between 0.8±1.0 mg/L and 4.1±3.2 mg/L, with a concentration of phosphate above 1 mg/L being considered a sign of ”poor” water quality (UKTAG, 2012). Environmental factors varying outside the optimal range for species largely explained the variance in the community composition of aquatic macrofauna (Chapters 3, 4).

RQ3. The responses of aquatic invertebrates exposed in situ in ditches bordering flower bulb fields were mainly determined by environmental factors, as identified by the General Linear Model (GLM). The performance of *Daphnia magna* and *Asellus aquaticus* was not affected by pesticides (expressed as Toxic Units). The reproduction of *C.sphaericus* was negatively correlated to pesticides which explained ~ 6% of variance in the reproduction output of *C.sphaericus*. Nutrients contributed largely to variance in the growth and reproduction of *D. magna*, whilst the survival and growth of *A.aquaticus* was dependent on dissolved organic carbon and temperature. Environmental factors largely affected the performance of aquatic invertebrates exposed in situ (Chapter 5).

RQ4. Imidacloprid induced a stronger effect on *Daphnia magna* supplied with phosphorus-deficient algae. According to previous studies, algae grown at low phosphorus conditions increase the width of the cell wall, as a mechanism of defense against poor external phosphorus conditions (Van Donk et al. 1997; DeMott & Van Donk, 2013). *D. magna*

in turn cannot digest such algae with thick cell walls and incorporate carbon needed for growth and development (DeMott & Van Donk, 2013). When not receiving enough food, daphnids allocate energy resources to filtering and not to growth and reproduction (Plath & Boersma, 2001). This possibly resulted in the reduced performance of daphnids supplied with nutrient-deficient algae. Food deficiency enforced effects of imidacloprid on *D. magna*. It can be concluded that food quality (in terms of the carbon: phosphorus ratio of algae) may affect the sensitivity of aquatic filter-feeding invertebrates to pesticides.

What do answers to the research questions mean for aquatic biota?

Are there effects of pesticides on aquatic biota in ditches around agricultural fields?

As found in the results of field work, the concentrations of pesticides (carbendazim, pirimiphos-methyl, imidacloprid, isoproturon, tolclophos-methyl and chlorprofam) in ditches bordering flower bulb fields were above the limits of detection. The concentration of pirimiphos-methyl exceeded NOEC (No Effect Concentration, 21 day with *D. magna*) at several sampling sites (Chapters 3, 5). Pirimiphos-methyl also contributed to the TU (toxic units) exceedings in the flower growing area in the previous decades (Chapter 2). Pesticides explained ~ 5% of the total variance in the total macrofauna community composition, ranging between ~ 1% and ~ 17% for different macrofauna groups (Chapter 3). Relative to the total variance in macrofauna community assumed to be 100 %, and ~25 % of explained variance, such contribution of pesticides to the total variance can be interpreted as substantial. Studies identifying the proportion of variance in the community composition of aquatic biota explained by toxicants area scarce. Setting procedure for such analysis would help to make results of the field studies comparable and enhance our understanding of the effects of toxicants on aquatic communities in the field.

What are the responses of different macrofauna taxonomic groups to pesticides?

Figure 1 shows the RDA triplot visualizing the relationships between the abundances of most common macrofauna species, concentrations of pesticides and environmental factors. Abundances of sensitive insects *Cloeon dipterum* (Ephemeroptera), *Ischnura elegans*, *Pyrrhosoma nymphula* (Odonata), *Chaoborus sp.* and *Chironomus sp.* (Diptera) were negatively correlated to nutrients. At the same time, abundances of species tolerant to pollution, such as molluscs *Physa fontinalis Valvata cristata* and *Valvata piscinalis* (Gastropoda), annelids *Stylaria lacustris* (Haplotaxida) and *Erpobdella octoculata* (Rhynchobdellida) and insects *Corixa punctate, Sigara striata, Plea minutissima, Notonecta glauca* (Hemiptera) were higher in ditches of the agricultural area (Figure 1). Abundances of these species were positively correlated to nutrients and pesticides.

Are there patterns in species trait composition of aquatic macrofauna in response to pesticides?

The trait modality distribution of several traits was also influenced by pesticides and environmental factors (Chapter 4). The biomass of predators (e.g., Odonata, Coleoptera,

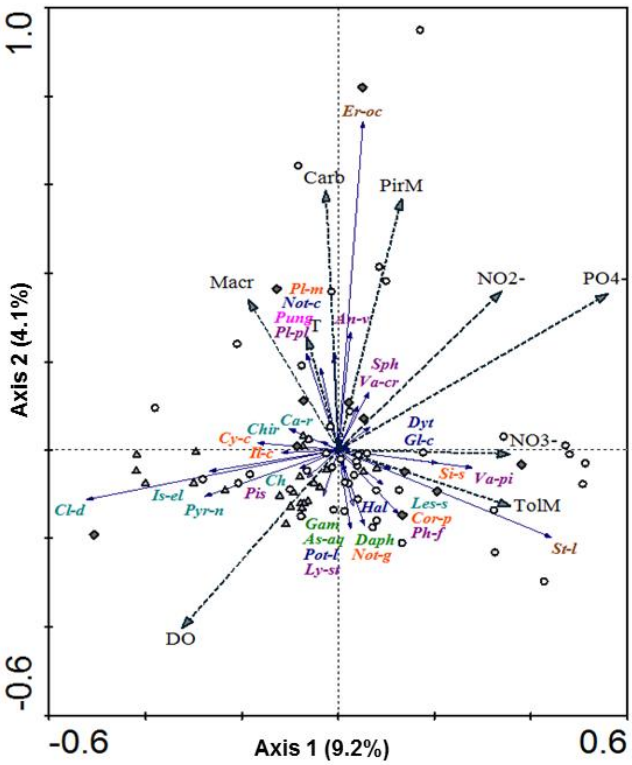

Figure 1. Redundancy analysis triplot showing relationships between Hellinger-transformed species abundances of the most common macrofauna species, environmental factors, and pesticides (the month and the year of sampling were included in the analysis as covariables). Explanatory variables having a correlation coefficient with the first two ordination axis above 0.2 are shown. The significance of the first canonical axis and the significance of all canonical axes (according to Monte Carlo test based on 999 permutations) are p = 0.004 and p = 0.084, respectively. Dashed lines represent pesticides and environmental factors, solid lines represent species abundances. PirM = pirimiphos-methyl, TolcM = tolclophos-methyl, Carb = carbendazim. Triangular = sampling sites in watersheds of nature reserve, circles = sampling sites in ditches next to flower fields, diamonds = sampling sites in ditches next to pastures. Abbreviations for the species names can be found in Table 1.

Rhynchobdellida, Gasterosteiformes) was higher in the watersheds of nature reserve than in agricultural ditches. The biomass of animals breathing through plastron (e.g., Diptera), i.e. exchanging oxygen and carbon dioxide in a thin air layer around the body, was negatively correlated to nutrients and pesticides. While the biomass of animals breathing through gills (e.g., Gasterosteiformes, Gastropoda, Bivalvia, Crustacea, Odonata), hydrostatic vesicle (e.g., Hemiptera, Coleoptera) and tegument (e.g., Rhynchobdellida, Haplotaxida) was not dependent on the water chemistry composition. Macrofauna on the sensitive pupa (e.g., Diptera) and larvae (e.g. Diptera, Odonata, Trichoptera and Coleoptera) life stages was found at higher

biomass in watersheds of nature reserves than in agricultural ditches. Semivoltine species (e.g., Gastropoda) were more abundant in the highly disturbed conditions of agricultural ditches.

Table 1. Abbreviations for the species names shown in Figure 1

Abbreviation	Species	Order	Class
Cor-p	*Corixa punctata*	Hemiptera	Insecta
Il-c	*Ilyocordis cimicoides*	Hemiptera	Insecta
Not-g	*Notonecta glauca*	Hemiptera	Insecta
Pl-m	*Plea minutissima*	Hemiptera	Insecta
Si-s	*Sigara striata*	Hemiptera	Insecta
Cy-c	*Cymatia coleoptrata*	Hemiptera	Insecta
Ch	*Chaoborus sp.*	Diptera	Insecta
Chir	*Chironomus sp.*	Diptera	Insecta
Ca-r	*Caenis robusta*	Ephemeroptera	Insecta
Cl-d	*Cloeon dipterum*	Ephemeroptera	Insecta
Is-el	*Ischnura elegans*	Odonata	Insecta
Les-s	*Lestes sponsa*	Odonata	Insecta
Pyr-n	*Pyrrhosoma nymphula*	Odonata	Insecta
Dyt	*Dytiscus sp.*	Coleoptera	Insecta
Hal	*Haliplus sp.*	Coleoptera	Insecta
Not-c	*Noterus clavicornis*	Coleoptera	Insecta
Pot-l	*Potamanthus luteus*	Coleoptera	Insecta
Pung	*Pungitus pungitus*	Gasterosteiformes	Actinopterygii
Er-oc	*Erpobdella octoculata*	Rhynchobdellida	Hyrudinea
St-l	*Stylaria lacustris*	Haplotaxida	Olygochaeta
As-aq	*Asellus aquaticus*	Isopoda	Malacostraca
Daph	*Daphnia sp.*	Diplostraca	Branchiopoda
Gam	*Gammarus sp.*	Amphipoda	Malacostraca
An-v	*Anisus vortex*	Basommatophora	Gastropoda
Ly-st	*Lymnea stagnalis*	Basommatophora	Gastropoda
Ph-f	*Physa fontinalis*	Basommatophora	Gastropoda
Pl-pl	*Planobris planobris*	Basommatophora	Gastropoda
Va-ma	*Valvata macrostata*	Heterostropha	Gastropoda
Va-cr	*Valvata cristata*	Heterostropha	Gastropoda
Va-pi	*Valvata piscinalis*	Heterostropha	Gastropoda
Pis	*Pisidium sp.*	Veneroida	Bivalvia
Sph	*Sphaerium sp.*	Veneroida	Bivalvia

Taxonomic and species trait composition of aquatic macrofauna in agricultural ditches and watersheds of nature reserve differed (Chapters 3, 4). Eutrophic conditions of agricultural ditches were unfavorable for sensitive macrofauna taxa. Other macrofauna taxa characterized by tolerance to pollution and species traits helping organisms to adapt to disturbances were found in high amounts in ditches of agricultural area.

What do answers to the research questions mean for water management?

The goal of the Water Framework Directive (WFD) is to protect the quality of surface and ground water in Europe (The EU Water Framework Directive, 2015). The WFD sets environmental quality standards for different substances and types of water bodies (amongst all, large drainage ditches, lakes, rivers, coastal waters and ground waters). Remarkably, the WFD is targeted at the protection of large water bodies (lakes larger than 50 ha and rivers larger than 10 km^2), while smaller water bodies (and connected waters) are not protected by the WFD. This lack of regulatory legislation makes control and management of ditches difficult and dependent on the regional water management authorities. Yet, aquatic biota in small waters is relatively rich in biodiversity. These small waters also contain high natural habitat diversity. Rare invertebrate species, not occurring in rivers, can be found in ditches. Aquatic macrophytes also maintain the water purifying function in ditches. Hence, the value of biodiversity in ditches is high. Small waters are also important with regard to ecosystem services: in addition to the main function of water level control, ditches represent an indispensable part of the landscape in the Netherlands and have high value for passive recreation.

As can be seen in the online tool (www.bestrijdingsmiddelenatlas.nl), high pesticide residue concentrations, often exceeding the water quality targets, are often found in surface waters around the flower bulb fields in the Netherlands. As seen in the results of the current study, pesticides in combination with environmental factors did affect community composition of aquatic biota (Chapters 3, 4). Hence, environmental management plan should be developed to reduce pesticide emissions in the study area. First, the emission routes of pesticides to surface waters should be identified. An inventory in the area can be done to determine possible sources of pesticide emissions (for instance, whether emissions occur due to the spray drift, runoff from the fields, or originate from point sources). As a next step, possible solutions to reduce emissions can be developed. The approaches to address the issue of the water quality in the area can be the following: 1) bottom – up approach, in which all actors in the field of agriculture, including water managers and regional stakeholders, combine their efforts in developing measures to reduce pesticide emissions or 2) top-down approach, in which initiatives aiming to reduce chemical emissions and impacts on the watersheds are taken by regulatory authorities. These types of approaches are discussed in

De Snoo & Vijver, 2012 (Chapter 14). As an example of a bottom-up approach, first, emission sources (point and non-point) can be identified, followed by the evaluation of existing policy regulations and setting agreements between the different actors so as to perform agricultural activities in a sustainable manner (Oommen et al., 2004).

As described in the literature, spray drift represents the main route of pesticide emissions in the Netherlands (Chapter 1, Van Linden et al., 2008). An example of a top-down approach to minimize spray drift in a very efficient practical way is to set buffer zones between crops and ditches (De Snoo & Vijver, 2012). A buffer zone is defined as an area typically located between the sensitive area (the ditch) and the crop where no spraying is done. The buffer zone can be covered with vegetation (grass, shrubs or trees) that creates a barrier between the crop and the ditch (Department of Environment and Primary Industries, 2014). Introducing buffer zones was shown to reduce the spray drift of pesticides, as applied on the fields, to ditches (De Snoo & De Wit, 1998).

The width of the buffer zone in the flower bulb growing area as currently prescribed by the policy guideline of Rijkswaterstaat Water (Article 3.8) should be a minimum of 150 cm. In addition, sprayers should be equipped with low drift nozzles, which should be located not higher than 30 cm above the plants during the spraying process. Such regulatory measures aiming to minimize spray drift should be followed. Such regulatory measures will benefit water quality in ditches, as well as aquatic biodiversity.

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Summary

"Effects of pesticides on aquatic macrofauna in the field" by Oleksandra Ieromina

Agricultural activities in the Netherlands are typically performed close to ditches, which inevitably leads to contamination of surface and ground waters with pesticides. The current PhD thesis addressed the effects of pesticides on aquatic macrofauna in the field. The study aimed to identify the extent of pesticide effects on aquatic macrofauna, given that aquatic ecosystems are influenced by abiotic and biotic factors that affect the performance of aquatic biota. Field research was performed in the flower bulb growing area of the Netherlands where pesticides are used intensively.

To address the study aim, the following research questions were formulated:

1. Did pesticide levels and aquatic macrofauna diversity in ditches of the flower growing region of the Netherlands change over the previous decades?
2. What proportion of the total variance in the community composition of aquatic macrofauna can be explained by pesticides, environmental factors (nitrate, nitrite, phosphate, dissolved organic carbon, dissolved oxygen, temperature, macrophyte coverage), presence of other biota, and time (seasonal and annual variation)? What is the predictive power of species trait approach in quantifying pesticide effects on aquatic macrofauna in the field?
3. What are the effects of pesticides combined with environmental factors on aquatic invertebrates exposed in situ in ditches bordering flower bulb fields?
4. Does food quality affect the responses of *Daphnia magna* to the insecticide imidacloprid?

Research question 1

To study temporal variation in pesticide levels and macrofauna diversity in ditches next to flower bulb fields, a dataset obtained from the Water Management Board Rijnland was analyzed (Chapter 2). The dataset consisted of pesticide concentrations measured at various locations over the years 1975 - 2010, and the species composition of aquatic macrofauna collected over the years 1983 - 2010 in ditches adjacent to flower bulb fields and in watersheds in a nature reserve. Pesticide levels in surface waters were expressed as toxic units (TU). In addition, TU were normalized by the number of pesticides measured per sample. Macrofauna diversity was estimated based on the Shannon diversity index. Normalized TU did not change in 1974 - 1998, followed by a decrease in 2000 - 2010. Concentrations of the most frequently measured pesticides (for instance, tolclophos-methyl, imidacloprid, chloridazon, isoproturon, chlorpropham, diuron, simazine, metoxuron) decreased over time. Macrofauna diversity in the flower bulb growing area and the nature reserve tended to

increase over time. Correlative analysis between pesticide levels and macrofauna diversity could not be performed because pesticides and macrofauna data were not consistent over time and location. Further field research is needed to elucidate causal relationships between pesticide levels in surface waters and macrofauna diversity.

Research question 2

To identify the relative contribution of pesticides to the total variance in the community composition of aquatic macrofauna, field work was performed in the flower bulb growing area of the Netherlands (Chapter 3, 4). Sampling and taxonomic identification of macrofauna, measurements of water chemistry and pesticide concentrations in ditches were performed during the years 2011 - 2012. Variance partitioning based on the partial redundancy analysis (pRDA) was applied to divide the total variance in macrofauna community composition into the variance explained by pesticides, environmental factors, the presence of other biota, and time. In addition, macrofauna species were classified into trait modalities of nine species traits. Sensitive insect species (Trichoptera, Diptera) were found at high abundances in watersheds of the nature reserve, while molluscs (Gastropoda) and annelids (Haplotaxida) were favored by eutrophic conditions of ditches adjacent to flower bulb fields. The largest proportion of variance in both taxonomic and species trait composition was explained by environmental factors, followed by pesticides and time.

Research question 3

To study the effects of pesticides in combination with abiotic factors on aquatic invertebrates, in situ exposure experiments with *Daphnia magna*, *Chydorus sphaericus* and *Asellus aquaticus* were deployed in ditches bordering flower bulb fields (Chapter 5). Relationship between survival, reproduction and growth of animals, pesticide concentrations and environmental factors (nitrate, nitrite, temperature, phosphate, dissolved organic carbon) was analyzed with a General Linear Model (GLM). Pesticides did not affect the performance of *D. magna* and *A. aquaticus*. Nutrients explained the largest proportion of variance in growth and reproduction of *D. magna*. Dissolved organic carbon and temperature contributed to the variance in survival and growth of *A. aquaticus*. Environmental factors largely determined the performance of aquatic invertebrates exposed in situ.

Research question 4

To study the combined effects of the insecticide imidacloprid and food quality (expressed as carbon: phosphorus ratio of algae) on the performance of *D. magna,* laboratory experiments were performed (Chapter 6). These experiments involved exposure of *D. magna* juveniles supplied with algae of varying nutritional quality to imidacloprid. A stronger effect of imidacloprid on the survival and growth of *D. magna* was observed at the conditions of food deficiency. It was shown in previous studies that algae grown at the conditions of low phosphorus concentrations tend to increase the width of the cell wall, which is a protective

mechanism against unfavorable conditions. Daphnids in turn cannot fully digest algae with thick walls and assimilate sufficient carbon as needed for growth. When obtaining insufficient amounts of carbon, daphnids spend more energy on filtering then on growth. Imidacloprid possibly acted as an additional stressor to daphnids, and strengthened the negative effects of food deficiency on the performance of animals. It can be concluded that food quality may affect the sensitivity of aquatic filter-feeding invertebrates to pesticides.

Conclusions

Pesticides residual concentrations in ditches of the flower bulb growing region of the Netherlands were found at detectable levels. Pesticides contributed to the total variance in aquatic macrofauna community composition. Environmental factors (amongst all nutrients, DOC, temperature) explained the largest proportion of variance in survival, growth and reproduction of aquatic invertebrates exposed in situ. Similarly, the highest proportion of variance in macrofauna community composition was ascribed to environmental factors. Based on the results it can be concluded that field-relevant factors should be considered in pesticide effect assessment.

Samenvatting

"Effecten van residuen van bestrijdingsmiddelen op aquatische macrofauna in het veld"

Proefschrift van Oleksandra Ieromina

Nederland is waterrijk, sloten omringen vaak intensieve agrarische activiteiten. Het is hierdoor onvermijdelijk dat verontreiniging van oppervlakte- en grondwater optreedt met bijvoorbeeld residuen van bestrijdingsmiddelen. In het voorliggende proefschrift worden de effecten van residuen van bestrijdingsmiddelen in oppervlaktewater op de aquatische macrofauna in de veldsituatie bestudeerd. Het doel van de studie is om de omvang van effecten van bestrijdingsmiddelen op de aquatische macrofauna gemeenschap in sloten te kwantificeren, waarbij expliciet rekening wordt gehouden met abiotische en biotische factoren in de veldsituatie. Het veldonderzoek is uitgevoerd in de bollenstreek van Nederland waar bestrijdingsmiddelen intensief worden gebruikt.

De volgende onderzoeksvragen zijn in dit onderzoek geformuleerd:

1. Hoe variëren de concentraties van bestrijdingsmiddelen en de aquatische macrofauna diversiteit door de jaren in sloten in de bollenstreek?
2. Kan de samenstelling van de aquatische macrofauna gemeenschap worden verklaard en welk deel van de variantie wordt verklaard door bestrijdingsmiddelen, milieufactoren (nitraat, nitriet, fosfaat, opgelost organisch koolstof, opgeloste zuurstof, temperatuur, macrofyten dekking), de aanwezigheid van andere biota en tijd (seizoensgebonden en jaarlijkse variatie)? Wat is de voorspellende waarde van het inschatteen van de bestrijdingsmiddelen effecten op aquatische macrofauna gebruik makend van soort eigenschappen?
3. Wat zijn de effecten van residuen van bestrijdingsmiddelen in combinatie met abiotische factoren op de sleutelsoort *Daphnia magna* indien in situ blootgesteld in sloten grenzend aan bollenvelden?
4. Is de voedselkwaliteit van invloed op de effecten van *Daphnia magna*, blootgesteld aan het insecticide imidacloprid?

Onderzoeksvraag 1

Om de temporele variatie in de bestrijdingsmiddelen concentraties en macrofauna diversiteit in sloten te bestuderen, werd een dataset geanalyseerd met veldwaarnemingen die verzameld is door het waterschap Rijnland (Hoofdstuk 2). De dataset bevatte bestrijdingsmiddelen concentraties gemeten op verschillende locaties over de jaren 1975 - 2010. Eveneens bevatte de dataset gegevens over de soortensamenstelling van aquatische macrofauna over de jaren

1983 - 2010 in sloten grenzend aan bollenvelden en uit stroomgebieden in een aangrenzend natuurgebied. Effecten van bestrijdingsmiddelen in het oppervlaktewater werden uitgedrukt als toxische eenheden (TU). Daarnaast werden TU genormaliseerd op basis van het aantal gemeten bestrijdingsmiddelen per monster. Macrofauna diversiteit werd geschat op basis van de Shannon diversiteit index. Genormaliseerde TU veranderde niet in de jaren 1974 - 1998, een afname van de TU werd gevonden in de periode 2000 - 2010. Concentraties van de meest gemeten bestrijdingsmiddelen (zoals tolclophos-methyl, imidacloprid, chloridazon, isoproturon, chloorprofam, diuron, simazine, metoxuron) daalden in de tijd. Macrofauna diversiteit in zowel de bollenstreek als in het natuurgebied vertoonde een licht stijgende tendens in de tijd. Correlatieve analyse tussen de bestrijdingsmiddelen concentraties en macrofauna diversiteit kon niet worden uitgevoerd omdat de datasets niet consistent waren qua tijd en locatie van bemonstering. Verder veldonderzoek is nodig om causale verbanden tussen de bestrijdingsmiddelen concentraties en macrofauna diversiteit te vinden.

Onderzoeksvraag 2

Om de relatieve bijdrage van bestrijdingsmiddelen aan de totale variantie in macrofauna samenstelling te identificeren, werd veldwerk verricht in de bollenstreek van Nederland. Bemonstering en taxonomische identificatie van macrofauna, metingen van waterchemie en concentraties bestrijdingsmiddelen werd uitgevoerd in de jaren 2011 - 2012 (Hoofdstuk 3, 4). Een partiële redundantie analyse (pRDA) werd toegepast om de totale variantie in macrofauna- gemeenschapssamenstelling te verklaren op basis van concentraties van bestrijdings-middelen, milieufactoren, aanwezigheid van andere biota en tijd. Bovendien werden soorten ingedeeld in een aantal soortenkenmerken. Gevoelige insecten taxa (Trichoptera, Diptera) werden gevonden bij hoge dichtheden in de stroomgebieden van het natuurreservaat, terwijl de aanwezigheid van weekdieren (Gastropoda) en ringwormen (Haplotaxida) werden begunstigd door eutrofe omstandigheden van sloten grenzend aan bollenvelden. Het grootste deel van de variantie in soortensamenstelling, uitgedrukt op taxonomische eigenschappen danwel op soorteneigenschap, werd verklaard door milieufactoren, gevolgd door bestrijdingsmiddelen concentraties en tijd.

Onderzoeksvraag 3

Om de effecten van blootstelling van ongewervelde waterdieren aan bestrijdingsmiddelen in combinatie met abiotische factoren te bestuderen, zijn in situ blootstellingsexperimenten uitgevoerd met *Daphnia magna*, *Chydorus sphaericus* en *Asellus aquaticus* (Hoofdstuk 5). Relaties tussen geschatte toxische eindpunten (overleving, voortplanting en de groei van de dieren), en concentraties aan bestrijdingsmiddelen en abiotische factoren (nitraat, nitriet, temperatuur, fosfaat, opgeloste organische koolstof) werden geanalyseerd met behulp van General Linear Modellen (GLMs). De bestrijdingsmiddelen concentraties hadden geen negatief effect op de fitheid van *D. magna* en *A. aquaticus*. Nutriëntconcentraties verklaarden het grootste deel van de variatie in groei en reproductie van *D. magna*. In water opgelost

organisch koolstof en de temperatuur droegen bij aan de variatie in overleving en groei van *A. aquaticus*. De fitheid van ongewervelden was vooral afhankelijk van milieufactoren.

Onderzoeksvraag 4

Om de interactieve effecten van het insecticide imidacloprid en de kwaliteit van het voedsel op de fitheid van *D. magna* te bestuderen, zijn laboratorium experimenten uitgevoerd (Hoofdstuk 6). Juveniele *D. magna* zijn opgekweekt met algen van verschillende nutritionele kwaliteit (uitgedrukt als C: P ratio). Daarna zijn de watervlooien blootgesteld aan imidacloprid. Een groter effect van imidacloprid op de overleving en de groei van *D. magna* werd gevonden bij watervlooien die werden gevoerd met P-deficiënte algen. Het mechanisme daarachter kan verklaard worden op basis van eerdere studies waarin werd aangetoond dat algen gekweekt bij lage fosfaatconcentratie de dikte van de celwand vergroten, als beschermingsmechanisme tegen ongunstige omstandigheden. De watervlooien kunnen algen met een verdikte celwand niet volledig verteren, hetgeen remmend werkt op hun groei. De imidacloprid blootstelling is in onze opzet dan ook een extra stressfactor voor watervlooien, en de interactie van een tekort aan voedsel en blootstelling aan bestrijdingsmiddelen heeft dan een versterkend effect op de overleving en groei van de watervlooien. Kortom, de voedselkwaliteit speelt een rol in de effecten van bestrijdingsmiddelen op ongewervelden.

Conclusie

Bestrijdingsmiddelen concentraties in de sloten van de bollenstreek van Nederland werden gevonden boven de detectie limiet. De bestrijdingsmiddelen residuen dragen bij aan de totale variantie in samenstelling van de aquatische macrofauna gemeenschap. Milieufactoren (onder alle voedingsstoffen, DOC, temperatuur) verklaarden het grootste deel van de variantie in de overleving, groei en reproductie van ongewervelde waterdieren die blootgesteld waren in het veld. Eveneens verklaarden de milieufactoren het grootste percentage van de variantie in macrofauna samenstelling op gemeenschapsniveau. Op basis van de resultaten kan worden geconcludeerd dat veld relevante factoren moeten worden meegenomen in de effect beoordeling van bestrijdingsmiddelen.

Curriculum vitae

Oleksandra Ieromina was born in Sevastopol (Ukraine) on September 6th 1986. From 2003 to 2008 she did bachelor studies in biology (specialization in "Plant Physiology and Biotechnology") at Tavrida National University named after V. I. Vernadskiy (Simferopol, Ukraine). Her bachelor thesis was conducted in collaboration with the Department of Ecological Physiology of Algae (Institute of Biology of the Southern Seas, Sevastopol, Ukraine) under the supervision of Dr. T.Y. Churilova and Dr. I.P. Oturina. Her Bachelor thesis focused on the dependence of microalgae photoadaptive responses on cell size. After finalizing her bachelor studies in 2008, Oleksandra was awarded with an Erasmus Mundus scholarship to participate in the joint Master course in Marine Biodiversity and Conservation (EMBC). The first two semesters of the Master course were based at the University of Algarve (Faro, Portugal). The courses of the next semester were undertaken at the University of Oviedo (Oviedo, Spain). Her Master thesis, supervised by Dr. L. Stemmann and Dr. L. Mousseau, was performed at the Oceanological Observatory of Villefranche-sur-Mer (UPMC/CNRS, France). In her Master thesis, Oleksandra investigated the effects of climate change on zooplankton communities of the North-West Mediterranean Sea. After completion of the Master course, Oleksandra joined the Institute of Environmental Sciences (CML, Leiden University) as a PhD researcher. Her PhD was part of the Environmental Chemoinformatics ECO project (Marie Curie Framework Program 7). From 2010 to 2015, Oleksandra conducted PhD research at CML under the supervision of Dr. M.G. Vijver, Prof. W.J.G.M. Peijnenburg and Prof. G.R. de Snoo. Her research focused on the effects of pesticides on aquatic macrofauna in the field.

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