Effects of pesticides on aquatic macrofauna in the field

Ieromina, O.

CHAPTER 6

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

O. Ieromina[†], W. J. G. M. Peijnenburg[†,‡], G.R. de Snoo[†], J. Müller[§], T. P. Knepper[§], M.G. Vijver[†]

[†]Institute of Environmental Sciences, Leiden University, P.O. Box 9518, 2300 RA, Leiden, The Netherlands; [§]Institute for Analytical Research, Hochschule Fresenius, University of Applied Sciences Fresenius, Limburger Straße 2, 65510 Idstein, Germany; [‡]National Institute for Public Health and Environment, P.O. Box 1, 3720 BA Bilthoven, The Netherlands

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Abstract

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

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

Introduction

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

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

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

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

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

Materials and methods

Test species and culture conditions

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

Preparing different phosphorus levels

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

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

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

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

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

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

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

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

Test set up

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

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

Preparing different imidacloprid concentrations

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

Analytical measurements

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

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

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

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

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

Estimated endpoints

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

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

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

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

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

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

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

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

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

Data treatment

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

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

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

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

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

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

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

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

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

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

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

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

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

Results

Effects of imidacloprid and phosphorus on the survival of Daphnia magna

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

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

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

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

Effects on the growth rate

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

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

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

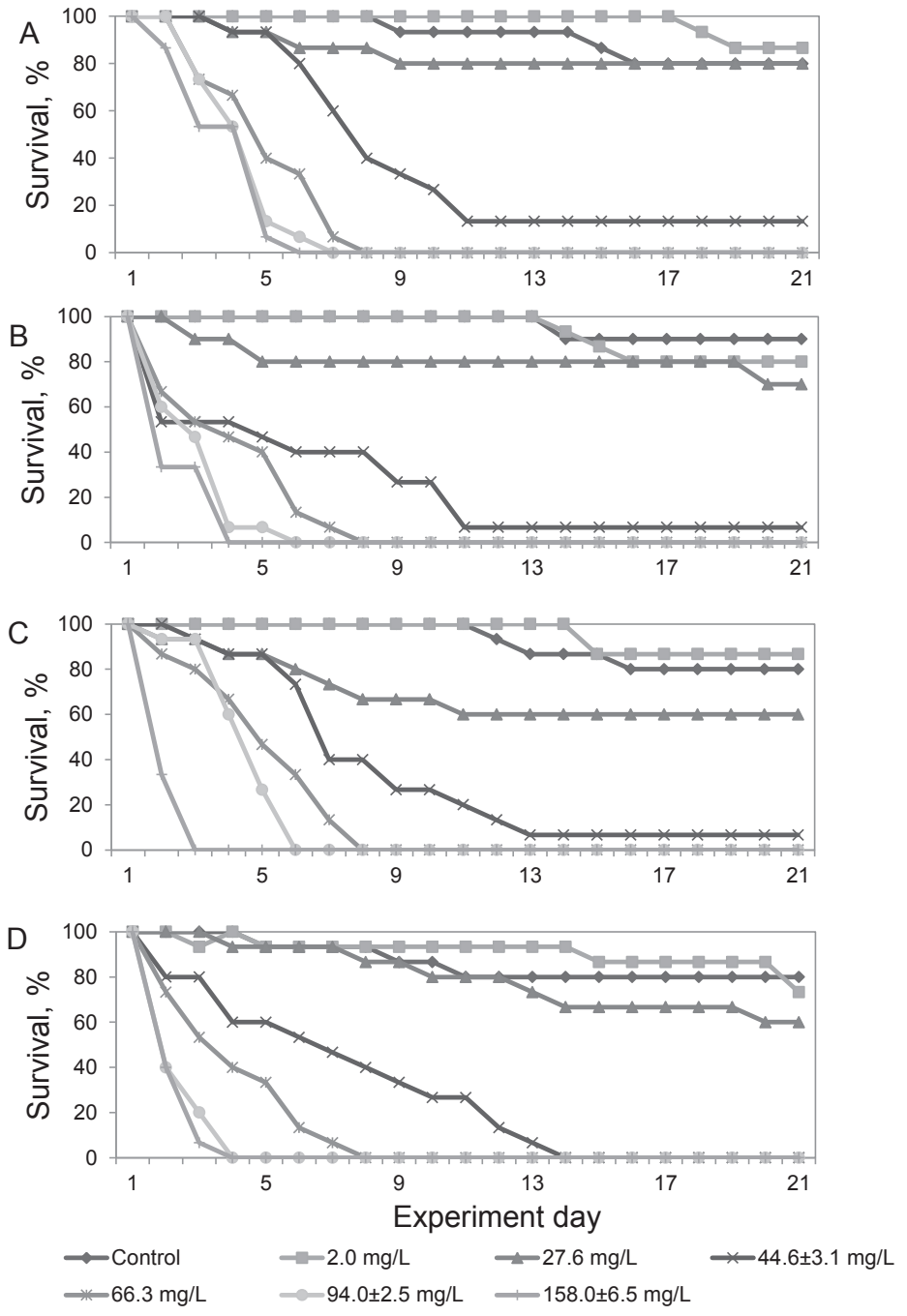

Figure 1. Effect of imidacloprid on the survival of *D. magna* supplied with different food regimes (Mean survival at C:P 35 (A), C:P 240 (B), C:P 400 (C) and C:P 1300 (D))

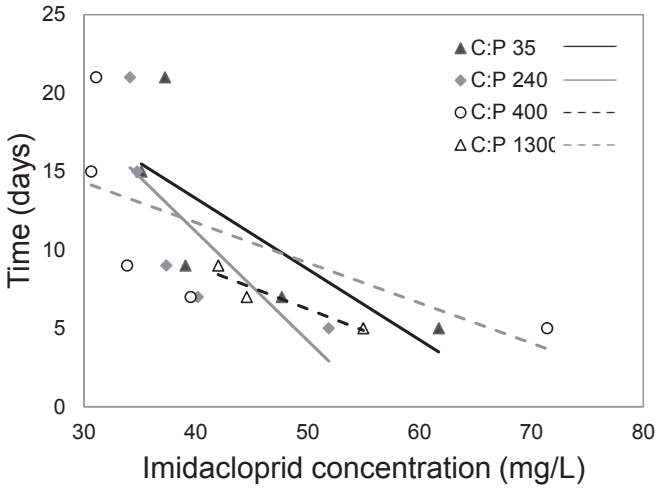

Figure 2. Time to 50% mortality of *D. magna* plotted versus imidalcoprid concentration

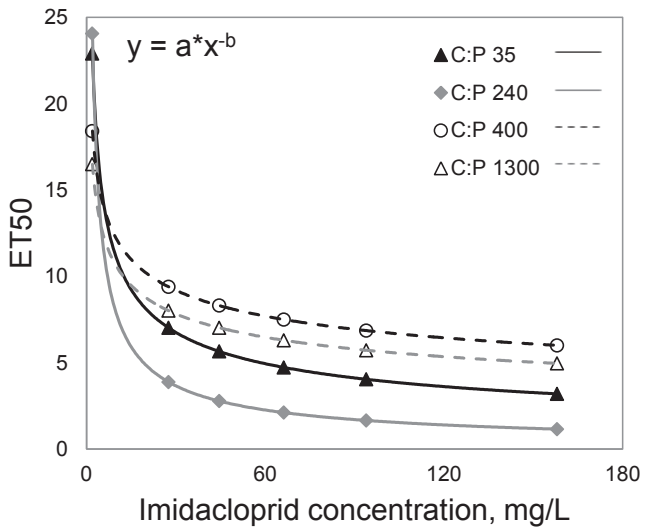

Figure 3. Relationship between median time to 50% effect (ET50) and imidacloprid concentration fitted with hyperbolic curve

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

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

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

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

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

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

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

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

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

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

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

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

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

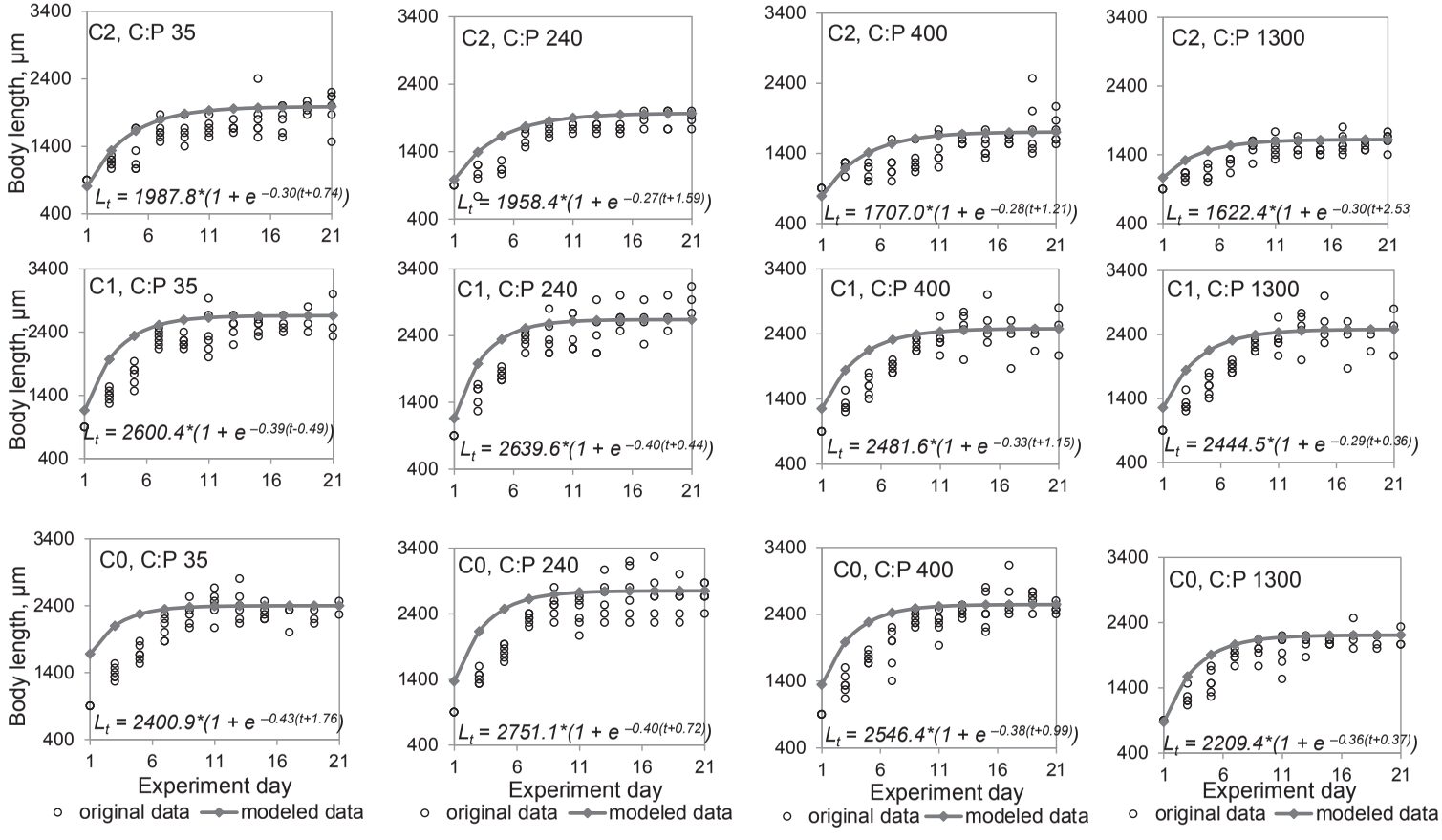

Figure 4. Body length of *D. magna* supplied with diets C:P 35, C:P 240, C:P 400 and C:P 1300 at control conditions (C0) and exposed to imidacloprid concentrations 2.0 mg/L (C1) and 27.6 mg/L (C2) fitted with Von Bertalanffy growth model

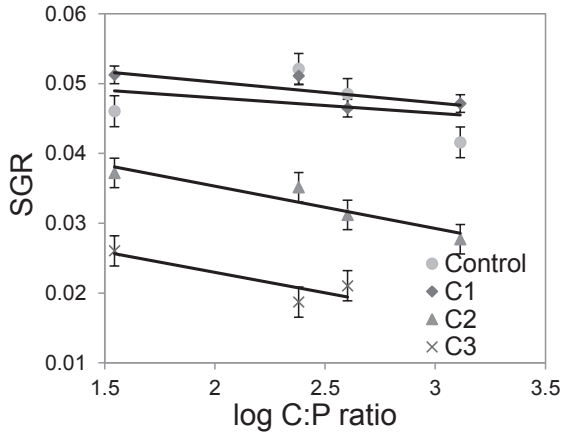

Figure 5. Somatic growth rate (µm/day) of *D. magna* exposed to a range of imidacloprid concentrations plotted versus log C:P ratios (shown on the graph are mean somatic growth rate and standard error). C1= 2.0 mg/L; C2= 27.6 mg/L; C3= 44.6±3.1 mg/L

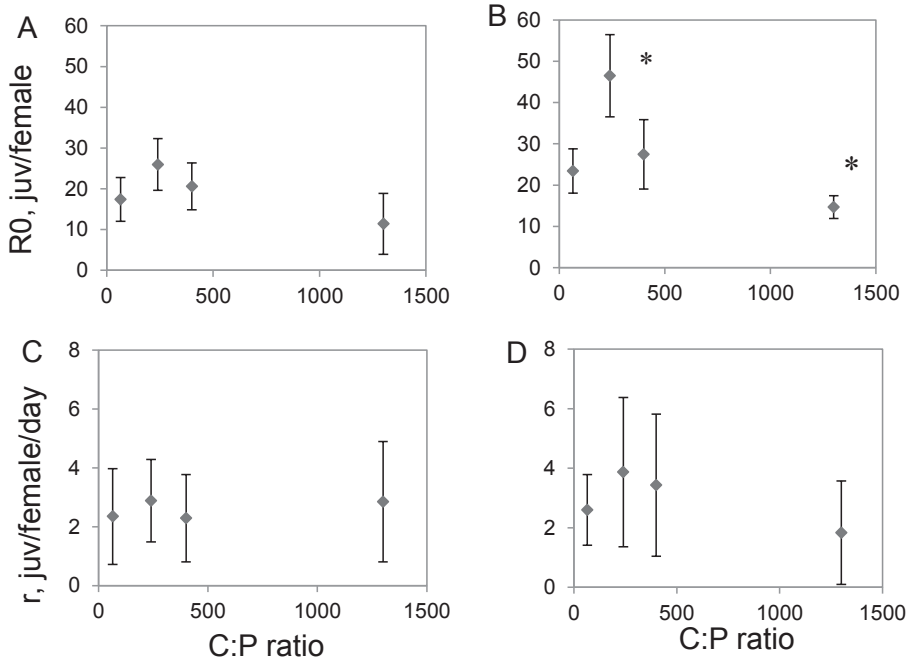

Figure 6. Net reproductive rate (R0, juv/female), average reproduction per day (juv/female/day) of *D. magna* at the control conditions (A, C) and imidacloprid concentration 2.0 mg/L (B, D) and age at maturity (E), * significantly different from other C:P levels at $p<0.1$

Effects on reproduction

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

Discussion

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

Effects on survival

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

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

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

Effects on the growth rate

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

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

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

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

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

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

Effects on reproduction

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

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

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

Conclusions

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

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Supplemental information

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

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

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

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

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

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

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

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

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

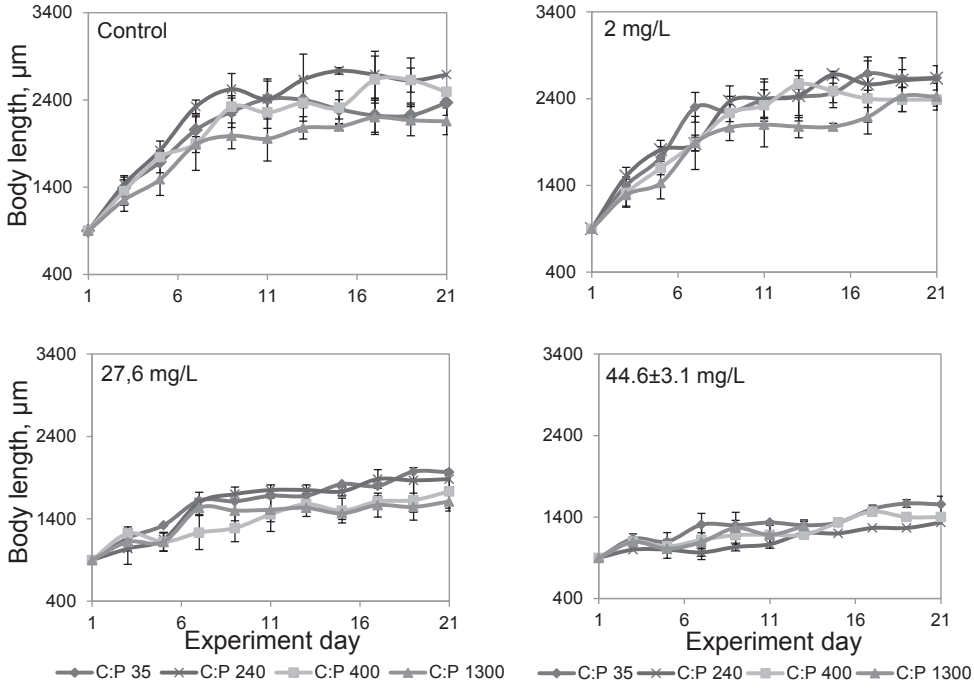

Fig. S2. Body length of *D. magna* at control and imidacloprid exposure concentrations 2.0 mg/L, 27.6 mg/L and 44.6±3.1 mg/L supplied with four food quality regimes