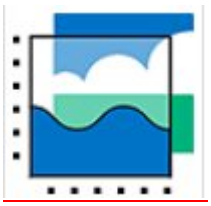

Marie Curie Initial Training Network

Environmental Chemoinformatics (ECO)

Final project report

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Assessing effects of pesticide mixtures in the aquatic environment

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Chapter 1. Introduction. Project Description

Aquatic environment is characterized by multiple abiotic factors and various interactions of chemicals and natural stressors. The goal of the current project is to quantify effects of pesticide mixtures in combination with abiotic stressors on aquatic macrofauna communities. Interactive effects of pesticides and abiotic stressors on aquatic biota were evaluated based on the results of field work in the area with intensive agricultural activities (flower growing region of the Netherlands). Pesticides applied in flower bulb crops can enter ditch systems through different routes: direct spray, leaching from the soil, runoff and spillage from pesticide containers.

Data on ecological effects of pesticides in natural environment is scarce. Ditch networks represent a typical aquatic ecosystem in the Netherlands. Ditches are characterized by extremely high connectivity. Toxicological effects on aquatic organisms are difficult to estimate because interaction of multiple abiotic and chemical factors lead to high data uncertainty.

In order to estimate impacts of pesticides in combination with biotic and abiotic factors on biodiversity, expressed as various taxonomic and species traits indexes macrofauna sampling and taxonomic identification were performed in 2011-2012. Additionally, water chemistry parameters and pesticide residue concentrations in water were monitored. Data were evaluated by multivariate methods and generalized linear model. Responses of aquatic key species to field-relevant stressors were investigated by means of in situ bioassays with three aquatic key species *D. magna, C. sphaericus* and *A.aquaticus*. In situ experiments were performed at 8 sites in ditches adjacent to flower fields characterized by different levels of pesticide emissions and 2 reference sites in ditches located in an upstream nature reserve not contaminated with pesticides (resulting in 10 sites in each experiment x 4 experiments in time. Cages were deployed in the period of intense agricultural activities (spring and autumn, years 2011 and 2012). Bioassay responses survival, reproduction and growth rate) of *Daphnia magna*, *Chydorus shpaericus, Asellus aquaticus* were analysed in relation to concentrations of pesticides and abiotic factors like nutrients, dissolved oxygen and dissolved organic carbon.

Furthermore combination effects of pesticides and nutrients were verified in the laboratory setting, by performing toxicity tests. Various combinations of commercial insecticide imidacloprid and algae containing different phosphorus concentrations, as well as their interactive effects on aquatic invertebrate species *D. magna* were analysed. Stronger impact of imidacloprid on the survival, growth and reproduction

output of daphnids was observed at phosphorus-deficient conditions. Results of the study indicated that toxicity of imidacloprid for *D.magna* increase when supplied with algae of low phosphorus content. Varying levels of nutrients in the natural aquatic environment influence the sensitivity of *D.magna* to the insecticide imidacloprid.

As a further work, during last year of PhD we plan to analyse historical database of WaterManagment board Rijnland that includes data on macrofauna and water chemistry/pesticide concentrations in surface waters in the province Southern Holland over the last 20 years. The aim is to reveal patterns in macrofauna taxonomic/trait diversity in relation to land use activities, soil types, chemical and abiotic stressors.

Results the project can be applied to improve current procedures in risk assessment of pesticides, that need adjustment considering various abiotic stressors present in the environment.

Concentrations of imidacloprid in the test chambers, and concentrations of pesticides in the surface water samples collected in Autumn 2012 were measured during the internship in the Institute for Analytical Research (Hochschule Fresenius, Idstein, Germany) in the period October-November 2012. The goal of the internship was to learn analytical method LCMS/MS after SPE.

Additionally I was also given the opportunity to supervise master students (2 student internships in 2011 and 2012) and participate in ECO schools that provided extensive training in REACH legislation and Environmental Risk Assessment (ERA). Significant attention was given to chemometrics (QSAR/QSPR modelling) and in vitro techniques as alternative tools to reduce/replace animal tests.

Collaborations: Water Management Board Rijnland, RNM (Bilthoven, the Netherlands), Institute for Analytical Research (Idstein, Germany)

Chapter 2. Effects of pesticides in combination with field-relevant stressors on freshwater invertebrate communities.

Manuscript in preparation

Aim: Quantify relative contribution of chemicals to the total impact in macrofauna compared to other stressors

INTRODUCTION

Loss of biodiversity in recent decades represents major environmental concern worldwide. Biodiversity plays important role in maintaining ecosystem functions under changing environmental conditions. Natural aquatic ecosystems are affected by chemical contamination including toxic pesticides that have potential to modify significantly community structure, reduce species diversity and population densities. Aquatic macrofauna plays crucial role in food web dynamics, nutrient recycling, biochemical fluxes in ecosystem. Diversity of aquatic macrofauna provides holistic view of the state of ecosystem and can be regarded as an early sign of disturbance.

A large variety of factors determine the fate and behaviour of pesticides in aquatic environment: chemical properties of pesticides, environmental characteristics (nutrient status, amount of precipitation, water chemistry), specific agricultural practices (spraying method, treatment program, landscape characteristics) (Wijngaarden, 2004). In a complex matrix of different stressors present in real environment it is difficult to accurately assess effects of pesticides on biota based only on first tier approaches. Moreover protection measures aiming to preserve natural populations are undertaken at higher organizational levels – population and communities. For this reason for the registration of pesticides under EU regulation a significant role is identified for field trials. OECD guidelines were set up for the higher tiered approaches (mesocosm semi-field studies) simulating realistic exposure conditions and covering a range of species (OECD, 2004). Numerous factors in the field affect aquatic biota apart from pesticides that results in uncertainty in risk estimation and can easily lead to wrong interpretation of toxic effects. Higher-tier procedures are being developed and implemented into practice to validate estimate of exposure and effects (Brock, 2006).

Another approach in risk characterization is based on chemical and biological monitoring data that provides important information on realistic levels of contamination, long term impacts of chemicals, indirect effects on biota, species interactions and recovery of communities. Water Framework Directive (WFD) states that surveillance data assess long-term changes in environmental conditions and levels of contamination (WFD, 2000/60/EC). Surveillance monitoring includes measurements of indicative biological, hydromorphological, physicochemical parameters (WFD, GD No

7). This is especially important to consider in the field studies aiming to assess effects of realistic exposure on biota, when parameters that are likely to affect community composition and properties of toxic compounds should be taken into account.

Several methodologies are being used to assess pollution in aquatic environment including taxonomic indexes, indicator species (Liess, 2005). Another approach based on species traits represents a promising tool in ecological risk assessment (Webb, 2010). Biodiversity indicators based on species traits have been used to study effects of land use, farming types, hydromorphology, heavy metal pollution on aquatic invertebrate communities (Magbauna, 2010; Ippolito, 2012; Charvet, 2000). Recently biomonitoring based on species traits started to be used in ecotoxicology research. Liess (2005) proposed index SPEAR (species at risk) that incorporated ecological traits sensitive to pesticides. SPEAR index was successfully used in assessment of pesticide impacts in rivers and had a predictive capacity also for other chemicals. Species traits approach allows to estimate sensitivity of aquatic communities to toxicants as well as recovery potential after toxic stress (or resilience of ecosystem) (Statzner, 2010). For instance, traits referring to locomotion type, dispersal ability determine potential of organisms to escape from polluted environment. In the current study we analysed physiological, life history, behavioural, ecological traits that determine vulnerability of ecosystem in response to pesticide mixtures.

The goal of the research was to investigate effects of pesticides on aquatic macrofauna communities in the realistic field setting considering relevant field factors (physico-chemical water parameters and habitat characteristics). Research question 1: are abundances of aquatic macrofauna and biodiversity measures of ecosystem affected by mixtures of pesticides in the realistic aquatic environment?

Results were obtained from field surveillance data collected from spring to autumn in the area were mixtures of pesticides were applied.

MATERIALS AND METHODS

Pesticides peak concentrations were measured during May-November 2011 within the research area. Selection of pesticides and water chemical parameters was done based on historical database of physic-chemical water properties (province Southern Holland, years 1990-2010) obtained from Water Management Board Rijnland and literature data on flower growing and cultivation. Sampling was done at sites representing watershed and ditches where organisms are collected. Concentrations of pesticides were measured in Omegam laboratoria BV. (Amsterdam, Netherlands) with liquid chromatography-mass spectrometry (LCMS-MS), gas chromatography-mass spectrometry (GCMS) and gas chromatography-electron capture dissociation (GC-

ECD). Functional groups of pesticides selected for measurements and DT50 values are presented in the Appendix Table A. Coordinates of sampling locations are presented in the Appendix Table B.

Water chemistry

Physico-chemical water quality parameters were measured at each sampling: temperature (°C), dissolved oxygen (DO, mg/L), oxygen saturation (%), pH, conductivity (mS) (Table N). Temperature and Oxygen were measured with Oxygen meter Z521 Consort. pH was measured with pH-meter Greisinger electronic. Conductivity was measured with conductivity-meter Eijkelkamp Agriresearch Equipment. DOC measurements were done with nondispersive infrared detector (NDIR). Important field conditions - width of the ditch, depth of the ditch, current velocity, macrophyte coverage were recorded in each sampling time.

Macrofauna sampling

Macroinvertebrate samples were collected with pond net 500µ mesh pushed over upper part of substratum. Multihabitat sampling strategy was followed. Surface area of each habitat type was estimated at each site and sampling units were collected from all dominating habitats. Larger organisms (mainly Gastropoda, some Coleoptera) as well as rare species (for instance, Libellula larvaes) were identified in the field and released. After all subsamples were collected, macrofauna sample was transferred to wide-mouth plastic sample jars. Samples were preserved with 4% formaldehyde or 70 % ethanol directly after sampling. In the lab samples were washed, sorted and identified to the lowest taxonomic level possible. Olygochaeta and Chironomidae were identified mostly to Family level. If organisms (mostly Crustacea and Olygochaeta) occurred in extreme high number, sub sampling (dilution) was done to estimate total number. After taxonomic identification samples have been stored in 90 % alcohol in the fridge.

Several invertebrate taxonomic indexes were determined: total number of species, abundance (total number of individuals), Shannon species richness, Pielous eveness, Fisher index, Brillouin index, Simpson index.

Macrofauna samples were collected at 14 sites monthly in the period May-November 2011: 2 sites in the nature reserve, 2 sites in pastures, 10 sites within flower growing area along the gradient of pesticide concentration. Three areas were selected to represent different stressors on aquatic macrofauna: sites in flower growing area with intensive pesticide application; sites in pastures receiving continuous inputs of nutrients from manure and fertilizers; control sites in the nature reserve area characterized by premium quality water. In total 86 macrofauna samples containing

111933 specimens belonging to 92 taxonomic groups (including all taxonomic levels identified) were analysed.

Statistical analysis

In order to assess impact of pesticides and other field relevant factors on diversity and abundance of macrofauna assemblages a number of relevant physico-chemical parameters as well as habitat characteristics were included in the analysis.

Association between macrofauna abundance, diversity, trait modalities and environmental characteristics was examined by Generalized Linear Model (GLM) assuming Poisson distributed error ε of counted N. GLM analysis resulted in a model for each taxonomic category (on the level of Class), each diversity index and trait modality.

Explanatory variables for GLM model were chosen based on their significance to determine community composition and abundance of invertebrate community and included 14 variables:

Physico-chemical water parameters: temperature (T), pH, conductivity (Cond), dissolved oxygen concentration (DO)

Nutrients: Nitrate, Nitrite, Phosphorus (P)

Pesticides: concentrations of individual pesticides present at concentrations above detection limit were included in the analysis: Chloorprofam (Chloor), Pirimifos-methyl (PirM), Tolclofos-methyl (TolM), Carbendazim (Carb), Imidacloprid (Imdc), Ethiofencarb (Ethfc)

Habitat characteristics: macrophyte coverage (Macroph)

Dependent variable were divided into three groups:

Taxonomic: abundance of classes Insecta, Maxillopoda, Branchiopoda, Malacostraca, Actinopterygii, Clitellata, Turbellaria, Gastropoda, Bivalvia, Amphipoda, Arachnida

Diversity: abundance (N), Shannon species richness (J), Pielous eveness (J), Fisher index (F), Brillouin index (H), Simpson index (L)

$$\log_e(Ni) = a + b1T + b2pH + b3Cond + b4DO + b5Nitrite + b6Nitrate + b7P + b8\ Chloor + b9\ PirM + b10\ TolM + b11\ Carb + b12\ Imdc + b13\ Mthc + b14\ Macroph + \varepsilon$$

Logarithmic link function between numerical abundances of main taxonomic groups, diversity indexes, trait modalities and environmental data was used in the GLM model in order to meet assumption of normal distribution. Dispersion parameter was estimated in the model. Generalized linear model assumes that explanatory predictor variables are not highly correlated therefore prior analysis environmental variables were examined for correlation structure and one of highly correlated variables was

removed from analysis. Stepwise regression algorithm was used to fit the model sequentially. Wald test was performed to test statistical significance of coefficients in the model. Macrofauna and environmental data sampled at different months were aggregated for analysis. GLM modelling based on macrofauna and pesticide data collected simultaneously. Statistical analysis was performed using GenStat software Version 13.1.0.4470 (VSN International Ltd).

Multivariate analysis

As a result of field work a large dataset containing diversity, trait and water chemistry data was obtained. In order to analyse dataset containing multiple variables and simultaneous observations multivariate statistic analysis was applied .

Multivariate approaches were used to examine species composition and abundance of macrofauna communities in different areas. Analyses were carried out using Primer Statistical software (Clarke & Warwick, 1994). Non-metric Multi Dimensional Scaling analysis (MDS) was performed based on abundance data to visualize similarities in the macrofauna assemblages between three areas: 1) nature reserve 2) pastures 3) flower growing fields. MDS analysis was based on Bray-Curtis similarity of log (X+1) transformed data. Macrofauna abundance data (counts of species) was used in MDS analysis. Two-dimensional MDS plots were obtained. Calculated stress value represents distances between the data (stress value <0,2 considered reliable).

ANOSIM ("Analysis of similarities") analysed significant differences between three areas identified for analysis (defined as ‘nature reserve – sandy dunes’, ‘pastures’ and ‘flower bulb fields’), using randomization permutation method based on rank similarity of samples. ANOSIM handled one way ANOVA analysis. In the output global R values and significance levels between groups were obtained.

SIMPER analysis (Similarity percentages - species contributions) was used to examine the contribution of each species to the average Bray-Curtis dissimilarity between groups of samples (groups defined ‘nature reserve – sandy dunes’, ‘pastures’ and ‘flower bulb fields’). It also determined the contribution to similarity within a group. CLUSTER analysis (hierarchical agglomerative clustering) was performed on macrofauna species trait data. CLUSTER algorithm defined groups of cases (sampling sites) based on the similarity of multiple variables (abundance of species) calculated for each site.

Multivariate analysis for different areas was done for samples collected on the same dates at all sampling sites.

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Chapter 3. Responses of *Daphnia magna*, *Chydorus sphaericus* and *Asellus aquaticus* to ambient pesticides contamination in ditches around bulb fields

Manuscript in preparation

Aim: Quantify effects of ambient pesticide concentrations on the performance of representative invertebrate species by means of 21 day in situ bioassay, contribution of pesticides to the overall effect

INTRODUCTION

The flower growing area in the province of Southern Holland (The Netherlands) represent a highly productive agricultural sector, in which relatively high amounts of pesticides are applied. Pesticides applied in flower bulb crops can enter ditch systems through different routes: direct spray, leaching from the soil, runoff and spillage from pesticide containers (Van Wijngaarden et al, 2004). Spray drift is considered to be the most important emission route (Van der Linden, 2006). Another important emission route is leakage from the baths where bulb are disinfected by fungicide treatments before planting that prevents infestation of the bulbs with fungal infections, such as botrytis (Van Kan, 2005).

Ecological effects of ambient concentrations of mixtures of different pesticides as can be found in ditches surrounding arable fields are poorly studied. Studies have focused mainly on the environmental fate of pesticides in the drainage ditches (Renaud et al, 2008; Wan et al, 2006) and measures to reduce risks of pesticides transferred from the agricultural fields to the surrounding water bodies (De Snoo et al, 1998; Margoum et al, 2006). One study addressed effects of pesticide contamination on aquatic communities in the ditch systems in Germany (Heckman, 1981). However to our knowledge no studies focused on individual responses of aquatic species to diffusive pesticide contamination in the ditches. Ditch networks in the Netherlands are characterized by extremely high connectivity. According to De Snoo and Vijver (2012) the total length of the ditches in the Netherlands reaches 300.000 km. As a result movements of aquatic organisms in those areas are negligibly limited by natural boundaries. This creates a potential for active dispersing organisms to escape from contaminated sites (Bilton et al, 2001). Moreover, ditch systems are highly dynamic in terms of hydrological and water chemistry parameters. Water level and excess of vegetation in ditches are continuously controlled by local authorities (Water Management Boards). Concentrations of nutrients and pesticide residues in surface waters are changing constantly depending on farming activities at the surrounding agricultural fields. Therefore ecological effects on aquatic biota are not easy to detect

because of the high data complexity and uncertainties arising from interaction of abiotic and chemical factors in the real field setting. In situ test were proven to reduce uncertainty in extrapolation of laboratory data on field responses as they are a step towards relevance to the realistic field situation (Burton, 2005; Schulz, 2003; Domingues, 2008; Rand, 2004; Arts et al, 2006). In situ experiments deployed in the field provide a basis to study two important components: 1) effects caused by chemicals and 2) their combination with other abiotic factors relevant in the realistic environment.

The current study aimed to evaluate invertebrate species responses to ambient concentrations of a mixture of pesticides as found in ditches around bulb fields. Our research question is: are there ecological effects on aquatic invertebrates *Daphnia magna, Chydorus shpaericus* and *Asellus aquaticus* of low, individually non-toxic concentrations of pesticides and other factors co-occuring in the field? Although individual pesticides levels are below critical values for *Daphnia magna, Chydorus shpaericus* and *Asellus aquaticus*, we hypothesize that the mixture of pesticides do cause impacts. 21 day in situ exposure experiments with *Daphnia magna, Chydorus shpaericus, Asellus aquaticus* due to the mixture of were performed in ditches around bulb fields where pesticides are applied. Cages were deployed in the period of intense agricultural activities (spring and autumn, years 2011 and 2012). In situ experiments were performed at eight sites in ditches adjacent to flower fields characterized by different levels of pesticide emissions and two reference sites in ditches located in an upstream nature reserve not contaminated with pesticides (resulting in 10 sites in each experiment × 4 experiments in time. Bioassay responses survival, reproduction and growth rate) of *Daphnia magna, Chydorus shpaericus, Asellus aquaticus* were analysed in relation to concentrations of pesticides and abiotic factors like nutrients, dissolved oxygen and dissolved organic carbon.

MATERIALS AND METHODS

Research area

The experimental area was located on the territory of two polders: Het Langeveld and Noordzijderpolder Noord of a surface area of about 15 km2 (Fig. 1). The area is intensively used for flower growing, also there are several patches of pastures and grasslands. Flowers in Southern Holland are grown on sandy-rich soil, which makes the run off of pesticides to the surface- and groundwater considerably high. Every agricultural field is surrounded by smaller ditches (2-4 m width). The water is subsequently collected in larger ditches (5-6 m width). Upstream of agricultural fields there is a nature reserve characterized by premium water quality. Water flows by a

natural gradient from the nature reserve to the flower bulb area. In situ experiments were deployed at eight sites on the ditches adjacent to flower bulb fields and two control sites on the channels within nature reserve. Experimental sites selected were on different distances from the nature reserve in order to obtain a gradient of emissions of agricultural usage, and thus pesticide residue concentrations. The average distance between experimental locations was 1 km. In the research area pesticides and fertilizers are applied sequentially during the crop cycle. Therefore ditches are subjected to continuous diffusive contamination. Seasonal crop rotation resulted in different pesticides that were applied continuously during the year in the period February – November, hence mixtures of 10 pesticides residues were expected to be found in the surface water. In situ experiments of 21 day duration were deployed in May and September during years 2011-2011 (4 experiments in total).

Pesticide measurements

Selection of pesticides for analytical measurements was based on the analysis of authorized pesticides as used in flower growing: e.g. information provided by DLV (Dutch national service for agricultural information); PPO (Dutch National Institute for Plant Production); Nationale Milieu Indicator for tulip and hyacinth growing (2004). Additionally, an historical database of physico-chemical water properties of Waterboard Rijnland (province Southern Holland, year 2010) was analysed (Van Rooden, 2011). Major pesticides applied in the study area were identified. Concentrations of pesticides were measured by Omegam laboratoria BV (Amsterdam, Netherlands) by means of GC-MS and LC-MS/MS.

Water chemistry

Physico-chemical water quality parameters were measured at the start (t=1) and at the end (t=21 day) of the experiment: e.g. temperature (ºC), dissolved oxygen (DO, mg/L), oxygen saturation (%) were measured with an Oxygen meter Z521 Consort. pH was measured with a pH-meter Greisinger electronic. Conductivity was measured with a conductivity-meter Eijkelkamp Agriresearch Equipment. Dissolved Organic Carbon concentrations were quantified using non-dispersive infrared analysis (NDIR). Phosphate and nitrate/nitrite were measured according to NEN 6663 and NEN-EN-ISO 13395 respectively (OMEGAM laboratory, Amsterdam, The Netherlands).

Justification of species selection

Selected species *D. magna* and *C. shpaericus* belong to the order Cladocera, have similar modes of feeding and respiration but have different body size and inhabit different compartments (water column and sediments respectively). Three species (D. *magna, C. shpaericus* and *A.aquaticus*) were selected as they are important macro

organisms in aquatic ecosystems. D. magna is a filter-feeding planktonic species that has high ecological importance serving as a food source for larger crustaceans and fish. Smaller-sized *C. shpaericus* (size male 0,3-0,5 mm) is a meiobenthic cladoceran that plays an important role in the food web by converting organic matter into biomass that becomes available for predators – invertebrates and small fish (Dekker, 2006). The aquatic Isopod A.aquaticus is an omnivore, grazing on algae and particulate organic matter (Vick and Blum, 2010). Additionally, crustacean species *D. magna* and *C. spaericus* and *A. aquaticus* were selected because their growth rate provides detectable increase in body length during 21 day test period.

Test animals

Juveniles of Daphnia magna, Chydorus sphaericus were obtained from the laboratory culture (National Institute of Public Health and Environment, RIVM, The Netherlands). Asellus aquaticus adult males and females were collected in the ditches around nature reserve areas and maintained in the laboratory during 1 month with a 16/8h light photoperiod and 20ºC. A. aquaticus juveniles and adults were fed with a diet consisting of dry leaves and fish food, rich in protein and minerals. Air was constantly supplied to each aquarium. In order to provide shelter, black plastic tubes or stones were placed at the bottom of aquaria. Once a week ditch water was filtered and 50 % was refreshed with Dutch Standard Water (DSW) in order to reduce bacterial and fungal growth. Every week A. aquaticus juveniles and adults were counted and isolated into separate containers. 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 hour old), *C.sphaericus* (36-48 hour 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* – dry leave and two pellets of fish food were added.

Response measurements

Initial size measurement of *D. magna* juveniles and *C. sphaericus* (36-48 hours old) subsampled from the permanent laboratory culture in RIVM was done prior to the field experiments. Initial size measurements of *A. aquaticus* juveniles (2-3 weeks old) was done 1 day before field deployment. An average body length at the experiment initiation was calculated based on 30 measurements. *D. magna* body length was defined as the distance from the most posterior point on the eye to the base of the junction of the tail spine with the carapace (Ranta, 1993). *C. sphaericus*: body length was defined as the distance from the posterior point on the eye to the end of carapace (Dekker, 2006). *A. aquaticus*: body length was defined as the distance between the base of the antennae until the top the of pleotelson (Vick and Blum, 2010).

Enclosures were retrieved after 21 days, surviving adults of the three species were counted and body morphometric parameters of surviving adults were measured. Moreover, juveniles of *D. magna* and *C. sphaericus* produced during the 21 day experiment were counted. For *C. sphaericus*, morphometric measurements were made for all organisms, and individuals larger than 350 µm were classified as adults. In natural environment *A. aquaticus* reach maturity in 20 weeks and does not produce juveniles during the 21 days exposure. Therefore endpoints such as survival and growth rate were estimated for *A. aquaticus*.

Survival (%) was estimated as the percentage of surviving *A.aquaticus* relative to the initial number of juveniles placed in the cage. Reproduction of *D. magna and C. shpaericus* was estimated as number of juveniles produced in 21 day of exposure. The total number of juveniles produced in 21 days and the total number of juveniles produced in 21 days/number of adults alive at day 21 were assessed.

The average body length at each site was calculated and used to estimate the daily growth rate. The Somatic Growth Rate (SGR) was calculated as:

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

where L i =final body length, L0=initial body length, d=total number of days (21 day).

Data treatment

Toxicity of pesticides at each sampling site was estimated in terms of Toxic Units (TU). The TU values were based on the NOEC (No Observed Effect Concentration), endpoint reproduction (21 day test) for *D. magna*:

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

where TUi(D. magna) 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). For each station, the sum of toxic units was determined and used in the data analysis (SumTU).

Principal Component Analysis (PCA) was used to reduce the dimensionality in the environmental data and identify parameters contributing to most of the variability in the dataset (first and second principal components). Environmental data for PCA analysis were log-transformed (all environmental variables except pH) in order to ensure normality and remove the effect of measurement units (log((1000*Conc)+1)). The graphs represent the PCA correlation for environmental variables (PC1 versus PC2).

In order to assess the impact of pesticides and other field relevant factors on survival, growth and reproduction of D*. magna, C. sphaericus* and *A. aquaticus* under field conditions, a Generalized Linear Model (GLM) was applied, following the general equation:

$$\log_e(Ni) = \alpha + \beta 1 * \text{Abiotic Factor1} + \beta 2 * \text{Abiotic Factor2} + \ldots + \beta N * \text{Abiotic Factor N} + \varepsilon,$$

where α=intercept; ε=error; Abiotic Factor1…Abiotic Factor N = explanatory variables (environmental parameters); Ni= response variable (estimated endpoints).

In order to avoid overfitting, every model was corrected for overdispersion. Data was assumed to have a Poisson distribution. A logarithmic link function between the response and explanatory variables was used. A stepwise regression algorithm was applied to fit the model sequentially. The Wald test was performed to test statistical significance of coefficients in the model. In order to account for overdispersion, a dispersion parameter was estimated for each model. All statistical analyses were performed in GenStat software Version 13.1.0.4470 (VSN International Ltd).

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Chapter 4. Application of trait-based theory to assess effects of pesticides on freshwater macrofauna

Aim: investigate effects of pesticides on the species trait composition of aquatic macrofauna

Manuscript in preparation

INTRODUCTION

Trait-based approach is a new tool to evaluate effects of pollution on aquatic ecosystems. Assessment of the state of ecosystem based on species traits was introduced several decades ago. Search on Web of Knowledge results in over 400 articles. Description of communities in combination with functional groups provides more complete understanding of ecosystem functioning.

Review of Magbauna et al (2010) focus on response of stream macroinvertebrates to different types of farming (conventional, integrated and organic). Authors estimated the impacts of farming type and management system on taxonomic and trait composition of macroinvertebrate community and relation to ecosystem functioning (measured as leaf breakdown rate and algal production). They found that invertebrate species traits are sensitive to agricultural practices and have same efficiency as taxonomic approach.

Statzner et al. (2010) give an extensive overview of invertebrates traits and the effect of multiple stressors on invertebrates in running freshwater ecosystems, including history, complications and technical framework of trait-based approach. Among the factors acting on the traits of macroinvertebrates are heavy metal pollution, cargo ship traffic, eutrophicaiton, climate change. A detailed description of use of functional feeding groups is provided to show that various stressors acting on the traits of invertebrates.

Vandewelle et al. (2010) gives an overview of functional traits as complementary biodiversity indicators, with the examples of several animal groups – benthic invertebrates, collembolans, some insects and birds. Authors introduce a new framework based on functional traits indices and underline that consideration of species traits can improve monitoring of the responses of biodiversity to different land use practices. In case study with benthic macroinvertebrates species traits changed in relation to land use/vegetation cover and hydromorphological features.

In the study of Doledec (2006) potential of species traits in quantifying effects of land use on invertebrate communities in grassland streams is clearly demonstrated.

Functional attributes of invertebrates as well as taxonomic composition were studied along the gradient of agricultural development (nutrients, sedimentation). As a result 5 of 60 individual species showed significant change in density in response to farming while species traits were more sensitive - 14 out of 53 traits modified. Therefore they conclude that species traits can complement classical taxonomic approach.

Menezes et al. (2010) give another overview of trait-based 'community descriptors' and raise a question if trait-based approach can offer an alternative to traditional taxonomy studies. They infer that species traits represent a promising method of biomonitoring in freshwater ecosystems.

However application of species traits of macroinvertebrates in research area related to field effects of pesticides (as a stressor) is not clearly documented. Field effects of pesticides on functional characteristics of macroinvertebrates require further understanding. Predictable changes in trait distribution were recorded for invertebrate communities along gradients of hydrologic disturbance (Vieira, 2008).

Gayraud (2003) found that reproduction traits, body form, feeding mode, dietary preference respond to anthropogenic stressors (including also organic and toxic pollution).

Species vary greatly in their sensitivity to environmental pollutants, and this variation can be described by constructing a species sensitivity distribution (SSD). The SSD is a statistical distribution estimated from a sample of toxicity data and visualized as a cumulative distribution function. From this distribution of species sensitivities, a hazardous concentration is inferred at which a certain percentage of all species is affected. However sensitivity of species to chemical varies considerably depending on functional characteristics of organisms of the species. Responses of organisms to chemicals can depend on developmental stage, dispersal ability, body size or other factors that should be taken into account.

GOALS

Comparison of different approaches (taxonomic and species trait indexes) used to evaluate toxic effects of chemicals on population and ecosystem level.

Application of "trait-based theory" introduced earlier in literature (mainly based on dynamic trait framework proposed by Webb (2010)) to realistic field data collected during year 2011 in the agricultural flower growing area. Investigate if inclusion of functional characteristics of species ("trait-based theory") can be applied to improve risk assessment of pesticides in freshwater ecosystems.

Proposal of new index based on functional characteristics of species ("trait diversity index").

To reach this aim we are going to study the impact of toxicants (pesticides) on the macrofauna communities in ditches bordering with agricultural fields. In order to test this, we are going to collect the following information:

- frequency distribution of species traits along the gradient of contaminant (gradient of pesticide concentration). Species traits divided in 4 groups: morphological, physiological, ecological and behaviour traits
- frequency distribution of species traits over time corresponding to different periods of flower growing cycle

Fundamental trait framework introduced in earlier publications (Webb, 2010) states that three major components of trait theory are:

- trait distribution: statistical distribution describing the frequency or probability of occurrence of each trait or trait category. In our case: frequency distribution of species traits weighted by abundance
- performance filter: relation between trait and local environment
- environmental gradient: environmental stressor acting to filter trait and form a filtered trait distribution. In our case: pesticide contamination and we also consider time as a factor

HYPOTHESIS

Invertebrates: we hypothesize that trait distributions as well as linkages with ecosystem functions will change along pollution (pesticide) gradient and over time.

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Chapter 5. Impact of imidacloprid on *Daphnia magna* under different food quality regimes

Submitted to the journal "Environmental Toxicology and Chemistry"

Aim: investigate combined effect of the insecticide imidacloprid and phosphorus limitation on the survival, reproduction and growth rate of *D. magna*

INTRODUCTION

The toxicity of pesticides in the aquatic environment is commonly assessed based on laboratory tests under controlled conditions: temperature, photoperiod, standardized feeding regime [1]. Opposite to the laboratory setting, the natural environment is characterized by fluctuating environmental conditions. Apart from physical conditions such as temperature, the amount of particulate organic matter in the water, also ecological condition of aquatic species varies, such as food quantity and quality.

Aquatic invertebrates of the subphylum Cladocera represent a dominant group of zooplankton mainly in freshwater ecosystems. The most well-known group is the daphnids, with common species *Daphnia magna*. The elemental food composition (estimated as C:P ratio) is one of the important factors influencing the performance of Cladocerans. The availability of phosphorus is an important factor controlling productivity of phytoplankton algae, which are primary producers in aquatic ecosystems [2]. Aquatic algae in turn serve as a main food source for filter-feeding aquatic invertebrates. Being filter feeders, Cladocerans have low ability to select better quality food. This makes them very vulnerable for changes in the food conditions [3].

Literature focuses mostly on sensitivity of aquatic invertebrates to either algae nutritional levels or chemical induced effects often at the physiological level (like growth rate [4, 5] and reproduction [5-8]) and effects at the biochemical level (like activity of enzymes involved in phosphorus and carbon metabolism [9] and the calcium balance [10]). So far the toxicity of the following chemicals to aquatic macro-invertebrate species supplied with different algae cell concentrations (estimated as number of cells in 1 mL) has been studied: fenoxycarb, chlorpyrifos [11], 3,4-dichloroaniline [11,12], endosulfan and esfenvalerate [13,14]. The comparison between the studies that do report interactions or combinations is not straightforward because chemicals selected have different modes of action and the food levels in the studies are often expressed as algae cell concentrations, not considering the algal phosphorus content. However, D.magna is sensitive not only for food quantity but also for food quality [3]. The effect of the algal phosphorus concentration on the toxicity of the following chemicals to aquatic

species was studied: the photosynthetic inhibitor WeatherMAX Roundup (referred as concentration of glyphosate) [15], fluoxetine [16], and hexavalent chromium [9].

Imidacloprid belongs to the group of neonicotinoid insecticides that block the nicotinergic neuronal pathway in invertebrates. Blockage leads to the accumulation of the neurotransmitter acetylcholine [17]. This results in paralysis of the insect, and consequently death. Imidacloprid is classified as moderately toxic based on its toxicity to mammals (weak antagonist of mammal nicotinic acetylcholine receptor) and it is highly toxic to invertebrates [17]. Therefore, imidacloprid is widely used in agriculture against a range of insect pests. Roessink et al. [18] found higher acute toxicity of imidacloprid to sensitive mayfly (order Ephemeroptera) and caddisfly (order Trichoptera) insect species compared to macrocrustaceans and insect species of orders Hemiptera, Megaloptera, Diptera.

The aim of the current experiment was to quantify the effect of the insecticide imidacloprid at a range of nutrional levels (defined as algae C:P ratios) for *D.magna* (Subphylum Crustacea, Suborder Cladocera). Toxicological endpoints used were survival, growth and reproduction, all relevant for population growth. We hypothesized that exposure to a range of imidacloprid concentrations at P-deficient conditions results in severe effects - reduced reproduction output, survival and growth rate of *D.magna* compared to P-high conditions. To mimic the differences in food quality in the field, four algal phosphorus levels were tested.

MATERIALS AND METHODS

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). Animals were cultured at standard laboratory conditions at 20°C and a 16h:8h light:dark photoperiod [19]. Adult *D.magna* were raised in 1 L plastic jars at M4 medium [20]. Culture medium was changed twice a week. Animals were fed with algal cells *Pseudokirchneriella subcapitata*. Algae were cultured in 2 L bottles in the Woods-Hole medium, the culture medium was replaced once a week. Algae were centrifuged at 2000 r.p.m. for 15 min in 50 mL falcon tubes, suspended in M4 medium and fed to D.magna.

In order to study the effect of the algal phosphorus content on *D.magna* responses, P-free Woods-Hole medium was prepared and divided between four 2L bottles. Four different amounts of K2HPO4 and algae *Pseudokirchneriella subcapitata* were subsequently added to create different phosphorus levels. Prior to the experiment, the algae were adapted to four different phosphorus conditions during

seven days in order to obtain algae cultures of different nutritional levels at the stationary growth phase (also in order to have sufficient algae biomass to initiate the nutritional experiment). Algae cultures containing four different phosphorus concentrations were kept in individual 2 L bottles with constant aeration and a 24 hour light period. Measurement of carbon and phosphorus in algae cultures were made after seven days adaptation. Table 1 depicts the nutritional levels used during the experiment.

C:P levels of algae were selected for the experiment based on the analysis of literature reporting C:P levels limiting performance of daphnids [7,8,9,15,16]. Because the aim of the current experiment was to study effects of nutritional levels (expressed as C:P ratios of algae) on *D.magna*, and not solely phosphorus, algae were grown at four different phosphorus levels during seven days. Phosphate is taken up by algae very fast, leading to decrease in extracellular phosphorus concentration [21]. At the same time algae cell density and internal phosphorus concentration increase [21]. For this reason after seven days we found similar concentration of dissolved phosphorus in four algae cultures (<0,05 mg/L), however concentration of total phosphorus differed (Table 1). Total phosphorus in turn includes all forms of phosphorus: dissolved and particulate phosphorus (bound to organic matter). Therefore after seven days most part of inorganic phosphorus was taken up by algae and transformed to the form of particulate phosphorus. Before feeding to daphnids, algae culture were centrifuged at 2000 r.p.m and only particulate fraction (algae cells dissolved in M4 medium) was used during the experiment.

HERE TABLE 1

For the determination of the organic carbon content, the 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 (NDIR). Total organic carbon concentrations were quantified by High Temperature Combustion/Direct Injection (HTC/DI).

The concentration of dissolved and total phosphorus in the algae culture was determined according to NEN 6663 (by the OMEGAM laboratory, Amsterdam, the Netherlands). The concentration of particulate phosphorus was determined as the difference between total and dissolved phosphorus concentrations. Because concentration of dissolved phosphorus was below the limit of detection at four treatments, half of the detection limit was used to calculate concentration of particulate phosphorus.

Test set up

D.magna neonates < 24 hours old were exposed for 21 day in accordance with the chronic toxicity experiment [19]. Additionally, body length measurements were performed and the somatic growth rate of *D.magna* was estimated.

Different exposure concentrations were prepared by diluting an imidacloprid stock solution in M4 medium [19]. The concentration of the stock solution was 400 mg/L, which is lower than water solubility limit of imidacloprid (610 mg/L) so no solvent was added [22]. The purity of the test substance as reported by the provider Sigma Aldrich Chemie BV (Zwijndrecht, Netherlands) was 99,7%. M4 medium containing a range of imidacloprid concentrations was transferred to the test chambers. Algae containing different four P concentrations and D.magna neonates were subsequently added to the test chambers. The experiment was performed in 100 mL test chambers, in total 50 mL medium in each test chamber. Six different concentrations of imidacloprid + control at four algal phosphorus levels were prepared. Each experimental treatment was performed in three fold (this resulted in 7×4×3= 84 test chambers). Five neonates were exposed in test replicate (three replicates each with five daphnids at every treatment). All experiments were carried out at a 16 h:8 h light:dark photoperiod and at 20°C.

Test chambers were not aerated during the experiment. M4 medium containing imidacloprid was renewed every three days to ensure continuous exposure to imidacloprid and to suppress bacteria/fungi growth. Feeding with algae cells P.subcapitata cultured at four phopshorus levels was done every three days at the day of the medium renewal. At four diets feeding was normalized based on the amount of total organic carbon (1 mgC/L). At each day of the medium renewal, another set of test chambers containing different concentrations of imidaclorid/phosphorus was prepared. Parent animals were transferred to the new medium with a pipette. Temperature, pH, oxygen saturation and water hardness were recorded three times during the experiment at the time of medium renewal and in freshly prepared medium.

Analytical measurements

Analytical measurements were performed in three test concentrations at four phosphorus levels (one replicate for each treatment) in freshly prepared medium and old medium (after three days exposure) in samples selected randomly in time. Measured concentrations were 44.6±3.1 mg/L; 94±2.5 mg/L; 158.0±6.5 mg/L respectively. Actual concentrations deviated from nominal concentrations in the range of 10% ± 8%. This deviation can be explained by dilution of the initial concentration during feeding (addition of algae to the test chambers). Because measured concentration of the test substance remained within ± 20% of the nominal, actual time-

weighted mean concentrations 2.0 mg/L, 27.6 mg/L and 66.3 mg/L were estimated assuming similar deviation from the nominal concentrations [19].

This concentration range was chosen based on reported acute and chronic toxicity data for imidacloprid: chronic 21 day NOEC *D.magna* with endpoint reproduction: 1.8 mg/L (No Observed Effect Concentration); acute 48 hour EC50 D.magna endpoint immobility 85 mg/L; EC50 P. subcapitata algae >100 mg/L [23]. 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 [24]). This allowed detecting effects on *D.magna* survival and growth on a relatively short time scale of 21 day.

Because the concentration of imidacloprid was expected to decline slightly over the period of three days between medium renewals (DT50 in microcosm= 14.8 days [23]), the time-weighted mean concentration was calculated as follows:

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

where TWConc – time-weighed mean concentration for the renewal period, time - number of days in the renewal period, Conc 0 – measured concentration of imidacloprid at the start of the renewal period, Conc 1 – measured concentration of imidacloprid at the end of the renewal period [19]. The average concentration per treatment was used in the statistical analysis [25].

Analytical measurements were performed using a 3200 Q Trap® LC/MS/MS (Applied Biosystems, Foster City, USA) in the Hochschule Fresenius (Idstein, Germany). The column used was: Aqua® C18 50 x 2.0 mm, 5 µm particle size, 125 Å pore size (Phenomenex, Torrance, USA). Eluent A used was 80 % MilliQ-Water and 20 % Methanol with 5 mM ammonium formiate, and Eluent B - 10 % MilliQ-Water and 90 % Methanol with 5 mM ammonium formiate. 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 blank. Multiple reaction monitoring of transition m/z 209 to 175 was used in mass spectrometry. The limit of quantification was 0.01 µg/L. Samples were diluted prior the analysis in the proportion 1:1000.

Estimated endpoints

Survival and reproduction of parent animals was counted daily during the 21 day experiment. Survival was estimated as proportion of surviving animals (animal was considered dead when no movement of antennae/appendages and no swimming behavior was observed). Offspring produced each day was counted daily and transferred to a new series of test chambers containing varying

imidacloprid/phosphorus concentrations. Survival of juveniles was also recorded. Number of juveniles produced daily was divided by the number of adults alive in each replicate. The net reproductive rate (R0) was determined as cumulative number of juveniles per adult produced in 21 day. The population growth rate (r) was determined as average number of juveniles produced per adult per day (according to [11]). The age at maturity was defined as the day when first reproduction occurred at each replicate. Average values for R0, r and age at maturity between three replicates and standard deviation were calculated.

Body length of the parent animals was measured every two days under a microscope STEM SR Zeiss fitted with a micrometer eyepiece. At least two randomly selected alive parent animals were measured at each test replicate (resulting in six size measurements per treatment, 6×4×7= 168 measurements every two days). When less than two animals were alive at test replicate, less measurements were done respectively. Alive animal was placed at the petri dish. Volume of water around was reduced with the pipet in order to immobilize the animal. After that photograph of the animal under the microscope was made. *D.magna* body length was defined as the distance from the most posterior point on the eye to the base of the junction of the tail spine with the carapace [26].

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

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

where L1=the average measured length of neonates at the day of the initiation of the experiment and L2=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. The Von Bertalanffy growth model was used to estimate growth rates for *D.magna:*

$$Lt = Lmax\ (1 + e\ –K(t-t0)),$$

Where Lt= body length of *D.magna* at time t; Lmax= length that can be reach at an infinite time, or a maximum potential length that can be reached at given conditions; K = growth rate; t= time (days); t0= theoretical age at Lt= 0. Von Bertalanffy growth model was constructed for control conditions, imidacloprid concentrations 2.0 mg/L and 27.6 mg/L, because at these treatments survival was maintained during 21 day that allowed comparison between food regimes.

Data treatment

One-way analysis of variance (ANOVA) was applied to test significant differences in EC10, EC20 values and reproduction output between the treatments

(95% confidence interval (CI)). Two-way ANOVA (95% CI) with replicates was performed to test the effect of two independent factors (imidacloprid and phosphorus concentrations) and the interaction between them on *D.magna* body length at days 3, 9, 15 and 21, net reproductive rate (R0), and age at first reproduction. For the two-way ANOVA analysis of body size measurements at control conditions (C0) and imidacloprid concentrations 2.0 mg/L (C1), 27.6 mg/L (C2), and 44.6±3.1 mg/L (C3) were used. For *D.magna* body length at age 3 and 9 days data for concentrations C0, C1, C2 and C3 were used. For *D.magna* body length at age 15 and 21 days concentrations C0, C1 and C2 were used because survival at C4 was not maintained during 21 day at all treatments.

Dose-response relationships between *D.magna* survival and imidacloprid concentration were analyzed. *D.magna* survival at days 9, 15 and 21 (for C:P 35, C:P 240, C:P 400 and C:P 1300) was plotted versus the corresponding imidacloprid concentration (log transformed). GraphPad Software (GraphPad Software, Inc, La Jolla, CA, USA) 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=bottom of the curve (minimum survival), max=top of the curve (maximum survival), x=concentration of imidacloprid, EC50=concentration of imidacloprid that causes 50% of *D.magna* survival, H=Hill slope (power of the curve).

EC10 and EC20 values were calculated using the following equation:

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

where ECF = EC10 or EC20, H = the Hill Slope value and F is 10 or 20.

EC10 and EC20 values were derived for 9, 15 and 21 days of exposure in order to compare effects of imidacloprid on *D.magna* fed with four diets at different age and to analyze whether the magnitude of effect changes depending on the duration of the exposure experiment (food quality expected to affect *D.magna* survival on the longer time scale).

RESULTS

Effects of imidacloprid and phosphorus on the survival of *Daphnia magna*

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 while at other diets it was maintained at 5% - 15% during the 21 day experiment (Fig 1). Differences in EC10 and EC20 values for 9 days between four food quality levels were not statistically significant ($p>0.05$) (Table 2). EC10 and EC20 values for 15 and 21 days did not differ

significantly between C:P levels 35, 240 and 400. However, mean values for EC10 and EC20 as well as Hill slope values were lower at P-deficient diet characterized by C:P 1300 (Table 2). This means that a stronger effect of imidacloprid on *D.magna* survival fed with algae containing very low phosphorus level was observed. However, comparison between EC10 and EC20 at different diets for 15 and 21 days is complicated because the 95% confidence intervals for these parameters at C:P 1300 could not be fitted.

HERE FIGURE 1

Hill slope values (H) at the C:P level of 1300 were significantly lower than at C:P 65, C:P 240 and C:P 400 (H=-49.61 for day 15 and H=-51.36 for day 21) (Table 2). A more negative slope means a more steep curve and faster response to changing exposure conditions.

HERE TABLE 2

Effects on growth rate

The Von Bertalanffy growth model fitted with the experimental body length data for D.magna showed that lowest values for growth rate (K) and maximum hypothetical length (Lmax) were reached at P-deficient diet C:P 1300, at control and imidacloprid exposure conditions (Table 3). At control conditions highest K was observed at C:P 35, however larger Lmax was attained at C:P 240. Similarly, at imidacloprid concentration 2.0 mg/L highest Lmax was observed at P-optimal conditions C:P 240 (Table 3, Fig.2).

HERE FIGURE 2

HERE FIGURE 3

At all diets, imidacloprid induced a negative effect on the D.magna growth rate K and somatic growth rate (SGR) (Fig. 2,3,4).

HERE FIGURE 4

Results of the 2-way ANOVA show significant effects of phosphorus, imidacloprid and their interaction on the body length of *D.magna* at age 3 and 21 days (Table 4).

HERE TABLE 3

Effects on reproduction

Production of juveniles was observed at control exposure conditions and at imidacloprid concentrations of 2.0 mg/L. There was no reproduction observed at the higher imdacloprid concentrations. The maximum net reproductive rate (R0) was observed at C:P 240 (optimal conditions). The lowest reproductive output was recorded for the P-deficient diet (C:P 1300). A similar trend was observed at the control and imidacloprid exposure conditions (Fig. 5A,B). 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 day test, endpoint reproduction) [23]. Because a low imidacloprid concentration was used, net reproductive rate (R0) and age at maturity for exposed animals did not differ significantly from the control. Two-way ANOVA revealed a significant effect of phosphorus on R0 ($p=0.02$) and on the age at maturity ($p=1.0E-08$) but effects of imidacloprid and imidacloprid-phosphorus interaction were not significant ($p>0.05$) (Table 4). The population growth rate (r) did not differ significantly for *D.magna* fed with different diets at control conditions and imidacloprid concentration ($p>0.05$) (Fig. 5C,D). At the control conditions and imidacloprid exposure delayed first reproduction was observed at *D.magna* supplied with P-low food (C:P 1300 and C:P 400) (Fig. 5E).

HERE TABLE 4

DISCUSSION

Varying environmental conditions, of which amongst all nutrient concentrations, are indispensable characteristics of natural aquatic ecosystems. Concentrations of nutrients in surface waters within agricultural areas vary significantly depending on local farming activities, fertilizers application, and the amount of precipitation. Exposure to a repeated dose of chemicals over time increases organism sensitivity and consequently the tolerance range to environmental factors narrows down [12]. At the conditions of multiple stressor exposure, the organism is more prone to food limitation or diet change. Extrapolation of results obtained in the laboratory to the field deals with high uncertainty [27]. This is one example of why earlier research demonstrated differences in toxicity between laboratory and field exposures range as a factor of 1.2 to 10 for the nutritional state [28].

Effects on survival

In the current study we found a larger effect of imidacloprid on *D.magna* survival at a phosphorus-deficient diet (C:P 1300) (based on EC10 and EC20 values for 15 and 21 days of exposure) (Table 2). In contrast to previous studies we did not observe significantly higher survival for *D.magna* fed with higher levels of food (expressed as the algae cell concentration in other studies) [11]. Differences between EC20 and EC10 values at C:P levels 35, 240 and 400 were not statistically significant, probably a result of high variation in survival between test replicates. *D.magna* supplied with algae of low nutrient level (C:P 1300) may have higher energy demand for maintaining physiological processes. This in turn affects the organism sensitivity [29]. The other hypothesis proposed by Brett&Muller-Navarra [30] showed that lower food quality of algae affected *D.magna* indirectly through the change in biochemical

composition of algae. 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 [31,32]. This was suggested to be a defensive mechanism of algae against grazing by D.magna at nutrient-deficient conditions [32]. Alternatively, *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. As a result, higher energy costs for filtering activity lead to a reduced growth rate and lower reproduction at a P-deficient diet [32]. This hypothesis can possibly explain our result: mean values for EC10 and EC20 were observed for *D.magna* supplied with P-deficient diet.

Effects on growth rate

A stronger effect of imidacloprid on the growth rate of *D.magna* was found at P-deficient conditions (Fig. 2,3,4). Aitchinson&Butt [33] found that at the conditions of starvation total cellular phosphorus content of green algae *Chlorella vulgaris* decreased. After 36 hours there was no polyphosphate found in algae cells [33]. In our study lowest total phosphorus content was measured for P-deficient algae *Pseudokirchneriella subcapitata* (C:P 1300) that explains its low nutritional quality. *D.magna* body phosphorus content in turn depends on algae C:P ratio [34]. Low nutrient content of *D.magna* may result in smaller body size, reduced mass of offspring. Results on daphnids growth rates as determined in our experiment especially at the high C:P level could therefore be possibly a result of reduced phosphorus uptake by *D.magna* fed with P-deficient algae. Also at P-deficient conditions values for both growth rate K and maximum hypothetical length Lmax derived in Von Bertalanffy model were lower compared to P-sufficient diets.

Phosphorus is stored in algae cells as polyphosphate [35,36]. Addition of K2HPO4 to the phosphorus-replete algae results in the increase of total cellular phosphorus and polyphosphate [37]. Faster grazing and higher growth rates were observed for *D.magna* fed with P-sufficient food [38]. However when supplied with algae of high phosphorus content feeding rate of *D.magna* were lower than at P-deficient conditions, because lower amount of energy was allocated to filtering [3]. In our experiment growth rates of *D.magna* fed with P-optimal (C:P 240) and P-rich (C:P 35) diet were higher than at P-deficient conditions also as a result of a larger amount of phosphorus incorporated by animals that possibly lead to increased body size. The observations supported the mineral limitation hypothesis introduced by Park et al. [39] stating that the growth rate of *D.magna* is positively correlated with phosphorus content of algae.

Urabe et al. [40] confirmed that phosphorus determine food quality for *D.magna* and estimated C:P ratio limiting algae growth (C:P= 300). It was suggested that *D.magna* fed with algae of C:P lower than 300 are not limited by phosphorus in food. This observation complies with our result: significantly lower growth at limited conditions of C:P 1300. Plath&Boersma observed reduced somatic growth rate at high C:P (approximately 30) [3]. Authors argued that this effect can be explained by lower incorporation of carbon by *D.magna* as a result of reduced feeding rate at P-rich conditions. In our study this result was not fully confirmed, however hypothetical body length Lmax derived from Von Bertalanffy model was higher at P-optimal conditions (C:P 240) then at P-rich (C:P 35). Additionally, in the study of Plath&Boersma significant reduction of somatic growth (about 3 fold) was observed at P-deficient C:P level of approximately 700. However, in other studies C:P limiting levels resulting in lower growth of *D.magna* were higher: C:P= 900 [34], C:P= 1000 [8], C:P= 1100 [15], C:P ranging from ~ 1037 to ~ 1360 [40]. In our experiment more negative effects on survival and somatic growth rate were observed at C:P 1300. Therefore possibly *D.magna* culture used in the experiment of Plath&Boersma had generally higher sensitivity to phosphorus. Additionally duration of experiment differed and was 6 days and K2HPO4 was added to algae cultures 24 hours before the start of the experiment. In our experiment algae were adapted to different nutritional levels during seven days and likely changed biochemical composition. However, negative effect of phosphorus on *D.magna* growth rate can happen when phosphorus concentration reach extreme level close to toxic.

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

Previous studies have suggested that the sorption of hydrophobic chemicals is positively correlated with octanol-water partitioning coefficient (Kow). In the study of Rose et al. [11] hydrophobic fenoxicarb caused higher toxicity to *D.magna* at high algae cell concentration. This was likely because larger amount of fenoxicarb was bound to algae cells and taken up by animals. Similar result was observed by Lessard&Frost for *D.magna* supplied with P-rich food [15]. Higher toxicity at a P-

sufficient diet was found for the pharmaceutical fluoxetine [16]. Alternatively, Barry et al. proposed that metabolism of hydrophobic chemical by algae can lead to lower effects on *D.magna* exposed at high food conditions [13].

Imidacloprid is a more polar insecticide that has a lower tendency to bind to organic matter (water solubility= 610 mg/L, logKow= 0.57). Therefore, irrespectively of the nutritional level (algae quantity and quality) imidacloprid is likely to induce a similar effect on the performance of *D.magna*. Only at the conditions of phosphorus deficiency (C:P 1300) effect of imidacloprid on the survival, growth and reproduction was more pronounced. Food limitation possibly acted as additional stressor on top of toxic stress and lead to higher toxicity of imidacloprid to *D.magna* supplied with algae of low nutritional quality. Following the concept of Van Straalen [42], under sufficient food conditions invertebrates likely withstand easier additional stress, as our results clearly show that at phosphorus-sufficient diets high imidacloprid concentration was easier to battle.

Effects on reproduction

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 (Fig. 5). At the conditions of P-deficiency, *D.magna* is likely to allocate more energy towards maintaining survival. Previous study suggested that lower food conditions increased the energy available to respiration and formation of carapace [43]. Consequently, the proportion of energy available for reproduction is reduced [9, 44]. This complies with the Dynamic Energy Budget theory (DEB) that allows calculating costs that are made by organisms to deal with various natural and anthropogenic stressors [29]. The energy obtained by the organism is balanced between somatic maintenance (growth) and reproduction: when high growth rate is reached, less energy is available for reproduction [29]. In the current experiment maximum number of juveniles per adult was produced at P-optimal conditions (C:P 240) whereas highest growth rates were maintained at P-rich conditions (C:P 35). High reproduction at C:P 240 was possibly maintained at the costs of the lower growth rate. At P-low conditions (C:P 1300, C:P 400) age at maturity was delayed that is possibly a consequence of lower growth rate and smaller body size of *D.magna* fed with algae of low nutritional quality. *D.magna* start reproducing when critical body size is reached. Because of the slower growth rate at P-deficient conditions *D.magna* attained critical body length later than at other diets that have led to delayed age at maturity, both at control and exposed treatments.

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 areas (see e.g. data waterboard Rijnland period 1993-2007 for Southern part of the Netherlands [45], or Gao et al. [46] period 2005-2006 for Southwestern China). Based on the results of the current study we can conclude that at the conditions of low P levels typical for nature reserves, imidacloprid pollution will result in more pronounced effects on crustaceans. Therefore, in more oligotrophic waters of for instance nature reserves located in the proximity of agricultural areas, imidacloprid causes stronger effects compared to more eutrophic waters.

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 it was concluded that toxicity of imidacloprid increased at a P-deficient diet as seen by observed effects on survival, growth rate and reproduction. Combined effects of toxicants and abiotic factors challenged the estimation of pesticides risks on daphnids populations in freshwater ecosystems. Result found can be applied to predict limiting ratios of Carbon:Nutrients for daphnids at the conditions of toxic stress. In the field situation by definition multiple abiotic factors are present and combined effects of chemicals and natural stressors can be expected. Interaction effects of resource limitation and toxic stress on organisms are important to consider in risk assessment of chemicals.

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FIGURE LEGENDS

Fig. 1. Effect of imidacloprid on the survival of *D.magna* exposed at different food regimes (Mean survival at C:P 35 (A), C:P 240 (B), C:P 400 (C) and C:P 1300 (D)). C0= Control, C1= 2.0 mg/L, C2= 27.6 mg/L, C3= 44.6±3.1 mg/L, C4= 66.3 mg/L, C5= 94.0±2.5 mg/L, C6= 158.0±6.5 mg/L.

Fig. 2. Body length of *D. magna* supplied with diets C:P 35 and C:P 240 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.

Fig. 3. Body length of *D. magna* supplied with diets C:P 400 and C:P 1300 at control conditions (A) and exposed to imidacloprid concentrations 2.0 mg/L (C1) and 27.6 mg/L (C2) fitted with von Bertalanffy growth model.

Fig. 4. Somatic Growth Rate (SGR) of *D.magna* exposed to a range of imdiacloprid concentrations plotted versus log C:P ratios (shown on the graph are mean SGR and standard error). C1= 2.0 mg/L, C2= 27.6 mg/L, C3= 44.6±3.1 mg/L.

Fig. 5. Net Reproductive Rate (R0, juv/female), Population Growth Rate (r, 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).

Figure 1

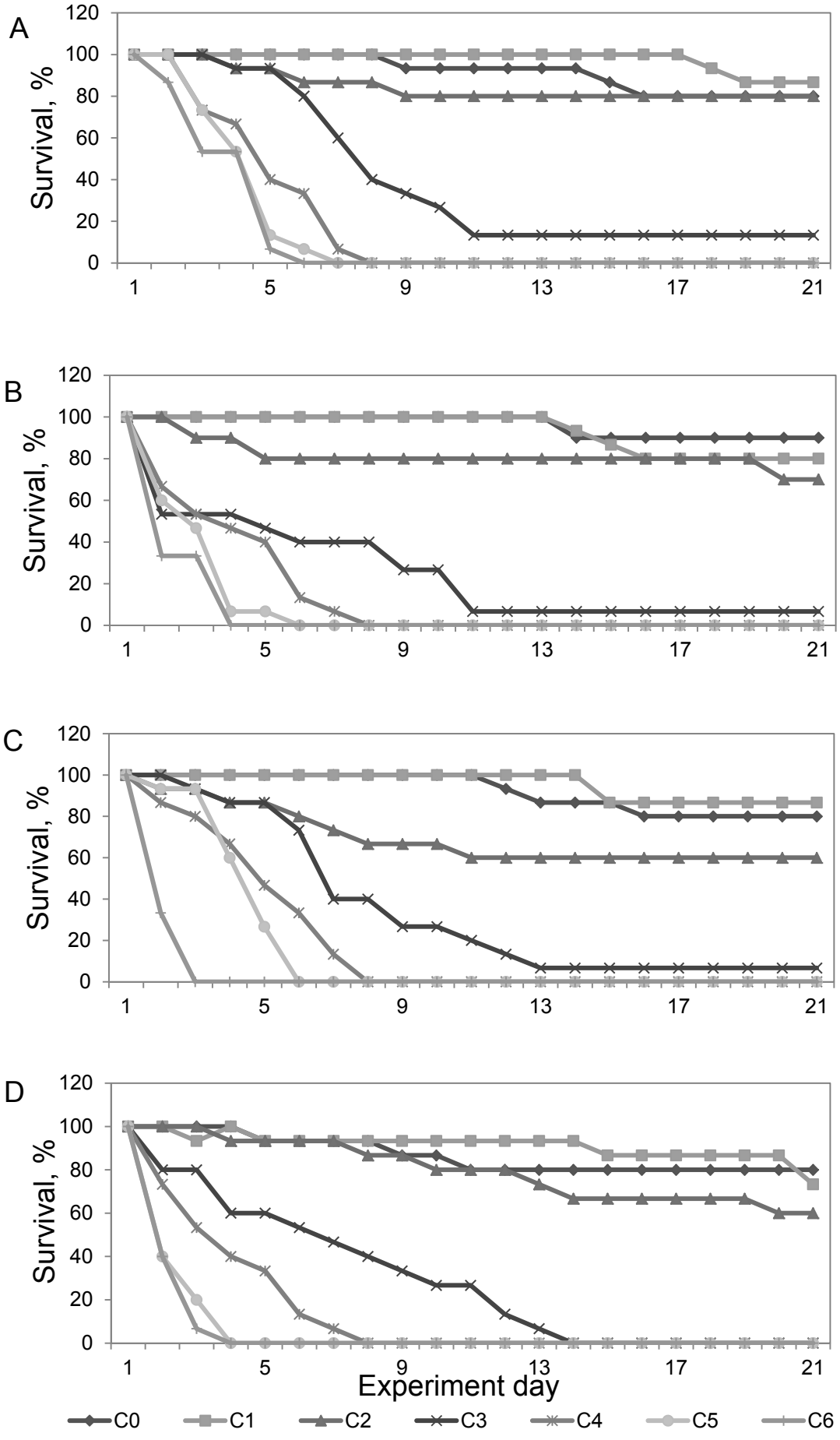
A
B
C
D
Survival, %
120
100
80
60
40
20
0
1
5
9
13
17
21
Experiment day
C0
C1
C2
C3
C4
C5
C6

Figure 2

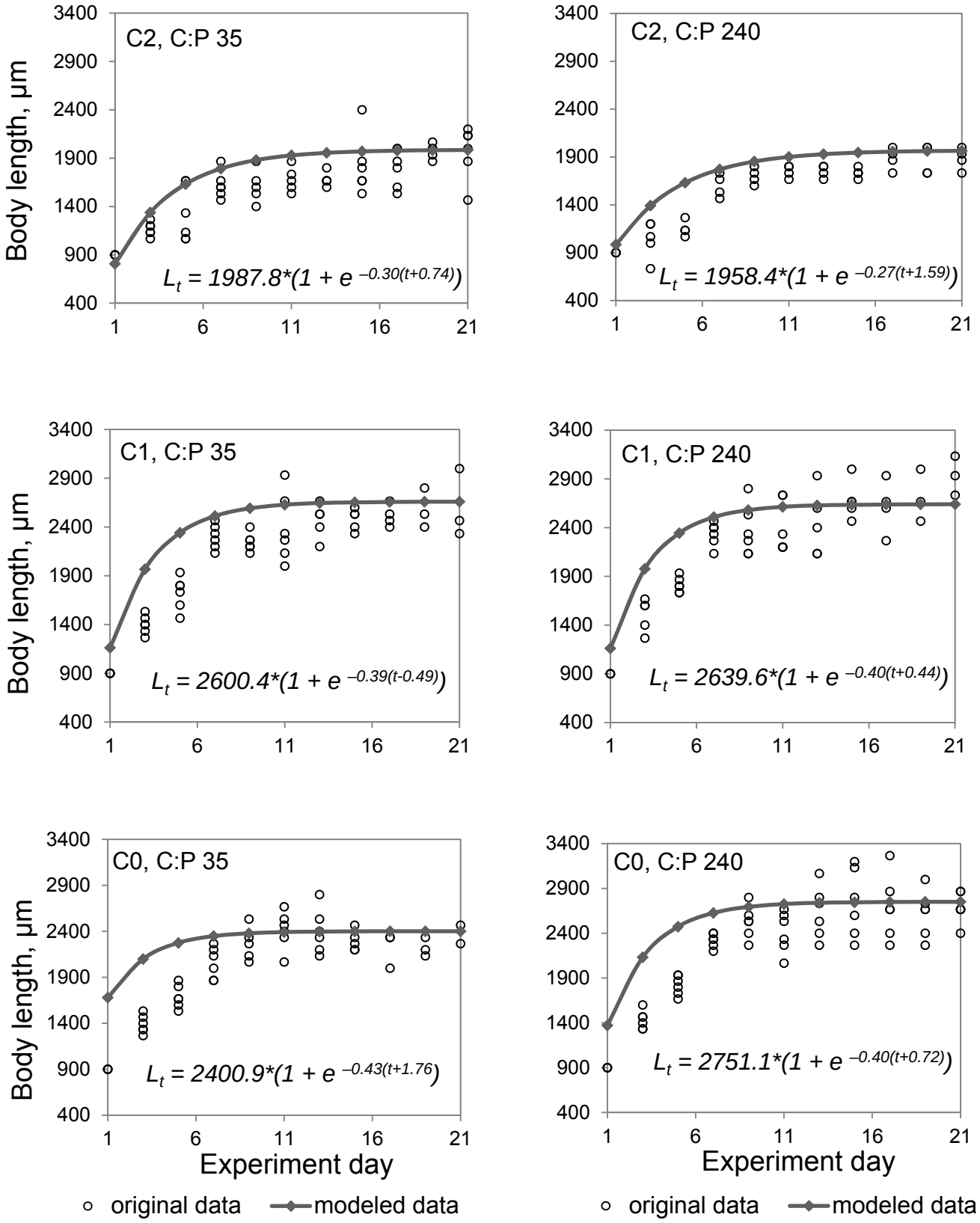
C2, C:P 35
$L_t = 1987.8*(1 + e^{-0.30(t+0.74)})$
C2, C:P 240
$L_t = 1958.4*(1 + e^{-0.27(t+1.59)})$
C1, C:P 35
$L_t = 2600.4*(1 + e^{-0.39(t-0.49)})$
C1, C:P 240
$L_t = 2639.6*(1 + e^{-0.40(t+0.44)})$
C0, C:P 35
$L_t = 2400.9*(1 + e^{-0.43(t+1.76)})$
C0, C:P 240
$L_t = 2751.1*(1 + e^{-0.40(t+0.72)})$
Body length, µm
Experiment day
original data
modeled data

Figure 3

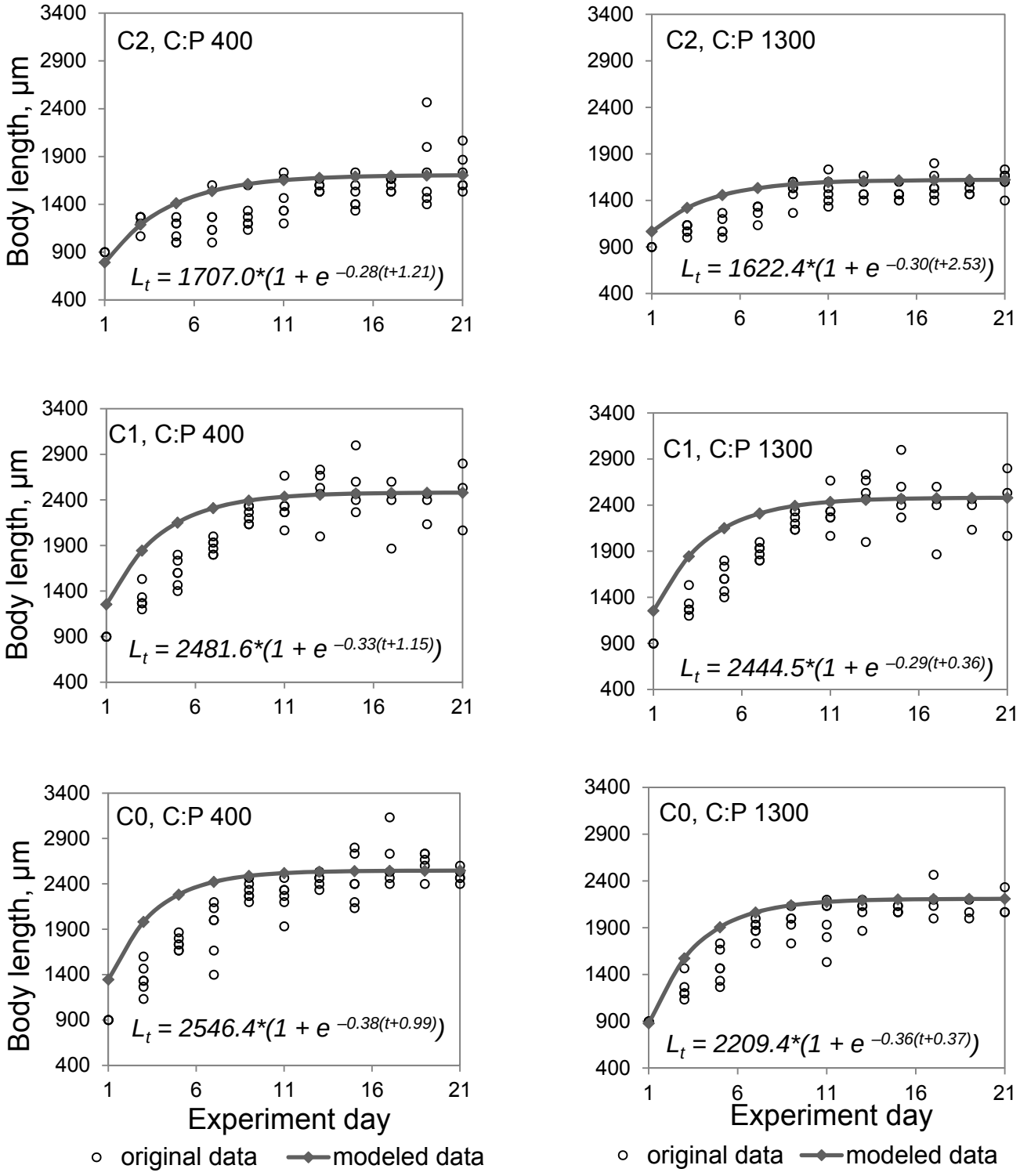
C2, C:P 400
$L_t = 1707.0*(1 + e^{-0.28(t+1.21)})$
C2, C:P 1300
$L_t = 1622.4*(1 + e^{-0.30(t+2.53)})$
C1, C:P 400
$L_t = 2481.6*(1 + e^{-0.33(t+1.15)})$
C1, C:P 1300
$L_t = 2444.5*(1 + e^{-0.29(t+0.36)})$
C0, C:P 400
$L_t = 2546.4*(1 + e^{-0.38(t+0.99)})$
C0, C:P 1300
$L_t = 2209.4*(1 + e^{-0.36(t+0.37)})$
Body length, µm
Experiment day
original data
modeled data

Figure 4

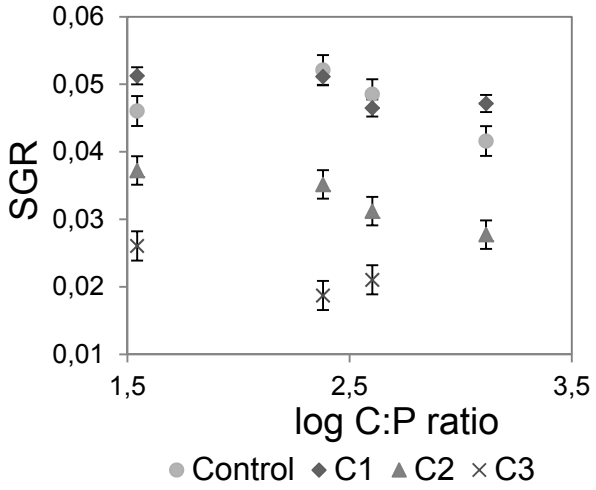
0,06
0,05
0,04
0,03
0,02
0,01
SGR
1,5
2,5
3,5
log C:P ratio
Control
C1
C2
C3

Figure 5

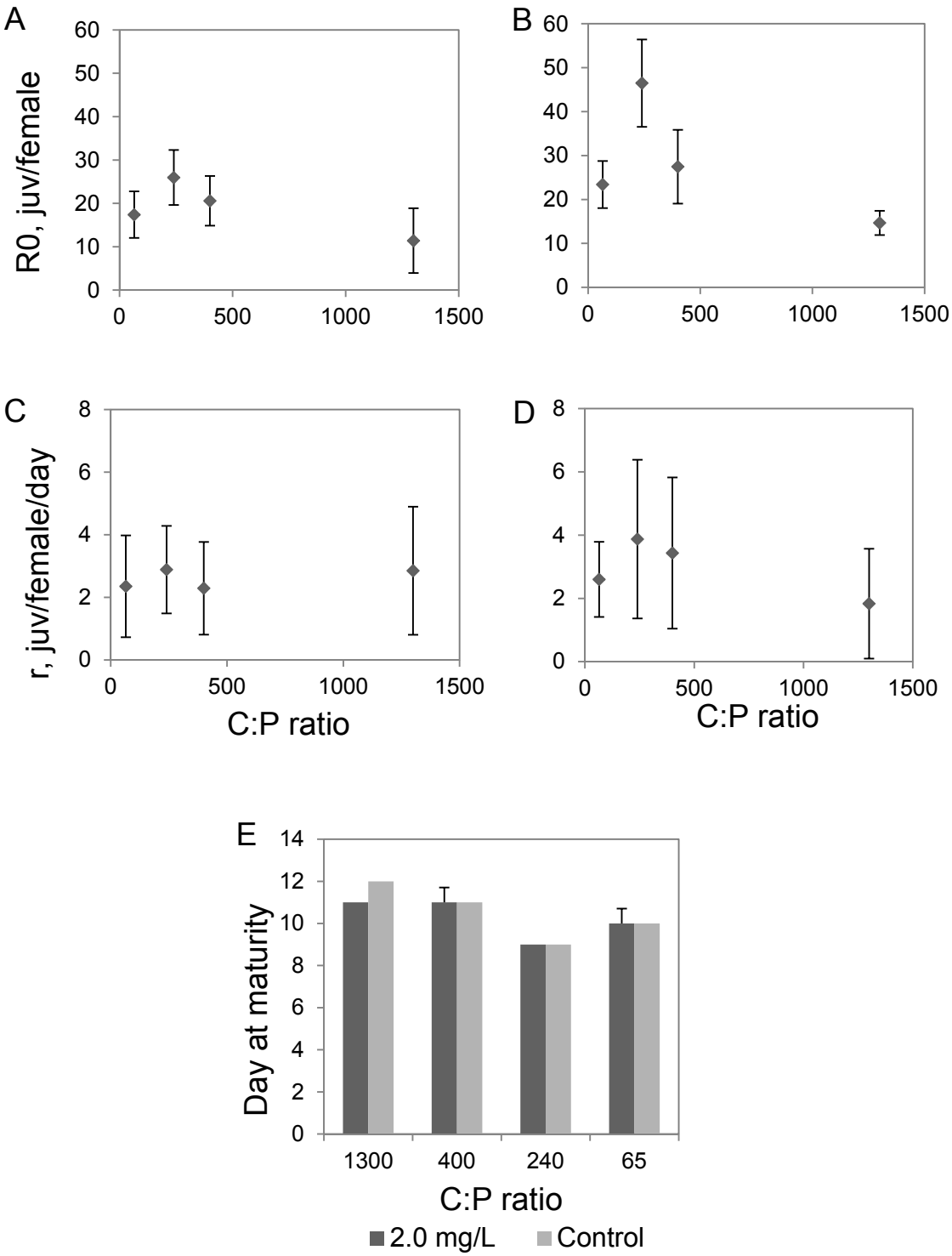
A
R0, juv/female
60
50
40
30
20
10
0
0
500
1000
1500
B
60
50
40
30
20
10
0
0
500
1000
1500
C
r, juv/female/day
8
6
4
2
0
0
500
1000
1500
C:P ratio
D
8
6
4
2
0
0
500
1000
1500
C:P ratio
E
Day at maturity
14
12
10
8
6
4
2
0
1300
400
240
65
C:P ratio
2.0 mg/L
Control

Table 1. Algae culture conditions and C:P levels used in the nutritional experiments with *D.magna.* Dissolved P= concentration of dissolved phosphorus, Total P= concentration of total phosphorus, Particulate P= concentration of particulate phosphorus, DOC= dissolved organic carbon concentration, TOC= total organic carbon concentration.

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	65
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
P-optimum	8.40	<0.05	1.00	0.98	32.79	87.50	240

Table 2. EC10 and EC20 values for 9, 15 and 21 days for *D.magna* exposed to imidacloprid at four food regimes (endpoint survival). H= hillslope value, d.f.= degrees of freedom, 95% CI= 95% confidence interval, * no CI= confidence intervals could not be fitted.

Day N		C:P ratio			
		65	240	400	1300
Day 9	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
	EC10	59.85	55.96	60.06	54.16
	95% CI	52.98 to 66.71	48.47 to 63.45	52.50 to 67.61	46.86 to 61.47
	EC20	51.54	48.21	48.61	49.31
	95% CI	45.63 to 57.45	41.76 to 54.66	42.50 to 54.73	42.66 to 55.96
Day 15	H	-7.47	-10.02	-6.69	-49.61
	d.f.	17	16	17	17
	R^2	0.9666	0.9382	0.9313	0.9768
	EC10	47.16	43.28	42.56	29.63
	95% CI	41.62 to 52.69	35.06 to 51.50	36.62 to 48.50	no CI
	EC20	42.31	39.92	37.70	29.15
	95% CI	37.34 to 42.27	32.34 to 47.50	32.44 to 42.96	no CI
Day 21	H	-9.30	-9.13	-6.86	-51.36
	d.f.	17	17	17	17
	R^2	0.9586	0.9503	0.9288	0.9591
	EC10	47.16	43.40	42.85	29.62
	95% CI	39.72 to 54.60	36.71 to 50.09	36.59 to 49.11	no CI
	EC20	43.22	39.71	38.07	29.16
	95% CI	36.41 to 50.04	33.59 to 45.84	32.51 to 43.63	no CI

Table 3. 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). 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.

C: P ratio	Estimated parameters	C0	C1	C2
C:P 35	K	0.43	0.39	0.30
	L_{inf}	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_{inf}	2751.1	2639.6	1958.4
	t0	-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_{inf}	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_{inf}	2209.4	2444.5	1622.5
	t_0	-0.37	-0.36	-2.53
	R^2	0.90	0.88	0.86

Table 4. 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. 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.

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

Chapter 6. Interannual variability of freshwater macrofauna in the areas characterized by different land use practices and soil types (province Southern Holland) – multivariate analysis of macrofauna community composition

Data being analysed

Aims

- Compare macrofauna community diversity in areas affected by land use practices on different soil types:

Types of land use: nature reserve, flower bulb crops, grassland, peat

Types of soil: sandy, light clay, peat

- Perform multivariate analysis of macrofauna community composition, investigate the similarities and patterns of macrofauna communities and environmental variables/pesticide concentrations in different areas over time scale 1997-1999, 2004-2007. Multivariate analysis used to detect trends in samples and explore the relationships between different datasets (different years, land use, soil types)

Hypothesis

There are differences in macrofauna community types depending on land use activities. In grassland macrofauna diversity is enhanced and high levels of species richness are maintained. Flower bulb growing coupled with application of pesticides decrease macrofauna diversity

Time plan

PhD project: September 2010- September 2014

September - December 2010: preparation of the working plan

January- March 2011: planning and practical preparation to field work

April - November 2011: field work in the flower growing area of Southern Holland including the following activities:

• Monthly sampling of aquatic macrofauna at 14-16 locations within the research area: 10 sites at the ditches boarding with flower growing fields, 4 sites bordering with pastures/grasslands and 4 sites in the ponds within nature reserve area, control sites

• Measurements of the water chemistry parameters in the field: T, pH, conductivity, dissolved oxygen concentrations. Further measurements in the lab: phosphate, nitrite, nitrate, pesticide concentrations (OMEGAM lab, Amsterdam), DOC (RIVM, Bilthoven)

• Sampling of *Asellus aquatic* in the field and culturing in the lab to be used in in situ experiments

• In situ exposure experiments with 3 species, duration 21 day: *Asellus aquaticus, Daphnia magna, Chydorus sphaericus* deployed at 10 sites in the field. Two experiments performed in May and September.

December 2011- January 2012: finalizing taxonomic identification of macrofauna samples collected in 2011

February- March 2012: evaluating results of the field work performed in 2011 April - **November 2012**: second round of field work (same activities as in 2011).

Additionally lab experiment performed to test combined effects of imidacloprid and nutrients on the performance of *Daphnia magna* in August- September 2012

October - November 2012: internship in the Institute for Analytical Research Hochschule Fresenius

December 2012- February 2013: finishing taxonomic identification of macrofauna samples collected in 2012, evaluation results of the field work performed in 2012, analysis of experimental data with imidacloprid- nutrients interactions

March - May 2013: Additional experiments performed in the lab to study combined effects of imidacloprid and nutrients on *Daphnia magna*

May- July 2013: Analysis of experimental data, paper writing

July 2013: Paper "Effects of imidacloprid on *Daphnia magna* under different food quality regimes" submitted to the journal "Environmental Toxicology and Chemistry" Further plans:

August-September 2013: Finalize paper on in situ exposure of three crustacean species to the ambient concentrations of pesticides in the field

Further plans:

October - November 2013: Finalize paper on effects of pesticides in combination with fieldrelevant stressors on freshwater invertebrate communities, based on macrofauna taxonomy data in relation to water chemistry collected in 2011-2012

January- February 2014: Finish paper on application of trait-based theory to assess effects of pesticides on freshwater macrofauna, based on macrofauna taxonomy data in relation to water chemistry collected in 2011-2012

March - April 2014: finish paper on interannual variability of freshwater macrofauna in the areas characterized by different land use practices and soil types

April- June 2014: write introduction to the PhD thesis and discussion

July 2014: send PhD thesis to the committee

Conferences and trainings attended

19-21 August 2013 – BASF Days Agro, Germany

25 February – 01 March 2013 - 3rd Winter School of ECO program. Linnaeus University (Kalmar, Sweden). Training in REACH requirements for model description and validation according to the OECD principles

December 2012 – Symposium “Ecosystem under stress”, Wageningen University. Oral presentation “Effects of pesticides on aquatic macrofauna under field conditions”

October – November 2012 - Internship in the Institute of Applied Sciences Fresenius (Idstein, Germany). Analysis of pesticide residues in the surface water samples using LCMS/MS after SPE. Analysis of imidacloprid in the experimental samples (see Chapter 5)

11 - 15 July 2012 - Marie Curie Actions Conference, Euroscience Open Forum (Dublin, Ireland)

11 - 15 June 2012 - 3rd Summer School of Marie Curie ITN "Environmental ChemOinformatics" (ECO) program. Verona, Italy. Focus on development and validation of QSAR models using both classical and new approaches

5 - 6 February 2012 - Netherlands Annual Ecology Meeting (NAEM), Lunteren, Netherlands. Poster presentation titled: “Toxic stress and species traits. Effect of pesticides on taxonomic and trait composition of macrofauna”

27 February – 2 March 2012 - 2nd Winter School of ECO program. Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Madrid, Spain. Focus on advantages and limitations of in vitro methods for toxicology studies

19 - 30 September 2011 - 2nd - Summer School of ECO program. Institute of Environmental Sciences, Leiden, Netherlands. Fundamental training on Chemical Risk Assessment & REACH legislation, bioassays, chemical analytics, chemometrics and QSAR/QSPR modelling

21 - 25 February 2011 -1 st Winter School of ECO program. Hochschule Fresenius (HSF), Idstein, Germany. Training in chemoinformatics

Teaching

February – July 2011 – Supervision of a Master student internship, on in situ exposure of aquatic key species

March – April 2012 – Supervision of a part of the Master student internship, on taxonomic identification of freshwater macrofauna, and multimedia fate modelling of pesticides applied in flower growing

December 2012 – Assistance in the course “Trends in Conservation Biology”

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