CHAPTER 5

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

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Published in Environmental Pollution 2014, 192: 196-203

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

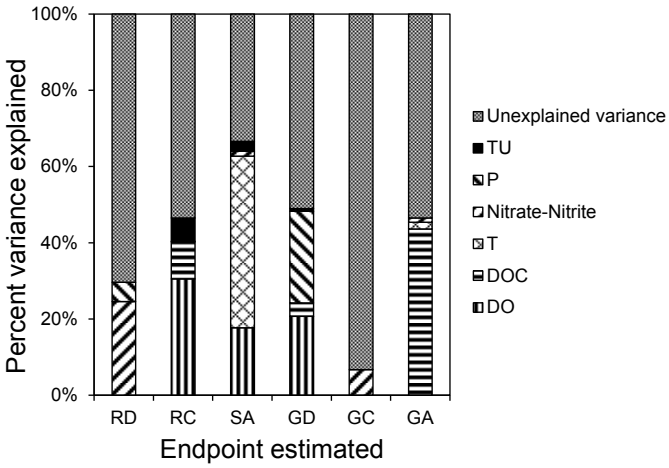

Figure 2. Histogram showing the contribution of each explanatory variable to the total variance explained by the general linear model (GLM). The figure is based on the results presented in the Table 2. RD = reproduction of *D. magna,* RC = reproduction of *C. sphaericus,* SA = survival of *A. aquaticus,*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

Acknowledgments

The authors are grateful to Water Board Rijnland for active discussions of experimental results and water system functioning in the research area. O. Ieromina is supported by the Environmental ChemoDatarmatics (ECO) project, Marie Curie ITN-EU Framework 238701. The authors thank Eric Gertenaar for assistance in the field work and Marja Wouterse for providing *D. magna* and *C. sphaericus* culture animals and DOC measurements.

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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

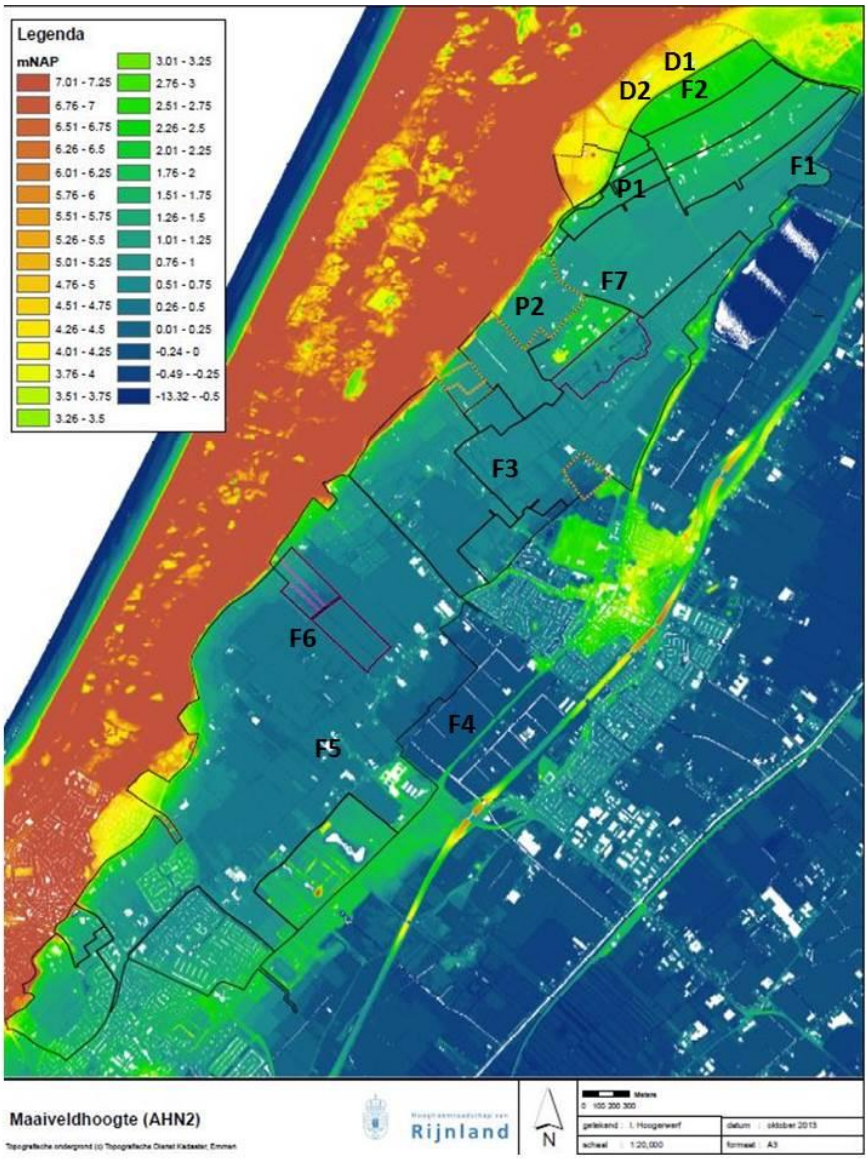

Figure S1. Topographic surface of the research area (source: Water Board Rijnland, October 2013)

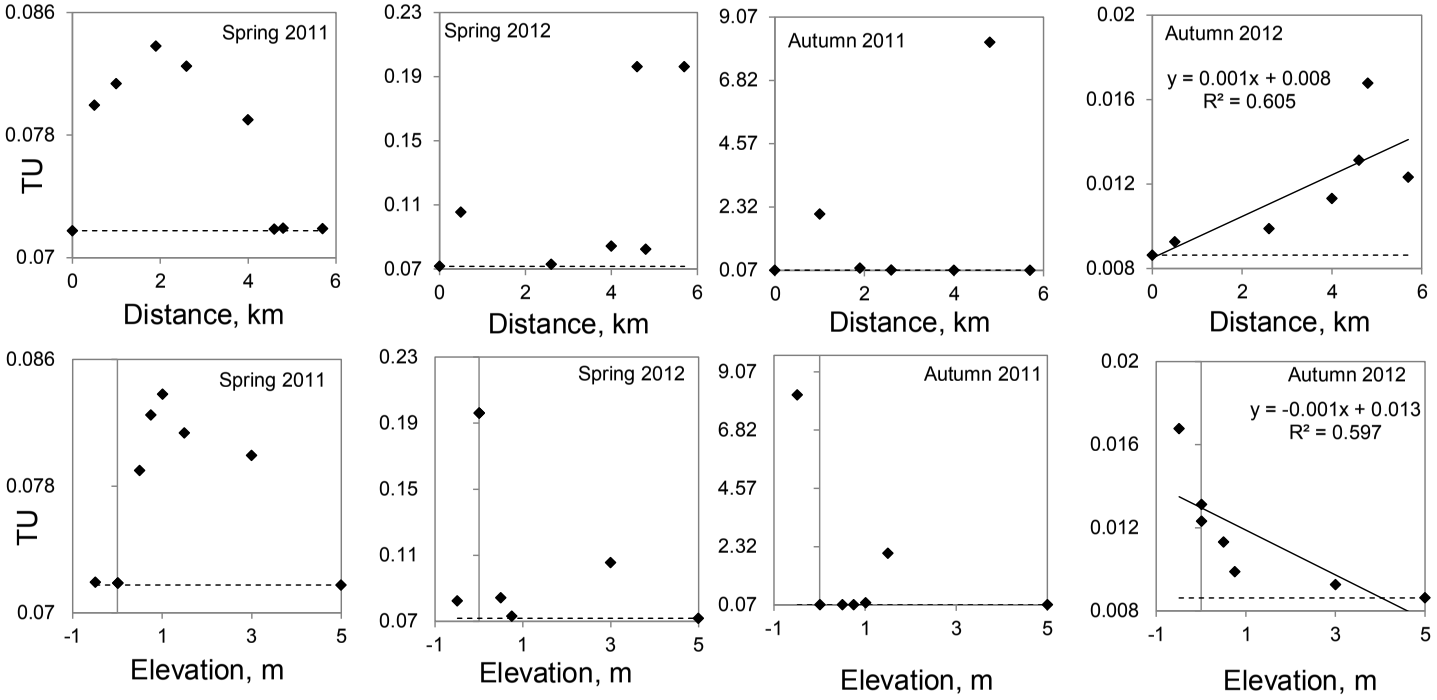

Figure S2. Toxic Units (TU) plotted versus the distance of the experimental location from the nature reserve and the elevation. Dotted line shows the limit of detection. When relationship between TU and distance/elevation was statistically significant ($p<0.05$), regression line and equation are shown

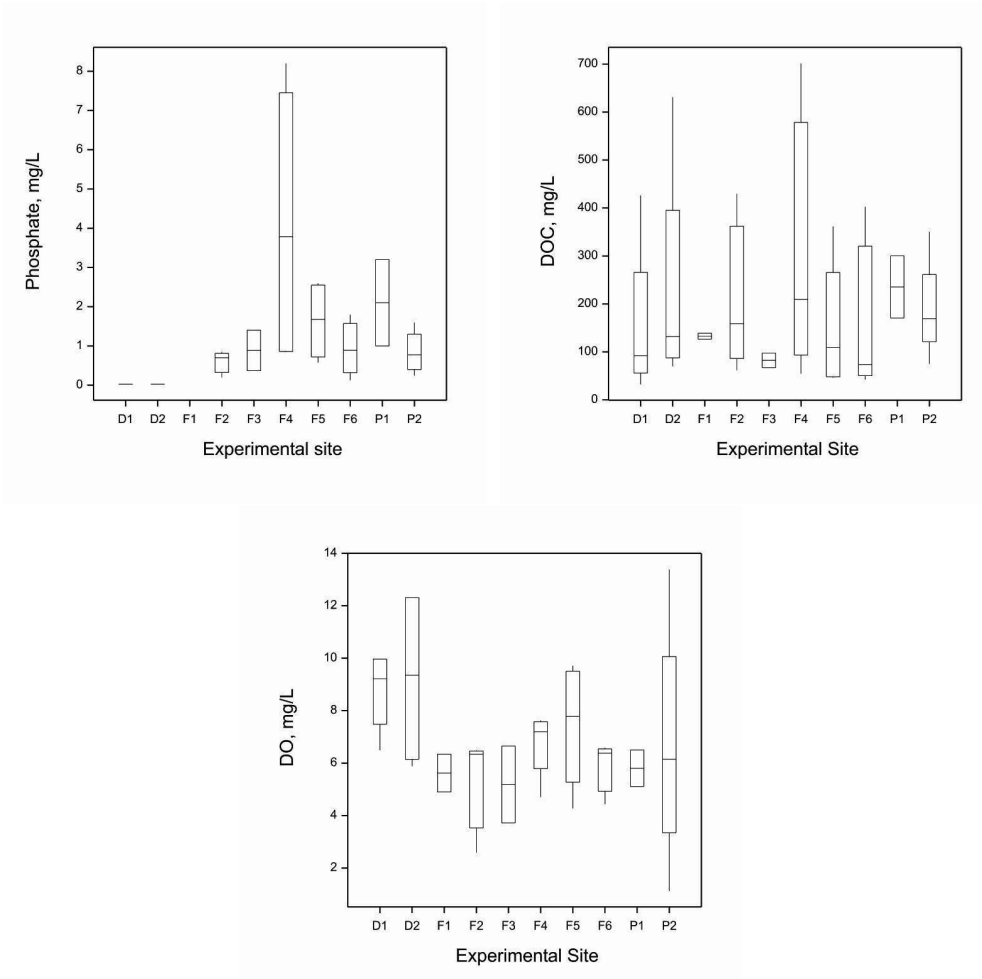

Figure S3. Boxplot of the phosphate (mg/L), Dissolved Organic Carbon (DOC, mg/L), Dissolved Oxygen (DO, mg/L) concentrations at experimental locations based on the data combined from the four experiments. D1 and D2 – experimental sites in nature reserve. F1, F2, F3, F4, F5, F6, P1 and P2 – sites in agricultural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

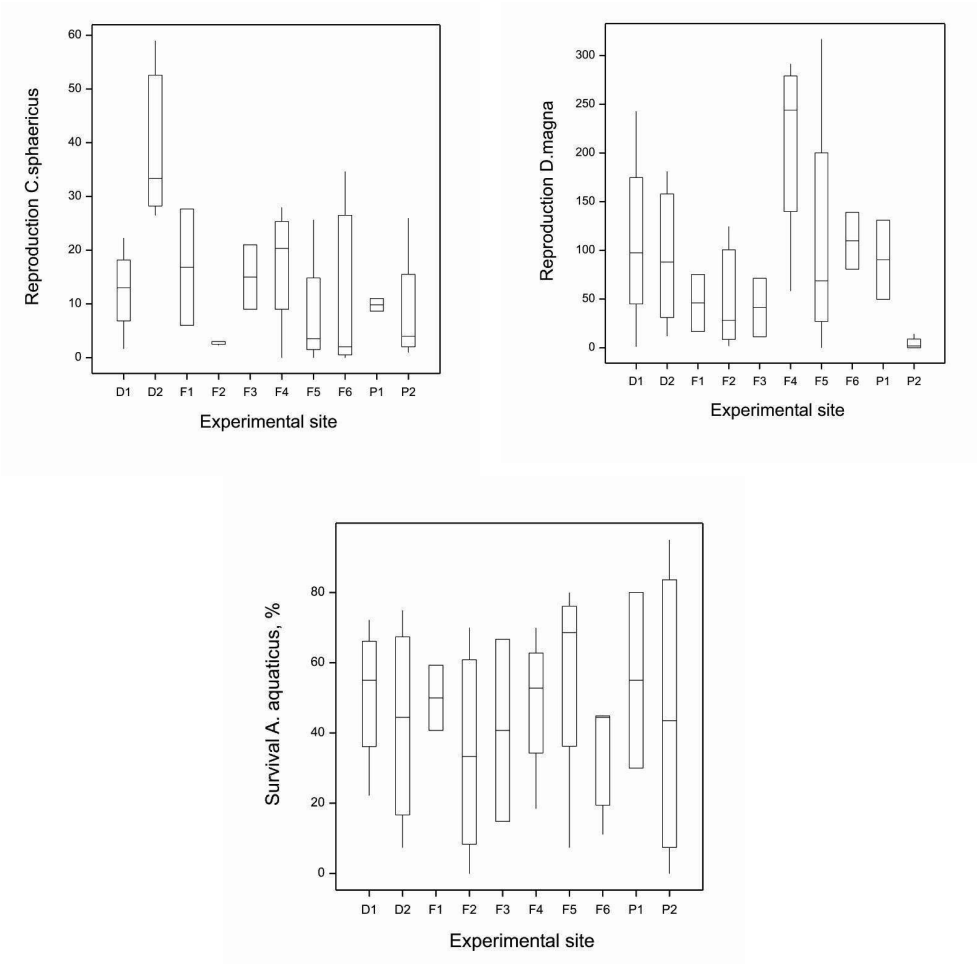

Figure S4. Boxplot of the reproduction output (number of juveniles at 21 day) for *D. magna* and *C.sphaericus* and survival of *A. aquaticus* (%) at experimental locations based on the data combined from the four experiments. D1 and D2 – experimental sites in nature reserve. F1, F2, F3, F4, F5, F6, 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)

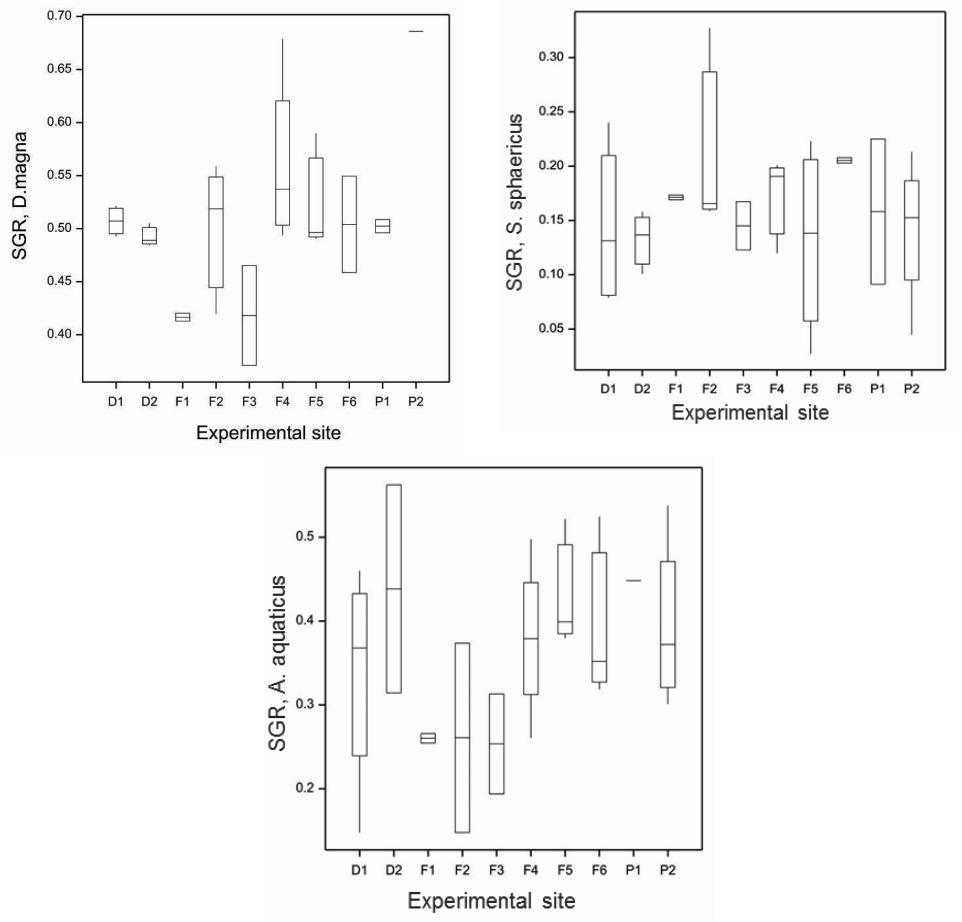

Figure S5. Boxplot of the Somatic Growth Rate (SGR, µm/day) for *D. magna* and *C.sphaericus* and *A. aquaticus* at experimental locations based on the data combined from the four experiments. D1 and D2 – experimental sites in nature reserve. F1, F2, F3, F4, F5, F6, P1 and P2 – sites in agricultural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

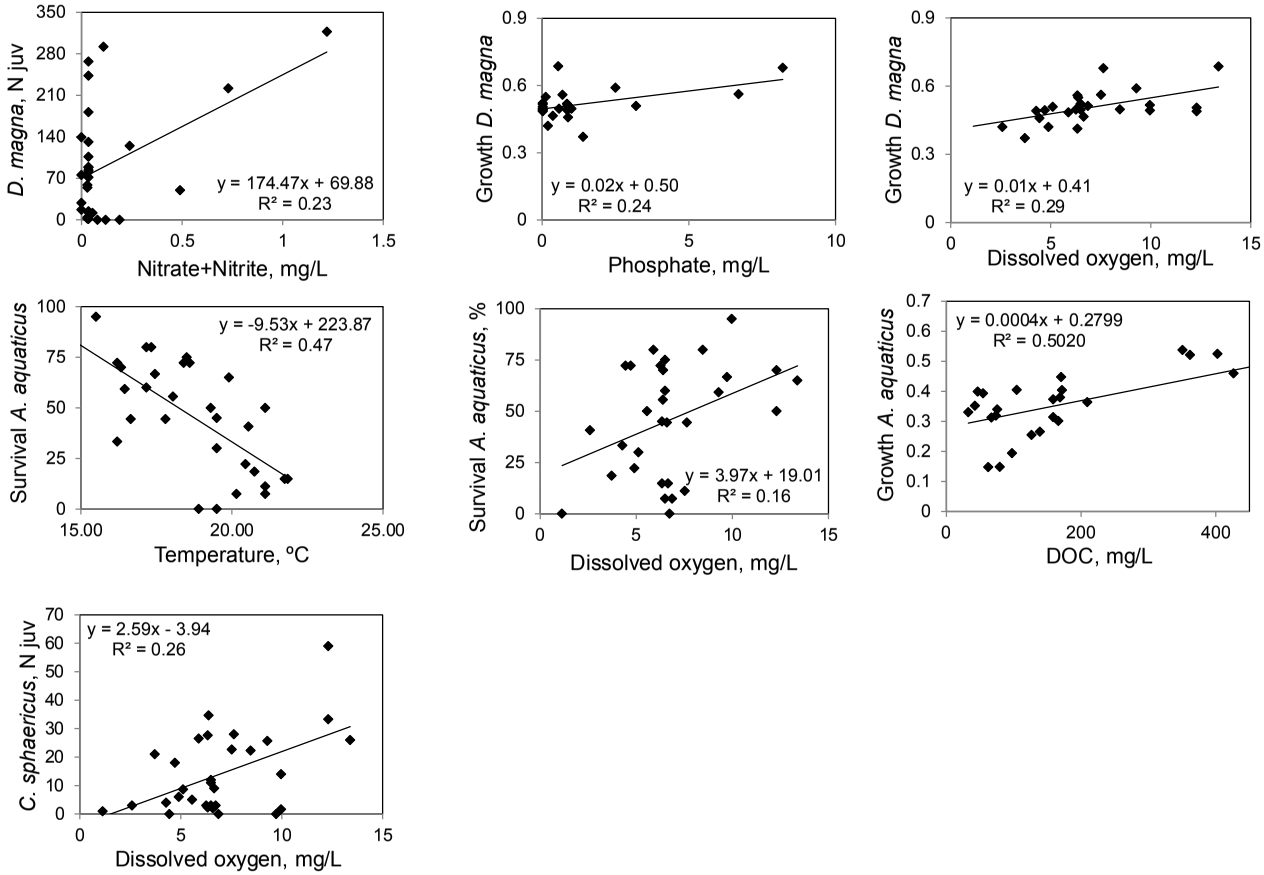

Figure S6. Linear regression analysis between the endpoints estimated for *D. magna, C. sphaericus* and *A. aquaticus* and environmental variables identified to be statistically significant in GLM model and explaining more than 20% variation in the endpoint