Effects of intra- and interspecific competition on the sensitivity of aquatic macroinvertebrates to carbendazim

Ana Isabel Del Arco [a,*], Gema Parra [a], Andreu Rico [b], Paul J. Van den Brink [b,c]

[a] Department of Plant Biology, Animal Biology and Ecology, Jaén University, B3-078 Campus de las Lagunillas s/n, 23071 Jaén, Spain
[b] Alterra, Wageningen University and Research centre, P.O. Box 47, 6700 AA Wageningen, The Netherlands
[c] Department of Aquatic Ecology and Water Quality Management, Wageningen University, Wageningen University and Research centre, P.O. Box 47, 6700 AA Wageningen, The Netherlands

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ABSTRACT

The Ecological Risk Assessment of pesticides and other potentially toxic chemicals is generally based on toxicity data obtained from single-species laboratory experiments. In the field, however, contaminant effects are ubiquitously co-occurring with ecological interactions such as species competition and predation, which might influence the sensitivity of the individuals exposed to toxicants. The present experimental study investigated how intra- and interspecific competition influence the response of sensitive aquatic organisms to a pesticide. For this, the effects of the fungicide carbendazim were assessed on the mortality and growth of the snail *Bithynia tentaculata* and the crustacean *Gammarus pulex* under different levels of intraspecific and interspecific competition for a food resource. Interspecific competition was created by adding individuals of *Radix peregra* and *Asellus aquaticus*, respectively. The interaction of competition and carbendazim exposure significantly influenced *B. tentaculata* growth, however, combined effects on survival and immobility were considered transient and were less easily demonstrated. Positive influence of competition on *G. pulex* survival was observed under low-medium carbendazim concentrations and under medium-high density pressures, being partly related to cannibalistic and predation compensatory mechanisms, enhanced under food limiting conditions. This study shows that intra- and interspecific competition pressure may influence the response of sensitive aquatic organisms in a more complex way (positive, non-significant and negative effects were observed) than just increasing the sensitivity of the studied species, as has generally been hypothesized.

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1. Introduction

Pesticides used in agriculture production constitute one of the most important sources of anthropogenic pollution into aquatic ecosystems (Parra et al., 2005; Stendera et al., 2012). Currently, the prospective Ecological Risk Assessment (ERA) of pesticides is mostly based on data obtained from single-species toxicity tests. Such an approach does not take into account ecological interactions between aquatic organisms such as competition or predation (Van den Brink, 2013; Brooks et al., 2009) and may therefore underestimate or overestimate pesticide risks for sensitive taxa and for the structure of aquatic communities (Beketov and Liess, 2006; Gui and Grant, 2008; Foit et al., 2012; De Laender and Janssen, 2013).

To date, only few studies have investigated the combined effects of toxicants and ecological interactions on the sensitivity of

aquatic organisms. For instance, Beketov and Liess (2006) studied the influence of simulated predation on *Artemia* sp. populations exposed to the insecticide esfenvalerate. They concluded that the vulnerability of *Artemia* sp. populations affected by the insecticide and predation is considerably higher as compared to the populations that were only exposed to the insecticide. This was mainly explained by the significant reduction of the population regulation capacity, described as the increased offspring production under low density conditions. Gui and Grant (2008) explored the responses of *Drosophila melanogaster* (Dipteran) populations to toxicants under different food availability scenarios. Results of their study indicated synergistic food-toxicant effects, but also indicated that compensatory mechanisms produced by toxicant exposure can occur at specific high competition levels due to density-dependent population processes.

Studies such as the ones described above are crucial to decide on whether, and under which conditions, ecological interactions should be incorporated in the intermediate tiers of ERA (De Laender et al., 2008). Furthermore, these studies provide empirical evidence of the mechanisms governing population and

community dynamics under chemical stress conditions and provide fundamental knowledge for the design of ecological scenarios and food-web models (De Laender and Janssen, 2013; Van den Brink, 2013). In this paper we investigate how competition for food affects the sensitivity of aquatic macroinvertebrates to pesticide exposure under laboratory conditions. For this, experiments were performed using the fungicide carbendazim and different levels of intra- and interspecific competition. The intraspecific competition experiments were performed by exposing two different species, the snail *Bithynia tentaculata* and the amphipod *Gammarus pulex*, to carbendazim under different density conditions, corresponding to different food limiting scenarios. The interspecific competition experiments were performed with two species, named as the focal and the competing species. The selected focal species were expected to show a higher sensitivity to carbendazim as compared to the competing species. This allowed us to establish asymmetries on the food competition process and to better observe the combined effects of the pesticide and competition stress over the focal species. The first experiment was performed using *B. tentaculata* as the focal species and the snail *Radix peregra* as the competing species. The second experiment was performed using the *G. pulex* as the focal species and the isopod *Asellus aquaticus* as the competing species. These aquatic organisms were selected because of their high abundance in aquatic ecosystems and their important ecological functions. For instance, snails account for up to 20–60% of the biomass of macroinvertebrates in rivers (i.e. Habdija et al., 1995), and amphipods and isopod crustaceans are considered crucial for ecosystem functioning due to their contribution to leaf break-down and organic matter decomposition (Graça et al., 1994; Zubrod et al., 2014). Carbendazim was selected as model compound because of its widespread use in a large number of crops (Daam et al., 2009), and because of its known toxicity to the selected aquatic organisms (Cuppen et al., 2000; Van Wijngaarden et al., 1998).

2. Material and methods

2.1. Test organisms

The snails *B. tentaculata* and *R. peregra* were selected based on their different sensitivity to carbendazim. According to Cuppen et al. (2000), and the results of a preliminary test (results not shown), *B. tentaculata* was expected to be more sensitive than *R. peregra*. In addition, their co-occurrence in natural drainage ditches was also an important factor for the selection of this species combination. Snails were collected from Dutch drainage ditches and ponds and acclimatized to the same laboratory environmental conditions as those used in the experiment (see Section 2.2). Only organisms in juvenile life stages and with similar length (*B. tentaculata*: 5.4 ± 0.7 mm; *R. peregra*: 5.0 ± 0.7 mm) were selected for the experiments.

The crustaceans *G. pulex* and *A. aquaticus* were also chosen due to their different sensitivity to carbendazim, with *G. pulex* being more sensitive to carbendazim than *A. aquaticus* (Van Wijngaarden et al., 1998). *G. pulex* and *A. aquaticus* organisms were collected from a freshwater pond where they naturally compete for food resources (Duno Pond, Renkum, The Netherlands, 51°58′9.31″N, 5°48′9.88″E) and were acclimatized to laboratory conditions for 7 days prior to the start of the experiment. Only young adults with similar length characteristics were selected for the experiments (*G. pulex*: 10.6 ± 0.5 mm; *A. aquaticus*: 7.7 ± 0.3 mm).

2.2. Experimental set-up

The intra- and interspecific competition experiments performed with the selected macroinvertebrate species and the fungicide carbendazim followed a factorial experimental design. All experiments were performed in triplicate ($n = 3$), with three levels of intra- and interspecific competition (i.e., control, medium and high) and four carbendazim levels (i.e., control, low, medium and high exposure concentrations). Both experiments consisted of a pre-exposure period, in which intra- and interspecific food competition were allowed to take place, and an exposure period, in which the combined effects of competition and carbendazim exposure were evaluated.

In the experiment performed with *B. tentaculata*, the competition controls (C) were stocked with 5 individuals of *B. tentaculata*. The treatments corresponding to the medium (intra-M) and high (intra-H) intraspecific competition levels were stocked with 10 and 20 individuals, respectively. The medium (inter-M) and high (inter-H) interspecific competition treatments were established by adding 5 and 10 individuals of *R. peregra*, respectively, to test units containing 5 individuals of *B. tentaculata*. The competition was allowed to take place during 21 days (pre-exposure period) prior to the carbendazim exposure. During the exposure period, the test medium was renewed once a week to avoid excessive water quality deterioration. Carbendazim was applied weekly for 21 days at a concentration of 400, 800 and 1200 µg/L to the low, medium and high exposure levels, respectively. These exposure concentrations were selected based on the results of the microcosm study performed by Cuppen et al. (2000), who found a chronic NOEC (population abundance) of 33 µg/L for *B. tentaculata*. Cucumber harvested from an organic farm was used as food resource. Cucumber pieces of 0.59 ± 0.05 g were added weekly to the test units. The cucumber weight provided to each test unit was calculated as the amount consumed by 5 *B. tentaculata* snails during one week in a preliminary feeding rate test. In case there were any cucumber leftovers during the experiment, they were removed before adding the next cucumber pieces.

In the experiment performed with *G. pulex*, the competition controls (C) consisted of five individuals of *G. pulex*. The medium (intra-M) and high (intra-H) treatments of intraspecific competition were established with a *G. pulex* density of 10 and 15 individuals per test unit, respectively. The medium (inter-M) and high (inter-H) interspecific competition treatments were set by adding 5 and 10 individuals of *A. aquaticus* to test units containing 5 *G. pulex* individuals. In this experiment, the pre-exposure period had a duration of 4 days (to avoid excessive cannibalism and predation effects), and the exposure period lasted for 21 days without medium renewal. Carbendazim was applied once at a concentration of 20, 40 and 80 µg/L to the low, medium and high exposure levels, respectively. These exposure concentrations were expected to approximate the LC10-LC50 range of the dose–response curve for *G. pulex*, based on previously published carbendazim toxicity data: acute (2 d) LC10 and LC50 for juvenile *G. pulex* were 27 and 77 µg/L, respectively, and the reported chronic (21 d) values for adults were 10 and 16 µg/L, respectively (Van Wijngaarden et al., 1998). In this experiment, pre-dried poplar leaves (*Populus* sp.) were weekly added to the test units as food resource. The amount used (0.7 ± 0.1 mg) was based on the results of a preliminary feeding rate test and approximates the amount consumed by 5 individuals of *G. pulex* per week.

In both experiments, the competition and carbendazim treatments were randomly assigned to the test units. The test units consisted of 1.5 L glass jars filled with 1 L of non-polluted pond water (the pond was located in Wageningen University, The Netherlands), previously filtered through a phytoplankton net (20 µm). The jars were placed in a water bath with a constant water temperature (20 ± 0.5 °C). High pressure metal halide lamps (Philips HPI-T, 400 W) were used to provide a daily photoperiod of 12 h, with a light intensity of approximately 500 µE/m²/s at the

jar's water surface. In the experiment performed with *G. pulex* and *A. aquaticus*, a stainless steel mesh was added to each jar in order to increase the available surface and to serve as a refuge for the test organisms.

The effects of competition and carbendazim exposure were assessed on the mortality and the growth of the focal (*B. tentaculata* and *G. pulex*) and competing species (*R. peregra* and *A. aquaticus*). Mortality was monitored on day 2, 4, 7, 14 and 21 after the (first) carbendazim application. Mortality of snails was assumed when they did not react after providing tactile stimuli on the soft part of their body with a laboratory needle. Mortality of *G. pulex* and *A. aquaticus* was assumed when they did not respond to any tactile stimuli. In both experiments, dead individuals were removed from the experiment. Snail and crustacean growth was quantified by measuring the relative changes in their shell or body length over time, respectively. Growth was assessed weekly in the experiment performed with snails. In the experiment performed with crustaceans growth was only measured at the end of the experiment to prevent overstress. In both experiments, growth was measured by taking high resolution pictures of the organisms in each jar (Microsoft LifeCam Studio) and analyzing them with AxioVision SE64 Rel. 4.8. After the growth measurements were taken, the organisms were returned to their original jars. Moreover, the immobility of both snail species was evaluated at the end of the exposure period (day 21). For this, the snails were individually placed in a circular area of 5 mm radius drawn on the surface of a Petri dish containing unpolluted water. Immobility was assumed when they did not move out of the drawn circle after a time span of 30 min. Immobility was not assessed in the *G. pulex* experiment.

During the experiments, temperature, pH, dissolved oxygen concentration and conductivity were measured weekly in the experimental medium at the start of the illumination period. Temperature, pH and dissolved oxygen concentration were measured with a WTW 340i multi-meter, and conductivity was measured with a WTW 315i meter. These data were used to rule out any potential effect of water quality differences on the evaluated biological endpoints.

2.3. Carbendazim application and analysis

Carbendazim stock solutions (100 mg a.i./L) were prepared using Derosal (50% carbendazim, w/v) and milli-Q water. Aliquots of the carbendazim stock solution were applied to the water surface of the carbendazim treatments and gently stirred with a laboratory spoon to ensure an homogeneous distribution of the pesticide over the water column.

Water samples of 2 mL were taken from the test jars after carbendazim addition to verify the nominal exposure concentrations and also weekly to assess its dissipation rate during the exposure period. A Perkin Elmer LC-90 UV detector was used to perform a direct analysis of the carbendazim concentrations in these samples. The mobile phase used was methanol:water (70:30), pumped at a flow rate of 0.7 mL/min with a Water M590 pump through a Waters Novapak C-18 column. This column was set in a Waters Temperature Control Module at 40 °C and with a wavelength of 285 nm. The retention time for carbendazim was 5 min in the device used to run the analysis. Calculation of the concentrations was based on external standard samples. The detection limit of this method was 2 μg/L.

2.4. Statistical analyses

The effects of species competition on the sensitivity of the focal species to carbendazim was assessed by (1) comparing the calculated EC50 or LC50 values among competition levels for each sampling day, and by (2) using Generalized Linear Models (GLMs). The calculation of the EC50 and LC50 values was carried out by means of log-logistic regression using the software GenStat 11th (VSN International Ltd., Oxford, UK), as described by Rubach et al. (2011). LC50 values were calculated for *B. tentaculata* and *G. pulex* at each sampling day and EC50 values were calculated for the growth of *B. tentaculata* at the end of the experiment (day 21). The GLM analysis was performed for each measured endpoint at each sampling day using the same software (GenStat 11th). The model used for the GLM analysis was adapted to the data distribution of the different measured endpoints. Immobility and survival were assessed using a binomial distribution and logit as the link function, while growth was evaluated by using a Poisson distribution and logarithm as the link function. The statistical model was defined by a constant, the exposure concentration, the competition level and their interaction, introducing both, the nominal carbendazim concentrations and the competition levels, as groups. The effects of the pesticide concentration, the competition or the interaction of both on the evaluated biological endpoints were considered to be significant when the calculated p-values were < 0.05, and were defined as moderately significant when they were between 0.05 and 0.10.

3. Results and discussion

3.1. Carbendazim concentrations and water quality

During the whole experimental period the average measured carbendazim concentrations were $91.3 \pm 13.9\%$ and $100 \pm 16.3\%$ of the nominal concentrations for the *B. tentaculata* and the *G. pulex* experiments, respectively (Table 1). Carbendazim was found to be very stable during the experimental period, with an average 7-day dissipation rate of $1.8 \pm 0.3\%$ in the *B. tentaculata* experiment, and $13.6 \pm 1.6\%$ in the *G. pulex* experiment (average $\pm$ SD Table 1). These results are in line with previous studies that have monitored carbendazim concentration dynamics under laboratory and semi-field conditions (Van Wijngaarden et al., 1998; Slijkerman et al., 2004). The average values of the measured water quality parameters were: temperature 20.9 ± 0.5 °C, pH 8.0 ± 0.5, dissolved oxygen 7.9 ± 1.9 mg/L, and conductivity 781 ± 54 μS/cm (average $\pm$ SD). There were no observable effects of the carbendazim exposure concentration or the organism density on the water quality parameters measured during the course of the experiments.

3.2. Toxic effects of carbendazim

In the *B. tentaculata* experiment, significant differences in mortality between controls and respective carbendazim levels were only found on day 1 for interspecific competition, and on day 21 for intra- and interspecific competition (Table 2, Fig. 1). However, the measured mortality rates on the last sampling day were not high enough to fit a dose–response model, and the LC50 values could not be calculated. Carbendazim exposure resulted in significant effects on *B. tentaculata* mobility on day 21 (Table 2), but the calculated EC50-21 d (immobility) value was above the highest tested concentration (Table 3). In close similarity to these results, Van Wijngaarden et al. (1998) calculated an EC50-28d (immobility) of 1641 μg/L (1169–2303) μg/L, which is expected to be in the range of our EC50 value if the experiment was continued for one week more.

Significant effects of carbendazim on *B. tentaculata* growth could not be demonstrated in either the intra- or interspecific competition tests (Table 2, Fig. 1b and d). Low energy input under toxic stress could affect important vital traits of snails such as

Table 1

Measured carbendazim concentrations in the experimental medium and calculated Average Exposure Concentration (AEC) during the whole exposure period. The reported concentrations for the experiment with *B. tentaculata* correspond to the measured concentrations approximately one hour after the first carbendazim addition (0 d), seven days after the first carbendazim pulse (7 d), and seven days after the second pulse (14 d). The reported concentrations for the experiment with *G. pulex* correspond to the measured concentrations approximately one hour after the carbendazim application (0 d), and 7 and 14 days after the application (7 d and 14 d, respectively). Concentrations are expressed as mean ± SD (μg/L).

Experiment	Nominal concentration	Measured concentration			AEC (Day 0–14)
		0 d	7 d	14 d	
B. tentaculata	400	374 ± 2	379 ± 2	366 ± 5	373 ± 2
	800	753 ± 2	734 ± 4	756 ± 2	749 ± 2
	1200	1127 ± 6	1105 ± 10	1122 ± 3	1119 ± 4
G. pulex	20	22 ± 1	15 ± 1	n.a.	21 ± 1
	40	41 ± 1	37 ± 2	42 ± 1	40 ± 1
	80	79 ± 1	72 ± 1	69 ± 1	78 ± 1

n.a.: not available.

mobility or feeding behavior in an attempt to optimize the new energetic balance. For example, Tripathi and Singh (2002) found a decrease in the glycogen concentration in snail tissues exposed to pesticides, which was attributed to the mobilization of this substance to meet the high energy demands required to mitigate toxic stress. Such response, however, was not observed in the measured endpoints of this study.

Significant effects of carbendazim on *G. pulex* mortality were observed on day 7, 14 and 21 after the start of the exposure period for the intra- and interspecific competition tests, as well as on day 2 and 4 as well for the interspecific competition test (Table 2, Fig. 2a and b). LC50 values were calculated for all sampling days, except for day 2, for which mortality was not high enough to fit a dose–response model (Table 3). The LC50-96 h for *G. pulex* in the competition control, 71 (36-139) μg/L, was found to be similar to the LC50-96 h value reported by Van Wijngaarden et al. (1998) for *G. pulex* juveniles: 55 (41–75) μg/L. The calculated LC50 values for the competition control on day 7, 14 and 21 (Table 3) also fall within the LC50 ranges reported by Van Wijngaarden et al. (1998) for the same exposure periods, confirming the previously reported

sensitivity of this species to carbendazim. Analyses of carbendazim impacts on *G. pulex* growth on day 21 could not be carried out due to the elevated mortality and the consequent insufficient amount of available data points.

3.3. Single and combined effects of competition and carbendazim

3.3.1. B. tentaculata experiment

Significant effects of intraspecific competition on mortality and immobility were not demonstrated (Table 2, Fig. 1a). However, intraspecific competition significantly affected growth rates of *B. tentaculata*, indicating that competition over resources was present (Table 2, Fig. 1c). Due to the low carbendazim effects on mortality observed in this experiment, LC50 values could not be calculated for the different competition levels (Fig. 1a, Table 3). However, the dose–response patterns on immobility showed that the EC50 values for the competition controls and for the intra-M competition level were approximately two times higher than the ones calculated for the highest competition level, indicating that higher competition under pesticide exposure could result in an increased snail immobility even though the respective 95% confidence intervals overlapped (Table 3). Combined effects of intraspecific competition and carbendazim on mortality, as calculated by the GLM analysis, were only detected at the start of the exposure period (Table 2). Such effects did not show a consistent dose–response pattern as they were mainly visible at the low carbendazim concentration (400 ug/L) and not at the intra-M competition level (Fig. 1a). This observation is consistent with the proposed theory of toxicant-induced reduction of intraspecific adverse effects (Liess, 2002). Liess (2002) studied the influence of intraspecific competition on a trichopteran (*Limnephilus lunatus*) population exposed to fenvalerate, and reported compensation of direct pesticide effects due to a reduction of indirect intraspecific pressure as compared to the competition controls. Such mechanism could explain the absence of increased mortality in the intermediate intraspecific competition level. Our results also suggest that above a density threshold (close to intra-H) those effects are no longer compensated by low pesticide toxicity. In addition, an EC50 could not be calculated in the control level (C), although the observed response was very similar to the intra-H competition level, and to a lesser extent, to the high intraspecific competition level (Table 3).

Table 2

Results of the GLM analysis (*p*-values) showing the effects of the carbendazim exposure, competition, and their interaction on mortality, immobility and growth in the different days of the exposure period for *B. tentaculata* and *G. pulex*. Bold values indicate significant ($p < 0.05$) or marginally significant ($0.05 \leq p \leq 0.1$) effects.

| Experiment	Independent variable	Days/endpoint (*p*-values)								
		Mortality					Immobility	Growth		
		2 d	4 d	7 d	14 d	21 d	21 d	7 d	14 d	21 d
B. tentaculata	Intraspecific competition	Carbendazim	0.99	0.47	0.44	0.30	**0.001**	**< 0.001**	0.39	0.56	0.65
		Competition	0.27	0.40	0.74	0.87	0.27	0.81	**< 0.001**	**0.01**	**0.01**
		Interaction	**0.02**	**0.001**	0.13	0.60	0.95	0.70	0.16	0.34	0.87
	Interspecific competition	Carbendazim	**0.01**	0.23	0.44	0.20	**0.03**	**< 0.001**	0.64	0.72	0.28
		Competition	**0.05**	1.00	**0.01**	0.12	0.31	0.29	**0.08**	**0.02**	**0.02**
		Interaction	0.10	**0.06**	0.97	0.30	0.82	0.29	0.93	**0.06**	0.44
G. pulex	Intraspecific competition	Carbendazim	n.c.	n.c.	**< 0.001**	**< 0.001**	**< 0.001**	n.m.	n.m.	n.m.	n.e.
		Competition	n.c.	n.c.	0.78	0.78	0.29	n.m.	n.m.	n.m.	n.e.
		Interaction	n.c.	n.c.	0.254	0.255	**0.06**	n.m.	n.m.	n.m.	n.e.
	Interspecific competition	Carbendazim	**0.06**	**0.001**	**< 0.001**	**< 0.001**	**< 0.001**	n.m.	n.m.	n.m.	n.e.
		Competition	0.97	0.83	**0.09**	**0.01**	**0.01**	n.m.	n.m.	n.m.	n.e.
		Interaction	0.29	0.55	0.89	0.47	0.47	n.m.	n.m.	n.m.	n.e.

n.m.: not measured.

n.c.: not calculated. Model could not be fitted due to the absence of partial effects on the different treatments.

n.e.: not evaluated. Mortality was too high and effects on growth could not be evaluated.
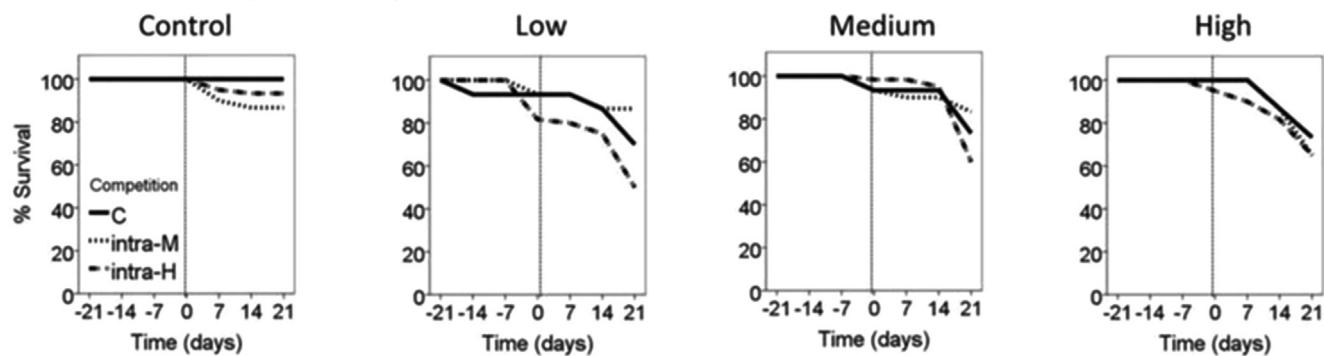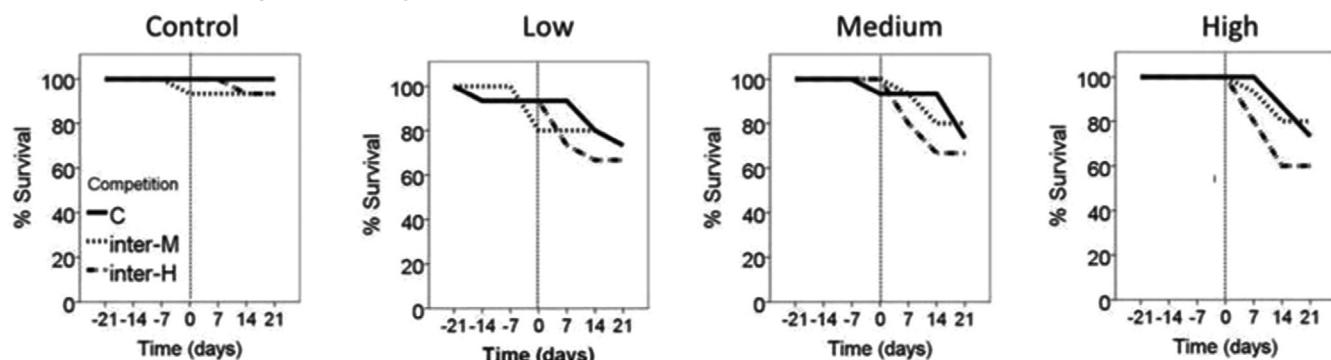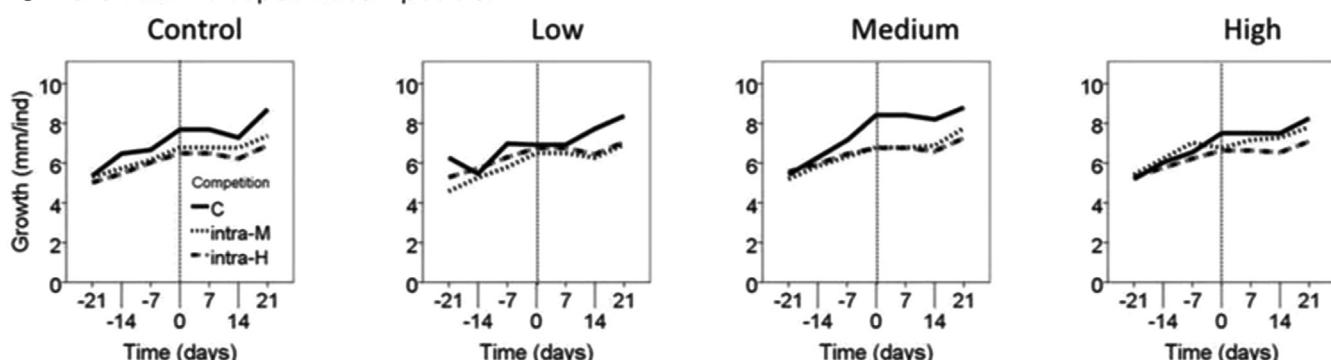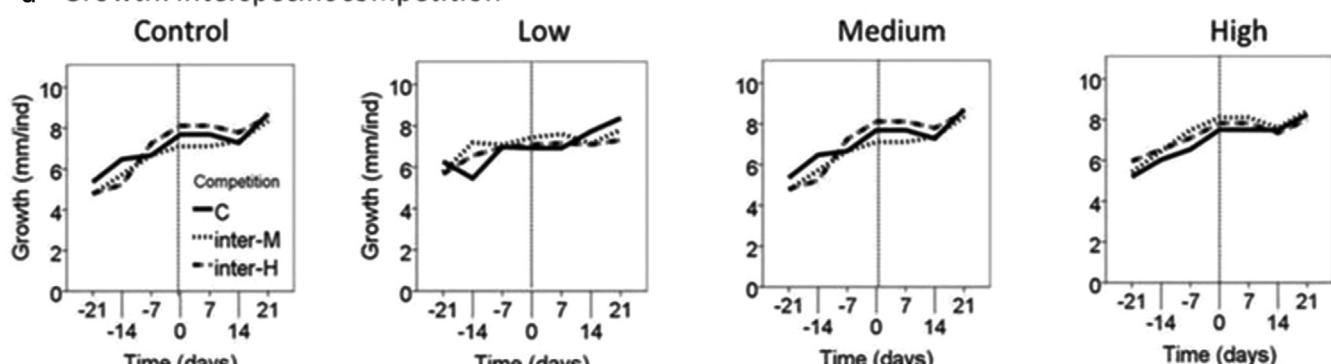
Fig. 1. Effects of carbendazim exposure and species competition on *B. tentaculata* survival and growth. The carbendazim exposure is represented as: Control, Low: 400, Medium: 800, and High: 1200 µg/L; C: control; intra-M: medium intraspecific competition; intra-H: high intraspecific competition; inter-M: medium interspecific competition; inter-H: high interspecific competition. The dashed vertical line indicates the start of the carbendazim exposure period.

Table 3
LC50 and EC50 values and their 95% confidence intervals for *B. tentaculata* and *G. pulex* calculated for each competition level. Concentrations are expressed in μg/L. C: control; intra-M: medium intraspecific competition; intra-H: high intraspecific competition; inter-M: medium interspecific competition; inter-H: high interspecific competition.

Experiment	Endpoint	Day	Species competition				
			Competition Control (C)	intra-M	intra-H	inter-M	inter-H
B. tentaculata	EC50 (immobility)	21	> 1119[a]	342 (233–502)	137 (34–551)	391 (192–769)	124 (3–5146)
G. pulex	LC50	2	n.c.	242 (36–1620)	n.c.	n.c.	115 (27–497)
		4	71 (36–139)	74 (49–110)	n.c.	n.c.	115 (27–497)
		7	30 (22–41)	n.c.	n.c.	38 (37–39)	25(19–33)
		14	30 (22–40)	38 (29–48)	34 (27–42)	38 (37–38)	17 (14–20)
		21	22 (17–30)	31 (22–44)	22 (17–29)	37 (36–37)	18 (17–19)

n.c.: not calculated. Model could not be fitted due to the absence of partial effects on the different treatments.

[a] Due to the absence of a concentration having a partial effect, no EC50 could be calculated for the control level.

Interspecific competition slightly influenced *B. tentaculata* growth both positively and negatively (Table 2, Fig. 1d) and significantly increased mortality on day 2 and 7 after the start of the exposure period. The positive or negative influence of interspecific competition on *B. tentaculata* growth depended on both the carbendazim concentration exposure and the sampling day. It may reflect density-induced mortality in the higher competition levels, decreasing competition pressures, and resulting in similar growth rates of surviving organisms under competition pressures and the controls. Combined effects of interspecific competition and carbendazim exposure on mortality were only marginally significant on day 4 (Table 2). Although a clear trend was observed towards higher mortality rates in the highest competition level during the whole experimental period (Fig. 1b), mobility on day 21 was not significantly affected by interspecific competition, nor by the interaction of interspecific competition and carbendazim (Table 2). Combined effects were detected on day 14 for growth (Table 2). Concerning the competing species, no LC50 or EC50 values within the tested concentration range could be calculated (Table A, Supplementary material).

3.3.2. G. pulex experiment

Although significant intraspecific competition effects on mortality were not detected at any sampling day for *G. pulex* (Table 2), a clear trend towards increased mortalities at higher densities was observed in the carbendazim controls (Fig. 2a). This trend is most likely related to food starvation and increased cannibalistic rates at high organism densities, since dead bodies of individuals were
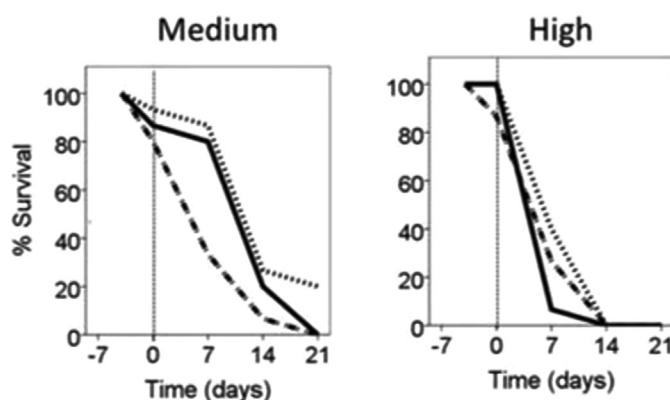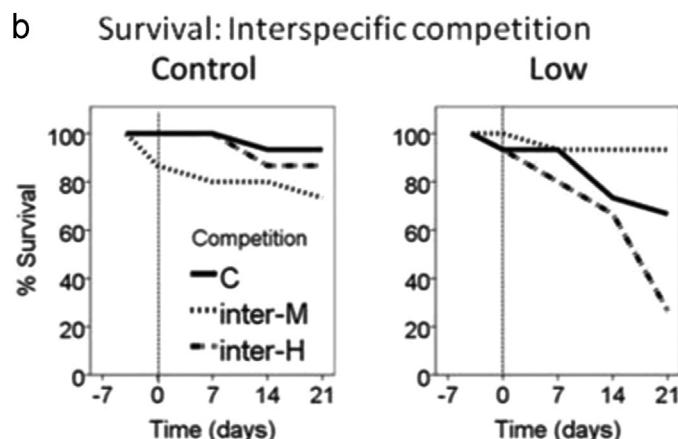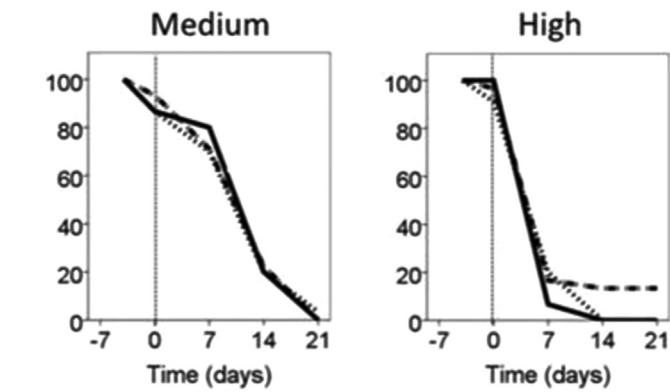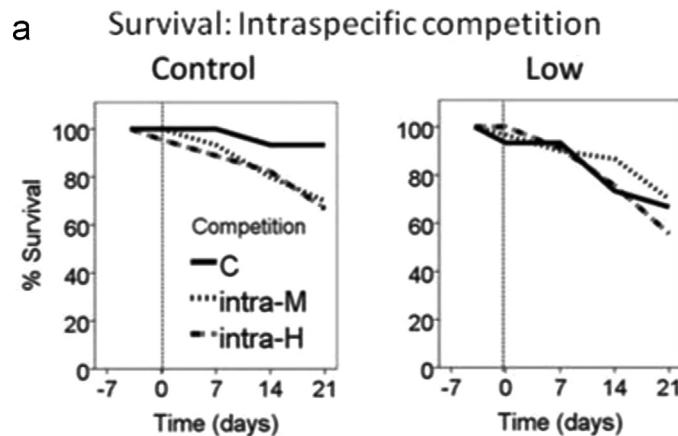
Fig. 2. Effects of carbendazim exposure and species competition on *G. pulex* survival. The carbendazim exposure concentrations are represented as: Control, Low: 20, Medium: 40, and High: 80 μg/L. C: control; intra-M: medium intraspecific competition; intra-H: high intraspecific competition; inter-M: medium interspecific competition; inter-H: high interspecific competition. The dashed vertical line indicates the start of the carbendazim exposure period.

often mutilated or not found in the sampled jars. Although calculated LC50 values for sampling days 14 and 21 were similar between intraspecific competition levels (Table 3), the combined effects of intraspecific competition and carbendazim stress on *G. pulex* mortality were found to be moderately significant at the end of the exposure period (Table 2). Such moderately significant effect is related to a reduced lethal effect of carbendazim at the medium and high intraspecific competition levels, as compared to the carbendazim controls (Fig. 2a). The first explanatory hypothesis for this was the reduced carbendazim availability at the highest density treatments, however, this was discarded after inspection of the carbendazim measured concentrations (data not shown). This reduced lethal effect at high densities was finally explained by the metabolism slow-down and immobilization behavior caused by carbendazim, particularly at the highest exposure concentration (Fig. 2a). Such behavior could have reduced the cannibalistic pressure, and outweighed the interactive food scarcity and carbendazim pressure.

Interspecific competition between *G. pulex* and *A. aquaticus* significantly affected *G. pulex* survival, but no significant interaction between interspecific competition and carbendazim exposure was observed on *G. pulex* mortality (Table 2). The high interspecific competition level had a negative effect on survival at the 20 and 40 µg/L exposure levels (Fig. 2b). This was also shown by the slightly lower calculated LC50 values for the high competition level (17 and 18 µg/L at days 14 and 21) as compared to the controls (30 and 22 µg/L at day 14) and the intermediate competition level (38 and 37 µg/L at days 14 and 21; Table 3). The lethal effects of carbendazim on *G. pulex* in the intermediate competition level were assumed to be alleviated by the presence of *A. aquaticus*, since *G. pulex* is known to strongly predate on *A. aquaticus* (Blockwell et al., 1998). This could be confirmed by the increased survival of *A. aquaticus* with increasing concentrations, probably because *G. pulex* predation rates were affected by the pesticide concentrations (Fig. S4, Supplementary material). This result demonstrated that competition stress combined with chemical exposures is able to influence species interactions. No effects of carbendazim on *A. aquaticus* were demonstrated within the tested concentration range (Table A, Supplementary material), showing a higher tolerance of this species to this compound as compared to *G. pulex*.

To sum up, the results of our experiments show the potential complexity of populations' responses under combined effects of competition and chemical exposure since the interaction of both pressures can vary depending on competition and toxicant concentration pressure levels. It was observed that at high interspecific competition pressure, the depletion of food availability combined with toxic effect cannot be compensated by predation benefits for *G. pulex* due to the presence of *A. aquaticus*. This could be a result of the higher density at initial conditions, which probably overstressed the population for food availability (inter-H, 5 *G. pulex* vs. 10 *A. aquaticus*; inter-M, 5 *G. pulex* vs. 5 *A. aquaticus*). The interactions between different levels of ecological interactions and the levels of toxicant exposure have previously been reported. For example, Linke-Gamenick et al. (1999) studied density-dependent effects of polycyclic aromatic hydrocarbons (PAHs) and of a fluoranthene (FLU) on survival, growth rate and reproduction of a polychaete (*Capitella* sp.) and found that at low food limitations and low toxicant concentrations the toxic effects were marginal, whereas at high toxicant concentration, food limitation intensified the toxic effects (synergistic effects). This also corresponds with the study by Barata et al. (2002), who found that at medium limiting food resources the negative toxic effects on population abundance drastically increase with increasing animal density, suggesting that compensation of toxicant impacts is related to mortality driven by competition stress. On the contrary, several

studies report negative effects of food limitation on species sensitivity. For instance, Stamplfli et al. (2011) found that the abundance of zooplankton species was more affected by a pesticide under food limited conditions. Foit et al. (2012) performed an experiment to test the competition effect on sensitivity and recovery capacity of two interacting populations (*Daphnia magna* and *Culex pipiens molestus*) and concluded that toxicant sensitivity was positively correlated to competition and delayed recovery. Therefore, there are evidences of both positive and negative combined effects of competition and toxicant effects. Effects of combined stressors (competition and pesticide) were expected since competition for food resources is one of the most relevant ecological interactions influencing mortality and development at the individual and populations levels (Van Buskirk, 1987; Gordon, 2000).

3.4. The importance of ecological interactions for risk assessment

Ecological interactions such as competition and predation are highly relevant for population responses in the field, so excluding them from ERA may lead to inefficient (over or under protective) regulations at both economic and ecological levels. Although it has been generally assumed that competition enhances the negative effects of a toxicant (Foit et al., 2012; Stamplfli et al., 2011), some studies have shown that population-level effects of food limiting conditions under chemical stress could be outweighed under medium-high population densities and low toxicant concentrations (Gui and Grant, 2008; Linke-Gamenick et al., 1999; Barata et al., 2002). The results of the *G. pulex* experiment are consistent with the latter hypothesis and indicate behavioral changes as the main drivers determining the effects of competition.

The results of the experiments here presented support the need to include both intra- and interspecific competition in ERA to better understand the combined effects of ecological interactions and toxic disruption on aquatic communities by, for example, assessing them with the help of food-web and meta-population models. These results also indicate that modeling approaches that intend to introduce food limiting conditions and species competition into prospective ERA should consider associated compensatory mechanisms such as species predation and cannibalism.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2015.05.001.

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