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# The relevance of biotic interactions to pesticide effects

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# The relevance of biotic interactions to pesticide effects

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II. Foit, K., Kaske, O., Liess, M., 2012. Competition increases toxicant sensitivity and delays the recovery of two interacting populations. *Aquatic Toxicology* 106: 25–31.

III. Foit, K., Chatzinotas, A., Liess, M., 2010. Short-term disturbance of a grazer has long-term effects on bacterial communities - Relevance of trophic interactions for recovery from pesticide effects. *Aquatic Toxicology* 99: 205–211.

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# Summary

Pesticides are amongst the few anthropogenic contaminants that are intentionally released in large quantities into the environment. Through rainfall, pesticides may enter surface waters and affect aquatic communities. Little is known about pesticide effects on aquatic communities, particularly in relation to biotic interactions.

This cumulative thesis addresses the influence of selected biotic interactions on pesticide effects, thereby contributing to a better ecological understanding of the risk assessment of toxicants. The selected biotic interactions are: (i) intra- and interspecific competition in a simple aquatic community (*Daphnia magna* - *Culex pipiens molestus*, chapter 3); and (ii) modified grazing pressure in a simple aquatic food chain (*Daphnia magna* - bacterial community, chapter 4).

To perform the experiments, the Nanocosm test system needed to be further developed (chapter 2). The Nanocosm test system enabled long-term investigations of populations of *Daphni*a and *Culex* larvae. The use of image analysis allowed a time-efficient, non-invasive and reliable detection of the abundance and size structure of both populations (chapter 2). Experiments with the Nanocosm test system revealed that biotic interactions may have an increasing and prolonging influence on pesticide effects: intraspecific competition increased the toxicant sensitivity of *Daphnia* and *Culex* larvae and thus amplified direct pesticide effects (chapter 3). Interspecific competition delayed the recovery of *Daphnia*, with positive indirect effects on the development of *Culex* larvae (chapter 3). Intraspecific competition delayed the recovery of *Daphnia* and thereby modified the grazing pressure on bacteria; the modified grazing pressure caused indirect pesticide effects on the trophic level of bacteria (chapter 4). Hence, natural communities might be disturbed for a longer period, at a higher intensity, and with more affected species than predicted with single-species tests alone.

# Zusammenfassung

Pestizide gehören zu den wenigen anthropogenen Schadstoffen, die absichtlich und im großen Maße in die Umwelt eingebracht werden. Bei Niederschlagsereignissen können Pestizide in Oberflächengewässer eingetragen werden und die Artgemeinschaft belasten. Es ist jedoch wenig bekannt über Pestizideffekte auf aquatische Gemeinschaften, insbesondere im Hinblick auf die Bedeutung biotischer Interaktionen.

Die kumulative Dissertation befasst sich mit dem Einfluss ausgewählter biotischer Interaktionen auf Pestizideffekte und trägt damit zum ökologischen Verständnis in der Risikobewertung von Pflanzenschutzmitteln bei. Zu den ausgewählten biotischen Interaktionen gehören (i) intra- und interspezifische Konkurrenz in einer einfachen aquatischen Gemeinschaft (*Daphnia magna* - *Culex pipiens molestus*, Kapitel 3) sowie (ii) ein veränderter Fraßdruck in einer einfachen aquatischen Nahrungskette (*Daphnia magna* - Bakteriengemeinschaft, Kapitel 4).

Zur Schaffung der technischen Voraussetzung ging den Untersuchungen die Weiterentwicklung des Nanokosmen Testsystems voraus (Kapitel 2). Die Nanokosmen ermöglichten Langzeitbeobachtung von Daphnien und Mückenlarven auf Populationsebene. Abundanz und Größenstruktur beider Populationen wurden mithilfe von Bildanalyse zeiteffizient, nichtinvasiv und zuverlässig erfasst (Kapitel 2). Die Untersuchungen mit dem Nanokosmen Testsystem zeigten, dass biotische Interaktionen einen verstärkenden und verlängernden Einfluss auf Pestizideffekte haben können: Intraspezifische Konkurrenz erhöhte die Schadstoffsensitivität von Daphnien und Mückenlarven und verstärkte dadurch direkte Pestzideffekte (Kapitel 3). Interspezifische Konkurrenz verzögerte die Erholung der Daphnien und förderte dadurch indirekt die Entwicklung der Mückenlarven (Kapitel 3). Intraspezifische Konkurrenz verzögerte die Erholung von Daphnien und veränderte dadurch ihren Fraßdruck auf Bakterien; der veränderte Fraßdruck führte zu indirekten Pestizideffekten auf unterer Trophieebene der Bakterien (Kapitel 4). Natürliche Gemeinschaften können daher durch eine Pestizidbelastung für längere Zeit, mit stärkerer Intensität und mit einer höheren Anzahl betroffener Arten gestört sein als durch Einzelarten-Testsysteme vorhergesagt.

# Chapter 1

## Introduction

*Pesticide use and the impact on surface waters*

Pesticides are amongst the few anthropogenic toxicants that are released into the environment intentionally and in great quantities. In Germany, nearly 52% of land area is used for agriculture (Statistisches Bundesamt, 2012) and about 40,000 tonnes of active substances are sold each year (UBA, 2012; Stand 2009/2010). From these figures, one can estimate a mean pesticide use in Germany of about 200 mg substance/$m^2$/year. During strong rainfall and runoff events, pesticides may enter adjacent surface waters and cause pulse exposures with short-term peak concentrations (Leu et al., 2004; Williams et al., 1995). Field investigations showed long-term effects of pesticides on the community structure of the macrozoobenthos: the higher the peak concentration of pesticides in surface waters, the lower the long-term proportion of sensitive species (Liess et al., 2008; Liess and von der Ohe, 2005; Schäfer et al., 2007). Furthermore, a negative relationship was revealed between pesticide use and biodiversity (Brittain et al., 2010; Geiger et al., 2010). However, only little is known about the mechanisms that impede a complete recovery of aquatic communities from pesticide stress.

*Direct and indirect pesticide effects*

Pesticide effects can be divided into direct and indirect effects. The direct effects of pesticides can be explained by the toxicity of the active substance itself. They comprise all toxicant-induced impairments of sensitive species with respect to behaviour, fitness, survival, growth or reproduction. Due to biotic interactions, these direct effects on sensitive species may initiate indirect effects on more tolerant species. One important biotic interaction is competition. For example, a toxicant-induced mortality of sensitive species may initiate a release from competition with positive indirect effects on the development of tolerant species (reviewed by Fleeger et al., 2003).

Indirect effects are suggested to be more common than direct effects (Fleeger et al., 2003; Relyea and Hoverman, 2006; Rohr et al., 2006) and to have a similar or an even greater influence on the structure of communities (Lampert et al., 1989; Menge, 1995). Consequently, failure to incorporate indirect effects into risk assessment paradigms may be a significant source of uncertainty in risk estimates (Preston, 2002).

*Ecotoxicological risk assessment: challenges and research needs*

EU legislation prescribes that pesticides should have no unacceptable effects on the environment, particularly no unacceptable impact on biodiversity and the ecosystem (EU, 2009). The approval process for pesticides is regulated by law and is based on internationally agreed testing methods (OECD, 2011). These methods are usually single-species tests at the individual level with test durations of up to 21 days. The determined threshold concentrations in the laboratory are extrapolated to ecologically safe concentrations in the field by safety factors. However, such extrapolations comprise a wide range of uncertainties (Chapman et al., 1998; Goodman, 2002), some of which are summarized below.

The laboratory differs from the field with regard to abiotic factors. In the laboratory, efforts are made to standardize and optimize abiotic conditions like water quality, food concentration and lighting. In the field, however, species are confronted with a constantly changing environment that may influence a species' response to pesticide stress. For example, it is well known that abiotic factors like temperature (Song et al., 1997), food limitation (Mommaerts et al., 2010), or UV radiation (Liess et al., 2001) may increase the sensitivity of a species to toxicants. The situation in the laboratory also differs from the field with regard to the number of species and their biotic interactions. Laboratory experiments are mainly conducted with a few species in test systems on an individual level. In contrast, in the field a large number of species is exposed to the pesticide. The community response to toxicants is therefore influenced by the traits of the species, like sensitivity and reproduction ability (Liess et al., 2008; Niemi et al., 1990), and particularly by biotic interactions.

The high significance of biotic interactions for pesticide effects is apparent, and their consideration is urgently required (Preston, 2002; Relyea and Hoverman, 2006). However, biotic interactions, while a major consideration in ecology, are still underrepresented in ecotoxicology. A comparison of keywords in the respective specialised literature demonstrates the different considerations of competition as an important biotic interaction (literature of the last 10 years, 1/2002 to 7/2012; Web of Science®). Publications in ecology that are tagged with the keywords "community" and "ecosystem" list, in 10% of cases, the additional keyword "competition". The same keyword search in publications of (eco-) toxicology returns the additional keyword "competition" only in about 1.7% of cases. To prevent an over- or underestimation of

the environmental risk posed by toxicants, more extensive knowledge about, and more research into environmental factors like biotic interactions are needed.

*Aim of the thesis*

The aim of this thesis is to assess the influence of selected biotic interactions on pesticide effects and the following recovery. The selected biotic interactions include: (i) intra- and interspecific competition in a simple aquatic community (*Daphnia magna* - *Culex pipiens molestus*); and (ii) a modified grazing pressure in a simple aquatic food chain (*Daphnia magna* - bacterial community). For the realization of the experiments, the test system Nanocosm was further developed.

## Problems and tasks of the publications

### I. Automated Nanocosm test system to assess the effects of stressors on two interacting populations (chapter 2).

*Problems*

In ecotoxicology, various test systems are used that differ in their degree of complexity. Accordingly, the strengths and weaknesses of these test systems differ as well. Single-species systems at the individual level are simple to establish, handle and observe, and the reproducibility and interpretability of results is high. However, these test systems lack ecologically important factors like biotic interactions. To predict effects on the ecosystem level, extrapolations are required that include a large range of uncertainties (Chapman et al., 1998; Goodman, 2002). Multi-species systems (pond mesocosms or artificial streams) include a wide range of biotic interactions. However, due to the complexity of the test system, the effort of handling and observation is increased, and the reproducibility and interpretability of the results are reduced. For the aims of this thesis, a test system of intermediate complexity was needed that enabled a simple and non-invasive long-term monitoring of two competing species by image analysis.

*Tasks*

(i) Development and calibration of an automated Nanocosm test system with two aquatic species (*Daphnia magna* and *Culex pipiens molestus*).

(ii) Application of the Nanocosm test system to quantify interspecific competition.

**II. Competition increases toxicant sensitivity and delays the recovery of two interacting populations (chapter 3).**

*Problems*

Competition is an important factor that affects the coexistence of species (Gordon, 2000). It can therefore be assumed that competition plays an important part in the recovery of species after pesticide stress. It is well known that a toxicant-induced mortality may initiate a release from competition with positive effects on the development of tolerant species (reviewed by Fleeger et al., 2003; Preston, 2002; Relyea and Hoverman, 2006). However, little is known about direct and indirect pesticide effects in the presence of persistently high competitive pressure.

*Tasks*

(i) Application of the Nanocosm test system to investigate direct and indirect pesticide effects in the presence of competition (*Daphnia magna* - *Culex pipiens molestus*).
(ii) Evaluation of direct pesticide effects under the influence of competition.
(iii) Evaluation of indirect pesticide effects under the influence of competition.

**III. Short-term disturbance of a grazer has long-term effects on bacterial communities - Relevance of trophic interactions for recovery from pesticide effects (chapter 4).**

*Problems*

After pesticide stress, populations may recover fast in terms of the total abundance. However, the size structure of the populations might be disturbed for unexpectedly long recovery times (Driskell et al., 2001; Johnston and Keough, 2005; Liess and Foit, 2010; Liess et al., 2006). Further research is needed to assess the field relevance of a changed population structure after pesticide stress. For example, little is known about effect propagations within the aquatic food chain.

*Tasks*

(i) Application of the Nanocosm test system to investigate indirect pesticide effects within a simple aquatic food chain (*Daphnia magna* - bacterial community).
(ii) Evaluation of indirect pesticide effects on the bacterial community due to a modified size structure of *Daphnia magna*.

## Main results

In the following, selected results of the thesis are summarized. The statistical analysis is briefly presented in brackets. For a detailed explanation of the statistical method and the results obtained, see chapters 2 to 4.

**I. Automated Nanocosm test system to assess the effects of stressors on two interacting populations (chapter 2).**

*(i) Development and calibration of an automated Nanocosm test system with two aquatic species (Daphnia magna and Culex pipiens molestus).*

The Nanocosm test system comprised populations of *Daphnia* and *Culex* larvae. Abundance and size structure of the two populations were quantified by image analysis. A comparison of the true abundance and the abundance detected with the Nanocosm test system showed a residual variance of 0.3% for *Daphnia* (n = 13) and 11% for *Culex* larvae (n = 11; residual variance after linear regression). The calibration of size structure was conducted with 12 size classes for *Daphnia* and 4 size classes for *Culex* larvae (n = 30 in each size class). For *Daphnia*, the mean variation coefficient of size measurement increased from 7% after manual measurement to 15.6% after detection; for *Culex* larvae, the mean variation coefficient increased from 7.2% after manual measurement to 19.6% after detection with the Nanocosm test system. Overall, the Nanocosm test system enabled a time-efficient, non-invasive and reliable long-term monitoring of the two aquatic populations.

*(ii) Application of the Nanocosm test system to quantify interspecific competition.*

To quantify the biotic interactions between *Daphnia* and *Culex* larvae, five Nanocosm test systems were established and monitored for 103 days. The biomass of the two populations oscillated, with distinct maximum values every 40 to 60 days. The maxima of the biomass of one species are negatively correlated with the biomass of the second species (Pearson's correlation test, $r = -0.86$, $n = 17$, $P < 0.001$). This negative relationship between the two species was observed over the whole testing period of 103 days. It was therefore independent from the temporal population density and food quantity and can be explained by interspecific competition.

**II. Competition increases toxicant sensitivity and delays the recovery of two interacting populations (chapter 3).**

*(i) Application of the Nanocosm test system to investigate direct and indirect pesticide effects in the presence of competition (Daphnia magna - Culex pipiens molestus).*
To investigate long-term pesticide effects under the influence of permanent high competitive pressure, a Nanocosm experiment with *Daphnia* and *Culex* larvae was performed. The two competing species were pulse-exposed to the pyrethroid fenvalerate (48-hour pulse with treatment concentrations of 0, 0.6, 0.8, 1, and 3 µg/L). The abundance and biomass of populations were quantified by image analysis. Adult mosquitoes were counted.

*(ii) Evaluation of direct pesticide effects under the influence of competition.*
Shortly after exposure, *Daphnia* showed a concentration-response relationship with an $LC_{50}$ of 0.9 µg/L. *Culex* larvae were slightly less sensitive with an $LC_{50}$ of 1.7 µg/L. For the two species, toxicant sensitivity increased with the population biomass of the respective species directly before exposure, which is explained by intraspecific competition (analysis of covariance: the explained variance is given separately for the exposure to fenvalerate ($EV_{Fenv}$) and the influence of intraspecific competition ($EV_{Comp}$). For *Daphnia*: $EV_{Fenv}$ = 71%, $EV_{Comp}$ = 7.6%; for *Culex* larvae: $EV_{Fenv}$ = 9.9% fenvalerate, $EV_{Comp}$ = 11%; for detailed results see chapter 3).

*(iii) Evaluation of indirect pesticide effects under the influence of competition.*
Several weeks after pulse exposure to 1 µg/L, the slight differences in sensitivity between the two species resulted in contrasting long-term effects due to interspecific competition. The recovery of *Daphnia* was impaired by interspecific competition, given as the survival of *Culex* larvae directly after contamination (linear regression analysis: intercept = 14.5, slope = −0.087, $r^2$ = 0.5, df = 18, $P < 0.001$). *Culex* larvae profited from the slow recovery of *Daphnia* and showed an increased success of emergence; the emergence success of *Culex* larvae was best explained by the biomass of *Daphnia* at the beginning of emergence time (linear regression analysis: intercept = 3.76, slope = −0.9, $r^2$ = 0.35, df = 30, $P < 0.001$).

**III. Short-term disturbance of a grazer has long-term effects on bacterial communities – Relevance of trophic interactions for recovery from pesticide effects (chapter 4).**

*(i) Application of the Nanocosm test system to investigate indirect pesticide effects within a simple aquatic food chain (Daphnia magna - bacterial community).*
To investigate the long-term transfer of pesticide effects from a higher trophic level to bacterial communities, a Nanocosm experiment with *Daphnia* was performed. The populations were pulse-exposed to the pyrethroid fenvalerate (48-hour pulse with treatment concentrations of 0, 0.8, 1, and 3 µg/L). The abundance and size structure of the populations were quantified by image analysis. Aquatic bacteria were monitored with regard to abundance (cell staining) and community structure (16S ribosomal RNA fingerprinting method).

*(ii) Evaluation of indirect pesticide effects on the bacterial community due to a modified size structure of Daphnia magna.*
Several weeks after pulse exposure to 1 µg/L, the size structure of *Daphnia* was modified: it was dominated by an increased percentage of small individuals (analysis of variance followed by Dunnett's multiple-comparison test, $P < 0.05$ at 1 µg/L fenvalerate). At the same time, the bacterial cell density was below control level (analysis of variance followed by Dunnett's multiple-comparison test, $P < 0.05$ at 1 µg/L fenvalerate). Likewise, the community structure of bacteria was modified, with the percentage of fast-growing bacteria reduced (analysis of variance followed by model simplification, simplified model: treatment concentration grouped in low disturbance vs. high disturbance: 43% vs. 34%; $P < 0.029$).

## Discussion

*The Nanocosm test system (chapter 2)*
The further developed Nanocosm test system enabled the long-term monitoring of pesticide effects in the presence of interspecific competition. The simplicity of the Nanocosm test system supported the replicability and interpretability of the results. Image analysis reduced the effort of monitoring. The Nanocosm test system enabled the experiments of chapters 3 and 4. This technique can be used to better integrate the

competition factor into environmental and ecotoxicological research, as well as for the assessment of risks due to stressors.

*Direct pesticide effects under the influence of intraspecific competition (chapter 3)*
Intraspecific competition increased the acute mortality of *Daphnia* and *Culex* larvae after pulse exposure to fenvalerate. The increased sensitivity due to intraspecific competition can be explained by a reduced food supply and thus reduced fitness of individuals. An increased sensitivity under low-food conditions is known from single-species test with *Daphnia* (Pieters et al., 2005). An increased sensitivity against pyrethroid due to intraspecific interactions was also observed for populations of *Limnephilus lunatus Curtis* (Liess, 2002) and *Daphnia* spp. (Knillmann et al., 2012a).

*Indirect pesticide effects on the same trophic level (chapter 3)*
Interspecific competition caused indirect pesticide effects between *Daphnia* and *Culex* larvae, two species of the same trophic level. Firstly, the recovery of *Daphnia* populations was delayed twice as long as observed in isolated populations without competitors (Liess and Foit, 2010; Liess et al., 2006). This difference in recovery time can be explained as follows: in isolated populations, the toxicant-induced mortality initiates a release from intraspecific competition with positive effects on the recovery process (reviewed by Hanazato, 2001). However, in the presence of competitors, this release from intraspecific competition is countered by interspecific competition and the recovery of the population is delayed. A prolonged recovery due to competing taxa was also observed for the recovery of *Daphnia* spp. populations after a short-term exposure to esfenvalerate (Knillmann et al., 2012b). Consequently, *Culex* larvae profited from the delayed recovery of *Daphnia* and showed an increased emergence success.

*Indirect pesticide effects between different trophic levels (chapter 4)*
A modified grazing pressure transferred pesticide effects from *Daphnia* to bacteria, two species of different trophic levels. A long time after the pesticide pulse, the size structure of *Daphnia* was still dominated by small individuals; at the same time, the bacterial abundance and percentage of fast-growing bacteria was reduced. Juvenile *Daphnia* are known to be more efficient grazers on bacteria compared to other size classes (see regression analysis in chapter 4; Brendelberger, 1991). The modified abundance and community structure of bacteria can therefore be explained as an indirect pesticide effect due to an increased grazing pressure of *Daphnia*.

*Conclusions*

In this thesis it was shown that biotic interactions have a significant influence on pesticide effects in the community context. Competitive pressure may intensify and prolong pesticide effects on the same trophic level (chapter 3). Grazing pressure may be an important mediator of pesticide effects between different trophic levels (chapter 4). Hence, natural communities might be disturbed for a longer period, at higher intensity, and with more affected species than predicted by single-species tests alone.

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# Chapter 2
# Automated Nanocosm test system to assess the effects of stressors on two interacting populations

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## Abstract

There is a great need in environmental research for test systems that include ecologically important factors and that are also easy to use. We here present the automated test system Nanocosm, which is composed of populations of *Daphnia magna* and *Culex pipiens molestus*. The Nanocosm system allows the investigation of stressed populations in the presence of interspecific competition, which is a very important factor involved in the dynamics of ecosystems. With the Nanocosm system, the abundance and size structure of populations of both species are quantified by image analysis. The technique enables a time-efficient, non-invasive and reliable long-term monitoring of interactions between two aquatic populations. We recommend the Nanocosm system as a novel tool for the simplified integration of competition into environmental and ecotoxicological research as well as for the assessment of risk due to stressors.

## Introduction

Competition is a very important factor involved in the development of populations and communities in the field. Therefore, it can be assumed that competition strongly influences the population and community responses to stressors such as toxicants. It is well known that the impairment or mortality of sensitive species after exposure to toxicants may result in a release from competition with positive effects on the development of non-sensitive species (reviewed by Fleeger et al., 2003; Preston, 2002; Relyea and Hoverman, 2006). However, only little is known about toxic effects and recovery in the presence of high intra- and interspecific competition. For example, after

exposure to toxicants, competing field populations may show unexpected long-term alterations in size structure (Driskell et al., 2001). Using a test system under laboratory conditions, similar effects of toxicants on the size structure of populations could be directly observed. The test system comprised of populations of *Daphnia magna* that were pulse-exposed to an insecticide and monitored by image analysis. The *Daphnia magna* population responded by a long-term increase of the abundance of small individuals that could be explained by high intraspecific competition (Liess and Foit, 2010; Liess et al., 2006).

At the community level, field investigations have shown unexpected long-term alterations in the community structure of macroinvertebrates following exposure to insecticides (Liess and von der Ohe, 2005; Schäfer et al., 2007). The mechanisms responsible for such long-term effects have yet to be revealed. Common test systems at the community level, such as pond mesocosms or artificial streams, often involve a multitude of biological species and therefore include complex interspecific interactions (Hanazato, 1998; Kennedy et al., 1995). This complexity impedes the identification and characterization of the individual mechanisms that are acting upon the community, and requires the time-consuming identifying and counting of large numbers of individuals. Hence, there is a great need for test systems that involve ecologically important factors and that are easy to use. To the best of our knowledge, a test system that allows two populations to be monitored non-invasively, in a time-efficient manner, and with detailed resolution of population size structures is not yet available.

The aim of this paper is to describe the novel test system Nanocosm, which can be used for monitoring two competing species by image analysis. The Nanocosm system is composed of populations of the cladoceran *Daphnia (Daphnia magna)* and the mosquito *Culex (Culex pipiens molestus)*. Both species are primarily filter feeders with a strong overlap of natural diets (DeMott, 1982; Merritt et al., 1992). Hence, *Daphnia* and *Culex* larvae compete for the same food resource which results in a negative relationship, as already observed in outdoor microcosm experiments (Duquesne et al., 2011; Stav et al., 2005).

A first version of the monitoring technique enabled the automated detection of *Daphnia* (Liess et al., 2006). The current version can detect and differentiate between both species. The appropriateness of the image analysis technique for the monitoring of population abundance and size structure is determined. The main interest of the Nanocosm system is its use in ecology and ecotoxicology. The Nanocosm system

allows the monitoring and analysis of the effects of stressors and of recovery processes over several generations in the presence of interspecific competition.

## Methods

*Aquatic populations*

We established five replicates of the test system Nanocosm, which were observed for a period of 103 days. Each Nanocosm system was initiated with 15 neonates of *Daphnia*, clone B (obtained from Bayer CropScience, Monheim, Germany), and 15 first-instar *Culex* larvae (obtained from the Federal Environment Agency, UBA, Berlin, Germany). The populations were cultured in 5.5-L cylindrical glass vessels (Harzkristall, Derenburg, Germany; Fig. 1). The glass vessels were filled with 4 L of Elendt M7 medium (OECD, 1997). Each glass vessel contained 500 g of washed aquarium sand (diameter, 1–2 mm) at the bottom, which served as a support for bacteria to promote self-purification of the test system. The populations were fed three times a week with a constant food concentration. The food was given as a suspension of ground dog biscuits (Hd-H biscuits, obtained from ssniff Spezialdiäten GmbH, Soest, Germany) mixed with stinging nettle (*Folia Urticae*, obtained from Caesar & Loretz GmbH, Hilden, Germany; weight ratio, 1:1; total carbon content, 0.9 mg/L). The populations adapted to the food supply by reaching the carrying capacity. The test vessels were covered with a net (polyester, 0.5-mm mesh size, obtained from Brettschneider, Heimstetten, Germany) to prevent the escape of adult mosquitoes. Two holes of 1 cm in diameter were made in the net to enable access to the populations (Fig. 1). Opening I was used to feed adult mosquitoes above the water surface. We closed the opening with a rolled-up pad of cotton wool that was soaked in a saturated solution of glucose and was replaced three times a week. Opening II was used to aerate the culture water three times a day for 15 min via silicone tubing (14 cm below the surface of the water; diameter, 4 mm; tapered end, 0.5 mm). The studies were performed at 20°C. The photoperiod was controlled (16:8 h light:dark), and lighting was provided by a 70-W, cool-white fluorescent tube that was situated 10 cm above the test vessels. The biofilm on the front window of the test vessel was removed once a week with a magnetic aquarium cleaner. The water quality was measured every month. The concentrations of ions were such that no negative effects on *Daphnia* or *Culex* larvae would be expected ($NH_4^+$, 0.01 ± 0.04 mg/L [mean ± SD; n = 20]; $NO_2^-$, 0.004 ± 0.006 mg/L; pH 7.7 ± 0.33;

dissolved $O_2$, 6.4 ± 0.46 mg/L; temperature, 20.5 ± 0.1°C; electrical conductivity, 810 ± 58 µS/cm).

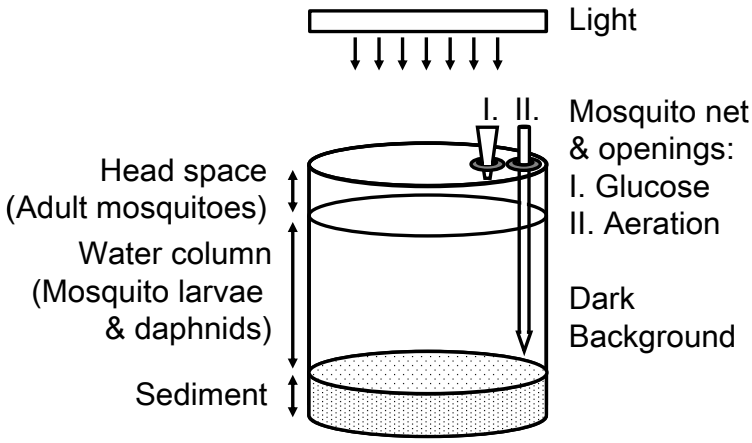


**Fig. 1.** Schematic illustration of the test system, which is composed of populations of *Daphnia magna* and *Culex pipiens molestus*.

*Photography of the populations*

The populations of the Nanocosm systems were monitored three times a week by non-invasive image analysis. The populations were photographed using a digital camera (Camedia C-4000 Zoom; Olympus, Melville, NY, USA). In order to obtain a high-quality image that was free from reflections, the camera was fixed to one end of a rectangular lightproof box (length, 0.7 m), whereas the opposite end of the box was fitted against the front surface of the test vessel. The organisms were illuminated from above (light intensity below net cover, ~46,400 lux). To increase the contrast of the illuminated organisms, a black plastic film was taped to the back of the test vessels.

To improve the distribution of individuals within the image, we photographed the two species one after another and influenced their distribution within the vessel as follows. First, we attracted individuals of *Daphnia* to the upper front side of the water column by utilizing their positive phototaxis, that is, their movement toward a weak light source (intensity below 1,500 lux). After individuals of *Daphnia* had gathered at the upper front side of the water column, we dispersed them by inducing negative phototaxis. The *Daphnia* swam downwards and three consecutive, time-lapsed (0.4 s) images were taken. When the *Daphnia* individuals reached the bottom of the test vessel, *Culex* larvae tended to leave the area. The *Culex* larvae swam upwards, gathered below the water

surface, and another three time-lapsed photos were taken. The digital camera had the following settings: image resolution 2,048 × 1,536 pixels, shutter speed 1/30 s, aperture F2.8, photosensitivity ISO 400, 3 x optical zoom, and focal depth in the middle of the test vessel.

*Image analysis – Overview*

The photographs were evaluated by image analysis. The image analysis technique consisted of two parts. In Part I, *Daphnia* and *Culex* larvae were detected as moving objects while swimming with algorithms adapted from Liess et al. (2006). In Part II, *Culex* larvae that had gathered in a motionless state below the water surface for breathing were detected. For the image analysis, we used the free software *ImageJ* (1997-2009).

*Image analysis – Part I: Daphnia and Culex larvae as moving objects*

The detection of moving objects was performed separately for each species and consisted of the following four steps (Fig. 2). Step 1: The three consecutive images of each species were converted to grayscale (8-bit, pixel values from 0 to 255) and subtracted from each other. After subtraction, only moving objects remained in the image and were distinguished from the dark background by setting a threshold (accepted pixel values > 45; Liess et al., 2006). Step 2: Owing to potentially high densities of *Daphnia*, we separated overlapping and touching individual *Daphnia* (watershed algorithm, *ImageJ)*. Step 3: The two species were distinguished according to their measured length/width ratio (i.e. the ratio of the primary to secondary axes of the best-fitting ellipse): *Daphnia* were defined as round objects with a length/width ratio of < 3.5; *Culex* larvae were defined as elongated objects with a length/width ratio of > 3.5. Using these rules, individuals of the respective species not under analysis were identified and removed from the image. Step 4: The individuals of the analyzed species were counted and their lengths were measured.

*Image analysis – Part II: Immobile Culex larvae below the water surface*

*Culex* larvae that had gathered motionless below the water surface were detected with the following eight steps. Step 1: The original image was converted to grayscale. The image was cropped and only the portion of the image that corresponded to the 5 cm immediately below the surface of the water was retained. Step 2: The structure of bright objects was separated from the dark background by applying an adaptive threshold

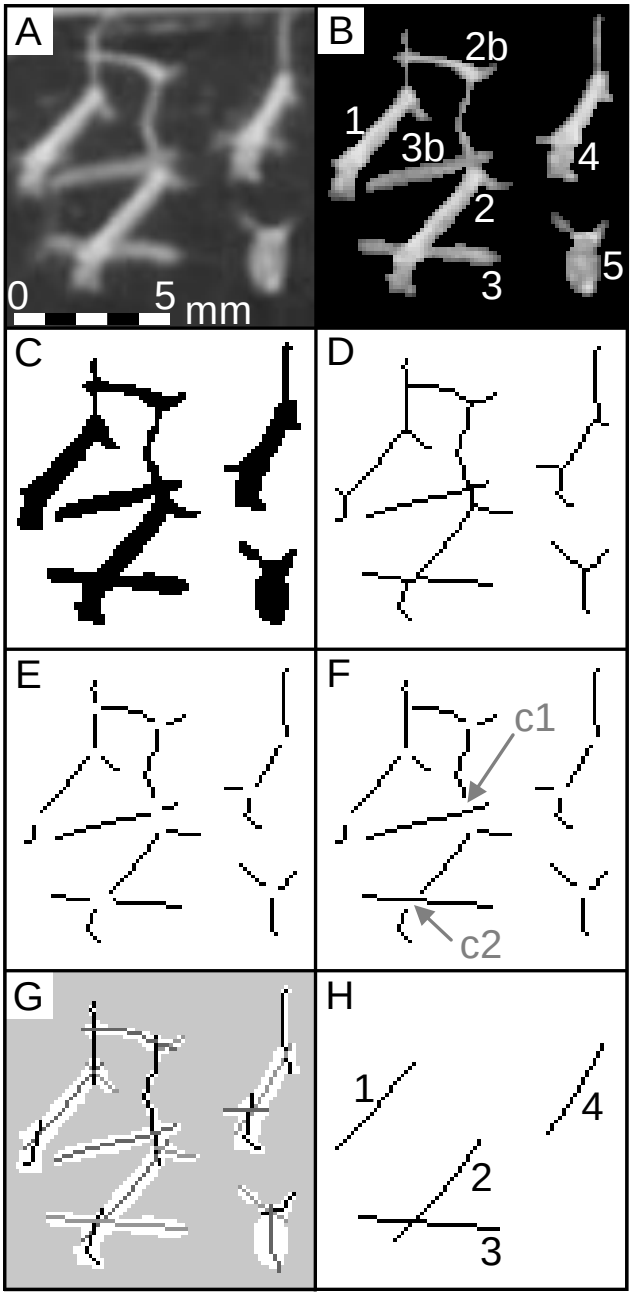


**Fig. 2.** Illustration of the stepwise detection of *Culex* larvae that had gathered motionless below the water surface. A: Detail of the original picture shown in grayscale; B and C: adaptive thresholding and polishing; D: skeletonization; E: deletion of junction points; F: reconnection of split objects (marked with arrows c1 and c2); G: extension of the objects within the area of the original biomass structure (see C); H: elimination of non-larval objects such as individual *Daphnia*, air bubbles, dead organic matter, breathing siphons, and mirror images in accordance with the rules listed in Table 1.

algorithm. Thereby, a smoothed version of the image (mean convolution filter, radius = 7 pixels) was subtracted from the original image followed by thresholding (accepted pixel values > 8). The structure obtained was polished by preprocessing steps of closing, erosion, and dilation. Step 3: The polished structure was skeletonised. Step 4: The skeleton was split into segments at its junctions. A junction was defined as a skeleton pixel with more than two adjacent skeleton pixels in a 3×3-pixel neighbourhood. Step 5: Segments of the split skeleton that belonged to the same larva were reconnected. Two segments belonged to the same larva when their end pixels were close together (distance < 5 pixels or 0.6 mm) and similar in angle (difference < 30°). End pixels were defined as the last five pixels (or 0.6 mm) of each segment. Step 6: Segments were extended until the border of the detected structure (see Step 2) was reached. Step 7: In the final analysis, non-larval objects such as individual *Daphnia*, bright air bubbles, dead organic matter at the water surface, breathing siphons, and mirror images of the *Culex* larvae were removed. The rules for removal are listed in Table 1 and are based on characteristics of the object, such as length, brightness, position, angle, mean width, and variance of local width. The local width of objects was calculated as the medial axis transform (MAT) by skeletonising the Euclidean Distance Map of the original biomass structure (Saito and Toriwaki, 1994; Zhang and Suen, 1984). The pixel values of the MAT preserved the local distance of the skeleton to the boundary of the original object. Step 8: The objects were counted and their lengths were measured. The abundance and size structure of the larval population were calculated by combining the results of Image analysis – Part I and Part II.

*Validation of the image analysis system*

We assessed the accuracy of the test system Nanocosm with respect to the measurements of abundance and size of individual organisms. To validate the measurement of abundance, 600 *Daphnia* and 250 *Culex* larvae were added to a test vessel. Batches of 25–100 individuals were randomly removed by sieving (nylon sieve, mesh-size, 200 µm; opening area, 63.75 $cm^2$). The size of the remaining population in the test vessels is referred to as $abundance_{True}$. The populations were photographed. Individuals in each photo were counted manually and detected by image analysis. We used linear regressions to analyze the relationships between $abundance_{True}$, the abundance visible on the photo, and the abundance determined from the photo by image analysis. The relationships were used for calibration, which resulted in the final values of $abundance_{ImageAnalysis}$.

**Table 1:** Image analysis of immobile *Culex* larvae: Rules for identifying non-larval objects (see Image analysis – Part II, step 7)[1].

| **Elimination Rule** | **Non-larval objects** |
|---|---|
| L < 1.2 | Small objects (e.g. dead organic matter; DOM). |
| L > 9 | Large objects (e.g. DOM). |
| L/W < 3.5 | Round objects (e.g. individual *Daphnia*). |
| L/W > 10 | Long objects (e.g. siphons). |
| L/W > 5 && W < 0.4 | Long and thin objects (e.g. siphons). |
| L/W > 8.5 | Long, bright, and highly structured objects (e.g. air bubbles). |
| V > 35 | High variance of shape-structure (e.g. air bubbles, DOM). |
| A = 0 | Object parallel to the water surface (e.g. air bubbles, DOM, mirror images). |
| B > 250 | Bright objects (e.g. air bubbles). |
| B > 220 && L/W < 5 && W > 0.5 | Bright and thick objects (e.g. DOM). |
| Br/V < 15 | Dark objects with high variance of shape (e.g. DOM). |
| Br < 20 && L < 2.6 | Dark and short objects (e.g. DOM). |
| Br < 50 && L < 1.9 | Dark and very short objects (e.g. DOM). |
| Br < 50 && L/W > 6 && W < 0.4 | Dark, long, and wide objects (e.g. DOM). |
| $L_{cross}/L \geq 2$ && L < 2.2 && $L_{cross} > 4.5$ | Breathing siphons. |
| $L_{Diff} < 1.3$ && $A_{Diff} < 0.4$ && $X_{Diff} < 1.3$ && $Y_{Diff} < 4.5$ | Mirror images. |

[1] Thresholds for length are given in mm and are based on a pixel resolution of 0.13 mm/pixel. The following characteristics were used: length (L) [mm], mean width (W) [mm], absolute brightness (B) [pixel value], relative brightness to the background (Br) [pixel value], coefficient of variation of local width (V) [%], angle (A) [radian], and length of largest larva crossed (Lcross) [mm]. The subscript 'Diff' indicates a comparison of larvae with their potential mirror images focusing on the length (LDiff) [mm], angle (ADiff) [radian], and distance between their closest endpoints in the x-direction (XDiff, parallel to the water surface) [mm] and the y-direction (YDiff, perpendicular to the water surface) [mm]. Several conditions are combined by the logical AND (&&).

To validate the measurement of the size of individuals, we monitored 12 size classes of *Daphnia*, which ranged from 0.8 to 4 mm, and four size classes of *Culex* larvae, which ranged from 2 to 6 mm (n = 30, in each size class). Batches of each size class were added and monitored separately. We used batches of 30 individuals to ensure a wide distribution of individuals in the water column. The variance of size measurements within a single size class was taken as a measure for the variance of size measurements within a population. The actual body sizes ($size_{True}$) of the *Daphnia* (i.e. the distance from the middle of the eye to the base of the tail spine) and the *Culex* larvae (i.e. the direct external distance between the head and the anal segment) were measured using a Leica MS 5 microscope equipped with a Leica DFC 300 F digital camera and a Leica KL 1500 LCD light source (Leica Microsystems, Solms, Germany). Thirty individuals per size class were added to a test vessel and photographed. Individual sizes were measured on the digital photo manually and then determined by image analysis. We used linear regressions to analyze the relationships between $size_{True}$, size measured manually on the photo, and size determined from the photo by image analysis. The relationships were used for calibration, which resulted in the final values of $size_{ImageAnalysis}$.

Furthermore, we investigated the potential interference of one species on the quality of detection of the other species. Therefore, populations of *Daphnia* (100 Ind./L) and *Culex* larvae (25 Ind./L) were cultured and photographed in the test system separately (one-species system, three replicates for each species) as well as in combination with the second species (two-species system, three replicates). Each replicate was monitored three times. We compared the mean $abundance_{ImageAnalysis}$ of the species photographed as a one-species system or as part of a two-species system using the Student's *t* test.

*Calculation of biomass*

The biomass of a population is given as the sum of individual dry weights W (µg). These individual dry weights W were calculated on the basis of the detected body lengths L (mm). For *Daphnia*, we used the relationship $W = 1.5\times10^{-8}\ L^{2.84}$ (Dumont et al., 1975). To find a corresponding relationship for *Culex* larvae we followed the standardized procedure described in Dumont et al. (1975), which gave the relationship $W = 4.4\times10^{-3}\ e^{0.8\times L}$ ($r^2$ = 0.98, n = 25, unpublished data). Owing to variance in the detected body sizes of the larvae (see Results), we defined a dry weight of 5.7 µg (larval length of 0.32 mm) as the lower limit and 1.1 g (larval length of 6.8 mm) as the upper limit for individuals.

*Emergence of mosquitoes*

The number of emerged adults can be measured accurately by counting and removing dead mosquitoes from the water surface. Alternatively, living adults can be counted through the window of the vessel without disturbing the system. Adult mosquitoes maintain the population by oviposition of the next generation. They die naturally after a few weeks. Due to this process of oviposition and natural mortality, populations of *Culex* develop self-sufficiently for many generations in the Nanocosm system (up to 250 days, paper in preparation).

*Analyses of population dynamics and interspecific interactions*

To investigate the population dynamics and interspecific interactions of both species, we monitored five replicates of the Nanocosm system for 103 days. The abundance and biomass of the populations of *Daphnia* and *Culex* larvae are given as moving averages with the mean value of three successive observations.

To investigate interspecific interactions, we correlated the biomass of one species with the biomass of the second species (Pearson's correlation coefficient). Long-term trends of biomass within each replicate and trend differences between replicates were removed by detrending. This was done by calculating a mean linear regression of biomass against time and using the residuals for further analysis. The biomass of the populations oscillated with distinct maximum values every 40 to 60 days. In order to further reduce data variability, we focused on analyzing these maximum values of biomass. For each species and replicate, we selected the maximum values of biomass within 40 to 60 days and related them to the biomass of the second species at that time.

Analyses were conducted with the free software R (R Development Core Team, 2010). A p-value of 0.05 was used to define significance for all statistical analyses.

## Results

*Validation of the Nanocosm system*

The measurements of abundance and size of individuals that were obtained by the Nanocosm system correlated well with manual measurements. The method comprised of two steps: i) photographing the organisms and ii) detecting from the photo by image analysis. We present the correlations of the measurements for the two steps separately.

The correlations of abundance of *Daphnia* were (i) abundance visible on the photo = 4.9 + 0.91 × $abundance_{True}$ (Ind./L, $r^2$ = 0.998, n = 13, $P$ < 0.001) and (ii) abundance detected from the photo = 1 + 0.97 × abundance visible on the photo (Ind./L, $r^2$ = 1, n = 13, $P$ < 0.001). After calibration, we obtained $abundance_{ImageAnalysis}$ with a residual variance of only 0.3% (Fig. 3A). The correlations for the size measurement of *Daphnia* were (i) size measured manually on the photo (pixel) = -0.2 + 4.2 × $size_{True}$ (mm, $r^2$ = 0.995, n = 12, $P$ < 0.001) and (ii) size detected from the photo by image analysis = 1 × size visible on the photo (pixel, $r^2$ = 1, n = 12, $P$ < 0.001). After calibration, we obtained $size_{ImageAnalysis}$ with a residual variance of 0.5%. Within the 12 size classes of *Daphnia*, the size measurement varied with a mean coefficient of variance (CV) of 7% for $size_{True}$ and 15.6% for $size_{ImageAnalysis}$ (n = 30 in each size class; Fig. 3B).

The correlations of abundance of *Culex* larvae were (i) abundance visible on the photo = 11.1 + 0.44 × $abundance_{True}$ (Ind./L, $r^2$ = 0.98, n = 11, $P$ < 0.001) and (ii) abundance detected by image analysis = -3.4 + 0.81 × abundance visible on the photo (Ind./L, $r^2$ = 0.89, n = 11, $P$ < 0.001). After calibration, we obtained $abundance_{ImageAnalysis}$ with a residual variance of 11% (Fig. 4A). The correlations for the size measurement of *Culex* larvae were (i) size measured manually on the photo (pixel) = 6.54 × $size_{True}$ (mm, $r^2$ = 0.97, n = 4, $P$ = 0.009) and (ii) size detected by image analysis = 3.4 + 0.84 × size measured manually on the photo (pixel, $r^2$ = 0.98, n = 4, $P$ = 0.007). After calibration, we obtained $size_{ImageAnalysis}$ with a residual variance of 6.5%. Within the four size classes of *Culex* larvae, the size measurement varied with a mean coefficient of variance (CV) of 7.2% for $size_{True}$ and 19.6% for $size_{ImageAnalysis}$ (n = 30 in each size class; Fig. 4B).

To reveal any interference of one species on the quality of detection of the other species, we monitored populations of *Daphnia* (100 Ind./L) and *Culex* larvae (25 Ind./L) separately and in combination. The monitored $abundance_{ImageAnalysis}$ of *Daphnia* was not affected by the presence of *Culex* larvae (mean abundance ± SE [Ind./L]: 97.2 ± 1.6 as a one-species system versus 100.7 ± 3.1 as part of the two-species system; $t$ test: df = 11, $P$ = 0.34). Likewise, the monitored $abundance_{ImageAnalysis}$ of *Culex* larvae was not affected by the presence of *Daphnia* (24.2 ± 1.7 as a one-species system versus 24.8 ± 2 as part of the two-species system; $t$ test: df = 15, $P$ = 0.83).

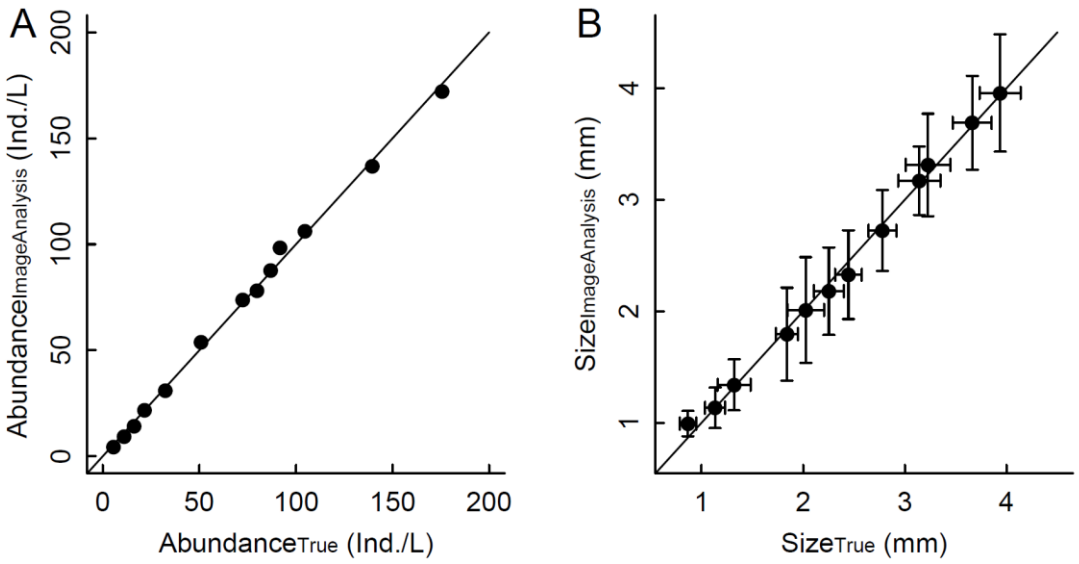


**Fig. 3.** Validation of the Nanocosm system for *Daphnia magna*. (A) Comparison between true abundance ($abundance_{True}$) and the monitored and calibrated abundance after image analysis ($abundance_{ImageAnalysis}$; $r^2$ = 0.997, n = 13). (B) Comparison between the true body size ($size_{True}$) and body size monitored and calibrated after image analysis ($size_{ImageAnalysis}$; $r^2$ = 0.995, n = 12). Error bars indicate ± standard deviations (n = 30 in each size class). The ideal correlation is represented by the diagonal line.

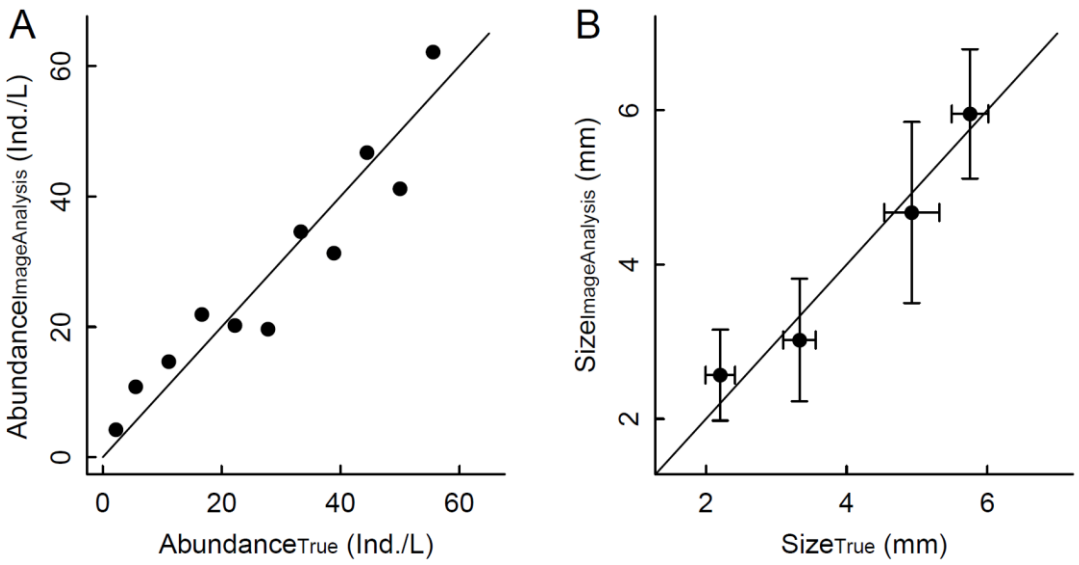


**Fig. 4.** Validation of the Nanocosm system for *Culex* larvae. (A) Comparison between true abundance ($abundance_{True}$) and abundance monitored and calibrated after image analysis ($abundance_{ImageAnalysis}$; $r^2$ = 0.89, n = 11). (B) Comparison between the true body size ($size_{True}$) and body size monitored and calibrated after image analysis ($size_{ImageAnalysis}$; $r^2$ = 0.935, n = 4). Error bars indicate ± standard deviations (n = 30 in each size class). The ideal correlation is represented by the diagonal line.

*Population dynamics and interspecific interactions of the two populations*

To quantify the population dynamics and interspecific interactions of the two populations, we established five Nanocosm systems with *Daphnia* and *Culex* larvae that were monitored for 103 days. On average, *Daphnia* reached a 5.1-fold higher level of abundance and a 0.3-fold lower level of biomass compared to *Culex* larvae (calculated from the time after the carrying capacity of the test system was reached, here from d 40 onwards, n = 26 time points). The abundance and biomass of both species increased with time (Fig. 5).

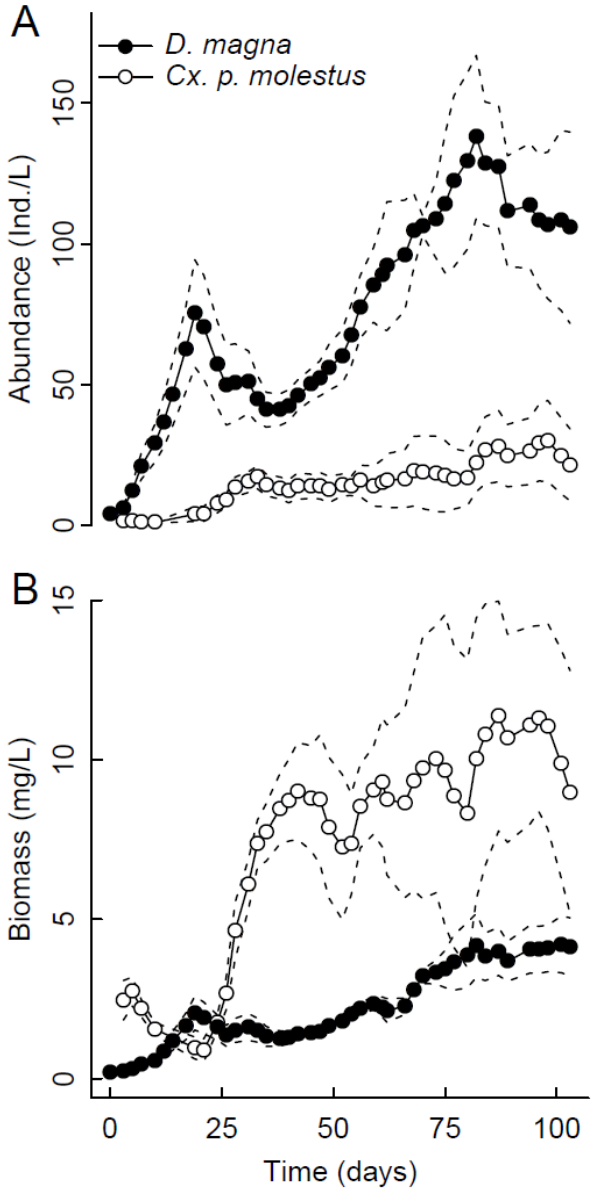


**Fig. 5.** Time courses of abundance (A) and biomass (B) of *Daphnia magna* (solid circles) and *Culex* larvae (open circles) coexisting in the Nanocosm test systems for 103 days. Values are given as a mean of five replicates (± SE, represented as dashed lines).

The population biomass of *Daphnia* and *Culex* larvae oscillated with distinct maximum values every 40 to 60 days (data of single replicates not shown). We observed a negative reciprocal relationship between the biomass of both species by correlating the maxima of one species every 40 to 60 days with the corresponding biomass of the second species (Pearson's correlation test, $r$ = -0.86, n = 17, $P$ < 0.001; Fig. 6). The linear correlation summarizes the following two relationships between the species: *Culex* = 4.5 – 4.5 × *Daphnia* (mg/L, $r^2$ = 0.57, n = 9, $P$ = 0.011) and *Daphnia* = 0.9 – 0.23 × *Culex* (mg/L, $r^2$ = 0.6, n = 8, $P$ = 0.014).

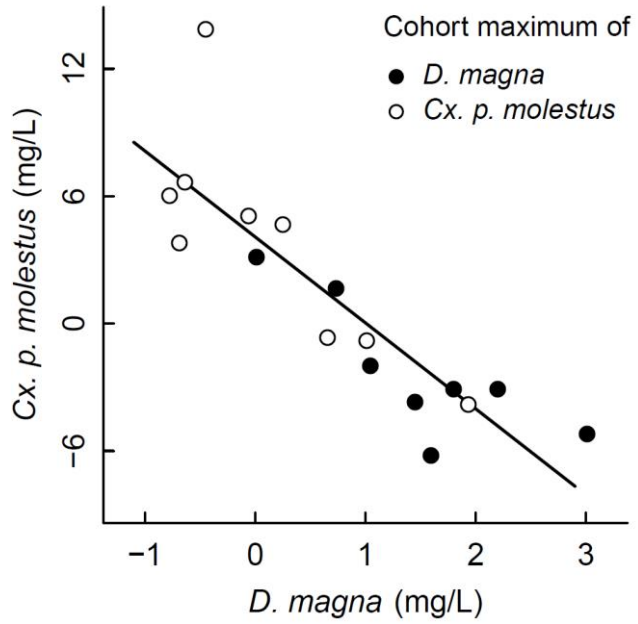


**Fig. 6.** Negative reciprocal relationship between the biomass of both species (Pearson's correlation test, $r$ = -0.86, n = 17, $P$ < 0.001). The maxima of the biomass of one species are negatively correlated with the biomass of the second species; the corresponding regression equations are *Culex* = 4.5 – 4.5 × *Daphnia* (mg/L, $r^2$ = 0.57, n = 9, $P$ = 0.011) and *Daphnia* = 0.9 – 0.23 × *Culex* (mg/L, $r^2$ = 0.6, n = 8, $P$ = 0.014). Biomass is shown as the detrended values of five replicates.

## Discussion

*Classification of the Nanocosm test system*

There is a long tradition of using test systems to determine the effects of stressors on specific processes that are relevant in natural systems. Test systems are model representations of the environment and differ with respect to their degree of complexity

(Boxall et al., 2002). Single-species systems are simple to establish and observe, and the results obtained are easy to reproduce and interpret. However, these systems lack ecologically important factors such as interspecific interactions. Therefore, values obtained using single-species systems need to be extrapolated carefully to predict the effects of chemicals on ecological communities (Cairns, 1983). Multi-species systems, such as pond mesocosms or artificial streams, often approximate the complexity of real systems and include a wide range of interspecific interactions, such as predation and competition (Hanazato, 1998; Kennedy et al., 1995). However, the increased complexity of multi-species systems results in increased effort to make observations, and the reproducibility and interpretability of the results are reduced. By comparison, the Nanocosm system is characterized by an intermediate level of complexity. In contrast to single-species systems, the Nanocosm system comprises two species and focuses on one ecologically important factor in the field, namely, competition. In contrast to mesocosm systems, additional species and further interspecific interactions are excluded. This simplicity of the Nanocosm system supports the reproducibility and interpretability of outcomes in the context of intra- and interspecific competition. The effort required to make observations is also reduced by using image analysis.

*The image analysis system*

The presence of adult mosquitoes required permanent covering of the test system with a mosquito net. Hence, traditional subsampling was impeded and the populations could only be monitored by non-invasive techniques such as image analysis. However, image analysis offers many advantages over subsampling: (i) it is less time-consuming, (ii) it enables more frequent collection of output data, and (iii) it involves no disturbance of the populations, as would result from sampling, species separation, counting, and size measurements. The Nanocosm system allowed the reliable measurement of the abundance and size structure of *Daphnia* and *Culex* larvae. The abundance and size structure of *Daphnia* have already been detected to a high level of precision in previous studies (Liess et al., 2006; Pieters and Liess, 2006). As in these previous studies, individuals of *Daphnia* could be controlled by phototaxis. This enabled us to take photographs that had an even distribution of individuals as well as to detect these individuals easily by their movement. In contrast, the distribution of *Culex* larvae could not be controlled by phototaxis, and photographed individuals were mainly found in a motionless state below the water surface. The larvae in the photographs differed in orientation and distance from the observer, which resulted in complex and overlapping

structures with different levels of brightness and clarity. The splitting of these structures into single larvae was performed on the basis of information about the shape and brightness of the objects. Hence, the detection of *Culex* larvae was more difficult than that of *Daphnia*, which explains the higher variance of measurements for *Culex pipiens*. However, failures to photograph and detect *Culex* larvae were of reproducible regularity and allowed successful calibration. To quantify the time-efficiency of the image analysis system, we measured the time needed for photography and the subsequent selection of photos. On average, photography and photo selection took 7 minutes per test system, each of which contained a mean number of 460 individuals to be monitored (calculated from the time after the carrying capacity of the test system was reached, here from d 40 onwards).

*Presence of interspecific interactions*

The Nanocosm system was used to analyze interspecific interactions between *Daphnia* and *Culex* larvae. We observed a negative reciprocal relationship between the detrended biomass of the two species. This negative relationship was visible over the whole testing period of 103 days and hence was exhibited at different levels of population densities and food quantities.

We explain the negative relationship between the two species by interspecific competition for food. Indeed, both species are primarily filter feeders with a high overlap of natural diets (DeMott, 1982; Merritt et al., 1992). In the Nanocosm systems, the food supply was renewed regularly and kept low. After the carrying capacity was reached, on average 115 individuals shared a daily food supply of 0.45 mg/L carbon; this corresponded to 0.004 mg carbon/individual/day. In comparison, the standardized *Daphnia magna* Reproduction Test requires a daily food up to 50 times higher (between 0.1 and 0.2 mg carbon/individual/day; OECD, 2008). Owing to the obvious lack of carbon sources, interspecific competition for food is assumed to be the main mechanism of the observed negative relationship between the two species. Additionally, stress due to interference competition and allelopathic effects might also contribute to the negative relationship that we observed between *Daphnia* and the *Culex* larvae (Larsson and Dodson, 1993; Navarro-Silva et al., 2009; Roberts, 1998). However, an exact quantification of underlying mechanisms is beyond the scope of this study, which was to create a worst-case situation in terms of high interspecific competition between controphics. The stress level was determined by the carrying capacity of the test system. A negative impact of *Daphnia magna* on the development of *Culex pipiens* has also

been observed in outdoor microcosm experiments (Duquesne et al., 2011; Stav et al., 2005).

*Application and limitations of the Nanocosm test system*

The Nanocosm system allows the long-term monitoring of direct and indirect effects of various types of stressor in the presence of competition. In ecological research, the Nanocosm system will be applied mainly to determine the basic effects of competition on stressed communities. The amount of competitive pressure and the initial conditions of the test system can be modified easily, for example, by artificial predation (Liess and Foit, 2010) or by exposing the population to contamination during a specific development phase (growing phase, peak density or declining phase; Pieters and Liess, 2006).

With respect to the risk assessment of toxicants, the Nanocosm system can be applied as an intermediate stage between lower-tier single-species tests and more realistic but complex higher-tier multi-species investigations. The system allows the relevance of long-term effects and recovery under competitive pressure to be assessed easily. The Nanocosm system is not designed to replace multispecies investigations owing to the following limitations. Relevant biological interactions, such as competition among more than two species, predation, and parasitism, are excluded. Similarly, the system does not reflect the natural variation in sensitivity among a multitude of species. However, the simplicity of the Nanocosm system allows high reproducibility and interpretability of outcomes. Furthermore, owing to the ease of handling of the test system, high numbers of replicates can be conducted, which increase the discriminatory power of hypothesis testing. Therefore, we believe that the integration of the Nanocosm system into the tiered risk assessment of toxicants as an intermediate stage would be of great value.

In summary, we recommend the Nanocosm system as a novel method and an efficient tool for environmental and ecotoxicological research and for the risk assessment of toxicants.

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# Chapter 3
# Competition increases toxicant sensitivity and delays the recovery of two interacting populations

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## Abstract

We investigated how persistent competitive pressure alters toxicant sensitivity and recovery from a pesticide pulse at community level. Interacting populations of *Daphnia* (*Daphnia magna*) and *Culex* larvae (*Culex pipiens molestus*) were pulse-exposed (48 h) to the pyrethroid fenvalerate. The abundance and biomass of the populations were monitored by non-invasive image analysis. Shortly after exposure, *Daphnia* showed a dose-response relationship with an $LC_{50}$ of 0.9 µg/L. *Culex* larvae were slightly less sensitive with an $LC_{50}$ of 1.7 µg/L. For both species, toxicant sensitivity increased with the population biomass of the respective species before exposure, which is explained by intraspecific competition. Several weeks after exposure to the highest treatment concentration of 1 µg/L, the slight differences in sensitivity between the two species were amplified to contrasting long-term effects due to interspecific competition: high interspecific competition impaired the recovery of *Daphnia.* Subsequently, *Culex* larvae profited from the slow recovery of *Daphnia* and showed an increased success of emergence. We conclude that, in natural systems where competition is present, such competitive processes might prolong the recovery of the community structure. Hence, natural communities might be disturbed for a longer period by toxic exposure than predicted from single-species tests alone.

## Introduction

Risk assessment of toxicants is often based on single-species tests under optimal laboratory conditions for growth and reproduction. In contrast, natural conditions are often non-optimal and the actual sensitivity and recovery of species might be substantially different (Heugens et al., 2001). As a consequence, the extrapolation of

toxicity data from the individual to the ecosystem level involves uncertainties that are usually compensated for by safety factors (European Commission, 2003). These uncertainties can be reduced by the identification of general patterns of context-dependent sensitivity and recovery of species after exposure to toxic stress.

At the individual level, the toxicant sensitivity of a species is known to be affected by various abiotic stressors. For example, acute sensitivity might depend on temperature (Song et al., 1997), salinity (Wildgust and Jones, 1998), oxygen level (Van der Geest et al., 2002), UV radiation (Liess et al., 2001), and the acquisition of food (Mommaerts et al., 2010).

At the population and community levels, species also experience biological interactions. One important biological interaction that affects the coexistence of species is competition (Gordon, 2000). Toxicant-induced mortality is frequently observed to reduce competitive pressure, which has positive effects on the subsequent recovery of affected species. At the population level, disturbed species may benefit from the release from intraspecific competition and recover rapidly (Beketov and Liess, 2005; Forbes et al., 2003; Liess, 2002; Linke-Gamenick et al., 1999; Moe et al., 2002; Muturi et al., 2010; Postma et al., 1994). At the community level, species may benefit from the release from interspecific competition that is caused by the mortality of more sensitive species (Hanazato, 1998; Johnston and Keough, 2003; Kesavaraju et al., 2010; Relyea, 2009). However, competition at the community level might also persist at a high level, especially when some dominant species are not affected by the toxicant. To the authors' knowledge, the process of species recovery in the context of persistently high competitive pressure has been shown only rarely, in two cases of phytoplankton communities (Fisher et al., 1974; Wang et al., 2011), and has not been investigated for heterotrophic species such as invertebrates or insects. In the present study, we investigated the effects of, and recovery from, a pesticide pulse in a simple model community with two competing species. We used Nanocosm, a test system composed of populations of the cladoceran *Daphnia* (*Daphnia magna*) and the mosquito *Culex* (*Culex pipiens molestus*). The Nanocosm system focuses on one important interaction of the field, namely competition. Both species are primarily filter feeders with a strong overlap of natural diets (DeMott, 1982; Merritt et al., 1992). Hence, *Daphnia* and *Culex* larvae compete for the same food resource which results in a negative relationship. The negative relationship between the two species was previously observed in a Nanocosm experiment without pesticide exposure (Foit et al., 2012) and in a few outdoor

microcosm experiments (Duquesne et al., 2011; Stav et al., 2005). Other ecologically important interactions like predation are excluded. However, the simplicity of the Nanocosm system improves the reproducibility and interpretability of the results. The effort required to make observations has also been reduced by using image analysis.
The aim of the study was to determine how intra- and interspecific competition may alter toxic effects and recovery after a pesticide pulse. The knowledge about pesticide effect and recovery in the presence of interspecific competition contributes to improving the risk assessment of chemicals by furthering consideration of the environmental context.

## Methods

We established 42 replicates of the test system Nanocosm, which were observed for a period of 47 days after pulse exposure to the pesticide fenvalerate. The Nanocosm system is composed of two interacting populations, namely the cladoceran *Daphnia* (*Daphnia magna*) and the mosquito *Culex* (*Culex pipiens molestus*), and a monitoring technique that is based on image analysis. The first version of the monitoring technique enabled the detection of *Daphnia* (Liess et al., 2006). The current version can detect and differentiate between both species and has been described elsewhere in detail (Foit et al., 2012).

*Aquatic populations*
Each Nanocosm system was initiated with 15 neonates of *Daphnia*, clone B (obtained from Bayer CropScience, Monheim, Germany), and 15 first-instar *Culex* larvae (obtained from the Federal Environment Agency, UBA, Berlin, Germany). The populations were cultured in 5.5-L cylindrical glass vessels (Harzkristall, Derenburg, Germany). The glass vessels were filled with 4 L of Elendt M7 medium (OECD, 1997). Each glass vessel contained 500 g of washed aquarium sand (diameter 1–2 mm) at the bottom, which served as a support for bacteria to promote self-purification of the test system. To maintain constant test conditions, the populations were fed three times a week with an unchanging amount of food. As the main source of food, we provided a suspension of ground dog biscuits (Hd-H biscuits, obtained from ssniff Spezialdiäten GmbH, Soest, Germany) mixed with stinging nettle (*Folia Urticae*, obtained from Caesar & Loretz GmbH, Hilden, Germany; weight ratio, 1:1; total carbon content,

0.87 mg/L). To support the development of *Daphnia*, we added a suspension of batch-cultured green algae *(Desmodesmus subspicatus*; 8.3 × 103 cells/ml; total carbon content, 0.03 mg/L). The green algae were cultured in algal medium (Grimme and Boardmann, 1972) that was aerated continuously (1% $CO_2$ added to air). The algal cells were harvested in exponential growth phase, centrifuged at 3,000 rpm for 10 minutes, and resuspended in Elendt M7 medium. The populations adapted to the given food supply by reaching the carrying capacity.

The test vessels were covered with a net (polyester, 0.5-mm mesh size, obtained from Brettschneider, Heimstetten, Germany) to prevent the escape of adult mosquitoes. Two holes of 1 cm in diameter were made in the net to enable access to the populations. Opening I was used to feed adult mosquitoes above the water surface. We closed the opening with a rolled-up pad of cotton wool that was soaked in a saturated solution of glucose and was replaced three times a week. Opening II was used to aerate the culture water three times a day for 15 minutes via silicone tubing (14 cm below the surface of the water; diameter, 4 mm; tapered end, 0.5 mm). The studies were performed at 20°C. The photoperiod was controlled (16:8 h light:dark), and lighting was provided by a 70-W, cool-white fluorescent tube that was situated 10 cm above the test vessels. The biofilm on the front window of the test vessel was removed once a week with a magnetic aquarium cleaner. The water quality was measured every second week. The concentrations of ions were such that no negative effects on *Daphnia* or *Culex* larvae would be expected ($NH_4^+$, 0.00 ± 0 mg/L [mean ± SD, n = 65]; $NO_2^-$, 0.007 ± 0.014 mg/L; pH, 7.7 ± 0.15; $O_2$, 6.8 ± 0.9 mg/L; temperature, 20.7 ± 0.5°C; electrical conductivity, 732 ± 44 µS/cm).

*Monitoring technique*

The populations of the Nanocosm systems were monitored three times a week by non-invasive image analysis. The populations were photographed using a digital camera (Camedia C-4000 Zoom; Olympus, Melville, NY, USA). In order to obtain a high-quality image that was free from reflections, the camera was fixed to one end of a rectangular lightproof box (length, 0.7 m), whereas the opposite end of the box was fitted against the front surface of the test vessel. The organisms were illuminated from above (light intensity below net cover, ~46,400 lux). To increase the contrast of the illuminated organisms, a black plastic film was taped to the back of the test vessels. The digital camera had the following settings: image resolution 2,048 × 1,536 pixels, shutter speed 1/30 s, aperture F2.8, photosensitivity ISO 400, 3 x optical zoom, and focal depth

in the middle of the test vessel. The photographs were evaluated by an image analysis technique that consisted of two steps. In the first step, *Daphnia* and *Culex* larvae were detected as moving objects during swimming with algorithms adapted from Liess et al. (2006). In the second step, mosquito larvae that had gathered in a motionless state below the water surface for breathing were detected. For a detailed description of the image analysis see Foit et al. (2012).

The Nanocosm system enables a reliable measurement of the abundance and size structure of populations of *Daphnia* and *Culex* larvae. A comparison between true abundance and the abundance monitored by the Nanocosm system showed residual variances of 0.3% for *Daphnia* ($r^2$ = 0.997, n = 13) and 11% for *Culex* larvae ($r^2$ = 0.89, n = 11). For a detailed description of the validation of the image analysis system see Foit et al. (2012).

The biomass of a population is given as the sum of individual dry weights W (µg). These individual dry weights W were calculated on the basis of the detected body lengths L (mm). For *Daphnia*, we used the relationship $W = 1.5\times10^{-8}\ L^{2.84}$ (Dumont et al., 1975); for *Culex* larvae, we used the relationship $W = 4.4\times10^{-3}\ e^{0.8\times L}$ with a defined dry weight of 5.7 µg (larval length of 0.32 mm) as the lower limit and 1.1 g (larval length of 6.8 mm) as the upper limit for individuals. For a detailed description of the calculation of biomass see Foit et al. (2012).

*Emergence of mosquitoes*

The number of emerged adults was estimated by counting through the glass of the vessel. Adult mosquitoes maintained the populations by oviposition of the next generation and died naturally after a few weeks. Due to the oviposition of adults, populations of *Culex* can be maintained without addition of individuals from external sources for many generations. This allows long-term investigations of *Culex* populations for more than 250 days in the Nanocosm system (paper in preparation). The possibility of competition between larvae and adults of *Culex* can be excluded here because the stages use a separate food supply and do not share the same space.

*Exposure to fenvalerate*

We exposed the populations of *Daphnia* and *Culex* larvae to a pulse of fenvalerate. Fenvalerate was used as a surrogate for the relevant pesticide class of pyrethroids. Fenvalerate is known for its high toxicity to *Daphnia* with a published 24-h $LC_{50}$ of 1.8 µg/L (Day and Kaushik, 1987). *Culex* species are known to be slightly less sensitive

(reviewed by Clark and Brooks, 1989). We exposed the populations of *Daphnia* and *Culex* larvae at the following nominal concentrations close to the 24-h $LC_{50}$ for *Daphnia*: control (nine replicates), 0.6 µg/L (ten replicates), 0.8 µg/L (ten replicates), 1 µg/L (ten replicates), and 3 µg/L (three replicates). Similar fenvalerate concentrations (up to 6.2 µg/L) were measured during runoff events in an agricultural headwater stream in Northern Germany (Liess et al., 1999). Fenvalerate [(RS)-cyano-3-phenoxybenzyl (RS)-2-(4-chlorophenyl)-3-methylbutyrate; Chemical Abstract Service no. 51630-58-1], was obtained from Riedel-de Haën [Seelze, Germany; high-performance liquid chromatography (HPLC) technical grade; purity, 99.9%]. Fenvalerate has a solubility in water of < 3 µg/L at 25°C, with a log $K_{OW}$ of 5.01 (Tomlin, 2003). As a consequence, dimethyl sulfoxide (DMSO) was used as a carrier solvent (Merck, Darmstadt, Germany; HPLC technical grade; purity, 99.8%). Aliquots of a stock solution were added directly to the test systems. The maximum amount of DMSO was 0.0025% by volume. The populations of *Daphnia* and *Culex* larvae were not fed on the day of the contamination to reduce sorption of the toxicant to particulate organic matter. Seventy-five percent of the water in all the vessels was replaced with fresh, uncontaminated M7 medium seven days after exposure. Exposure to 3 µg/L fenvalerate resulted in the complete extinction of both populations; hence, these results are not shown here.

*Validation of exposure concentration*

We quantified the actual concentrations of fenvalerate in the culture water 5 minutes after exposure to nominal concentrations of 0.6 µg/L, 0.8 µg/L, 1 µg/L, and 3 µg/L fenvalerate. We took 200-ml samples from three randomly selected replicates per treatment. To improve the detection limit of fenvalerate, the samples from each treatment were pooled. The concentrations of fenvalerate were determined by solid phase extraction of the samples with C18 columns (Baker, Phillipsburg, NJ, USA), followed by elution and quantification in acetonitrile using an HPLC system (PerkinElmer Life and Analytical Sciences, Shelton, USA) that was equipped with a quaternary liquid chromatography (LC) pump, LC autosampler, diode array detector, vacuum degasser, and Peltier column thermostat. The HPLC columns used were CC 250/4 Nucleodur 100-5 $C_{18}$ ec (Machery Nagel, Düren, Germany). The analysis was performed by Kommunale Wasserwerke Leipzig GmbH (Leipzig, Germany). On average, the measured concentrations were 16.8% less than the nominal concentrations and reached 94.7% of the nominal concentration at 3 µg/L, 108.2% at 1 µg/L, 75.1% at 0.8 µg/L, and 54.6% at 0.6 µg/L. Despite deviating measurements at low

concentrations, all exposure concentrations are given as nominal values. A previous investigation with a similar test design demonstrated the temporal reduction of a nominal concentration of 3.2 µg/L to percentages of 77, 26, 18, 11, and 4% at 1, 24, 48, 96, and 144 h after exposure, respectively, (Pieters and Liess, 2006).

*Statistical analyses*

All analyses and construction of plots were conducted with the statistical software R (R Development Core Team, 2010). Functions that were not implemented in the standard packages *base* and *stats* are given in parentheses. A p-value of 0.05 was used to define significance for all statistical analyses.

The abundance and biomass of *Daphnia* and *Culex* larvae are given as a moving average that corresponds to the mean values of three successive observations (interrupted at the time of exposure to fenvalerate). Differences between the means of the control and the exposed treatments were calculated for each sampling day using one-way analysis of variance followed by Dunnett's *post hoc* multiple-comparison test (time-by-time ANOVA, adapted from Diggle et al., 1994; R-package *multcomp*, function *glht*). In the few cases where the conditions of data normality (Shapiro–Wilk test) were violated, differences between means were calculated using the non-parametric Kruskal–Wallis test, followed by a non-parametric multiple-comparison test (R-package *pgirmess*, function *kruskalmc*; Siegel and Castellan, 1988). An observation was regarded as significantly different from the control if an adjacent observation also showed a significant deviation. This requirement reduced the likelihood of a type I error.

The emergence success of *Culex* larvae was calculated by dividing the total number of emerged mosquitoes by the maximal abundance of *Culex* larvae. Data normality and homoscedasticity of the emergence success were violated. As a consequence, we present the values as medians and compared the high cumulative emergence success at 1 µg/L with the pooled emergence success for the other treatments by a non-parametric Wilcoxon test (one-sided).

The short-term effect of fenvalerate on *Daphnia* and *Culex* larvae is given as acute mortality within 48 h after the pesticide pulse. The median lethal concentration ($LC_{50}$) for acute mortality was calculated by fitting a log-logistic function (R-package *drc*, function *drm*). The influence of competition on acute mortality was investigated by analysis of covariance (ANCOVA). We selected (i) acute mortality as a response variable, (ii) the concentration of fenvalerate as a categorical predictor, and

(iii) competitive pressure as a continuous predictor, which was represented by the population biomass of both species one day before exposure. ANCOVA was performed by stepwise model simplification (Crawley, 2005).

Long-term effects of fenvalerate on *Daphnia* and *Culex* larvae were investigated by multiple regression analysis and stepwise model simplification according to Crawley (2005). To avoid multicollinearity, correlating predictor variables were split into different subsets and each subset was used to define a maximal model. *Daphnia* showed, in general, distinct population growth after pulse exposure at the highest concentrations (0.8 and 1 µg/L). For these two concentrations, we selected the population growth of *Daphnia* as a response variable that was given as the percentage increase of population biomass from day 2 to day 28 after the pesticide pulse. The population growth of *Daphnia* was square root-transformed to achieve normality and homoscedasticity of residuals after regression analysis. Negative values were avoided by adding a constant of 40. To explain the recovery of *Daphnia*, the significance of the following predictor variables were tested by multiple regression analysis separately for each species: maximal biomass before exposure, acute mortality during exposure, and weekly mean biomass after exposure.

Distinct long-term effects on *Culex* larvae were observed in terms of emergence success until day 47 after exposure (end of observation period). The emergence success of *Culex* larvae was square root-transformed to achieve normality and homoscedasticity of residuals after regression analysis. To explain the emergence success of *Culex* larvae, the significance of the following predictor variables were tested by multiple regression analysis separately for each species: maximal biomass before exposure, acute mortality during exposure, weekly mean biomass after exposure, and biomass at the time of the first emergence of *Culex* larvae. In five populations, no emergence of mosquitoes occurred (number of replicates without emergence: control, 1; 0.6 µg/L, 2; 0.8 µg/L, 1; 1 µg/L, 1). These replicates were treated as outliers and removed from the regression analysis.

## Results

*Acute mortality of Daphnia and Culex larvae*

Directly after exposure to fenvalerate, the abundance of *Daphnia* decreased in a dose-dependent manner with a median lethal concentration (48-h $LC_{50}$) of 0.9 µg/L (95%

confidence interval, 0.84 to 1 µg/L). *Culex* larvae were slightly but insignificantly less sensitive, with a 48-h $LC_{50}$ of 1.7 µg/L (95% confidence interval, 0.86 to 2.5 µg/L).

For both species, analysis of covariance (ANCOVA) indicated that acute population mortality after two days depended on the exposure to fenvalerate and, in addition, on the population biomass of the respective species one day before exposure. For *Daphnia*, the acute population mortality was influenced strongly by all four concentrations of fenvalerate (explained variance, EV = 71%), and less by the population biomass of *Daphnia* before exposure (EV = 7.6%, ANCOVA, adjusted $r^2$ = 0.79, df = 31, $P < 0.001$). For *Culex* larvae, the acute population mortality was influenced to a minor extent by fenvalerate (EV = 9.9%) and equally influenced by the population biomass of *Culex* larvae before exposure (EV = 11%, ANCOVA, adjusted $r^2$ = 0.21, df = 34, $P < 0.01$). As acute mortality was similar at all concentrations of fenvalerate, the concentration levels were pooled to simplify the model. For both species, acute mortality did not depend on interspecific competition.

*Long-term effects of fenvalerate on Daphnia magna*

After acute mortality, *Daphnia* showed a distinct population growth and the reduced population biomass recovered to control levels by days 9 (0.6 µg/L), 17 (0.8 µg/L), and 31 (1 µg/L, Fig. 1A). We investigated the influence of competition on the population growth of *Daphnia* after exposure to 0.8 and 1 µg/L fenvalerate. The population growth of *Daphnia* is given as the percentage increase in population biomass within 28 days after the pesticide pulse. The period of 28 days was chosen because the greatest variation of population growth between different concentrations was observed at this time. Regression analysis showed that the population growth of *Daphnia* was reduced by interspecific competition with *Culex* larvae. The population growth of *Daphnia* within 28 days after pesticide pulse was best explained by the survival of *Culex* larvae directly after exposure (intercept = 14.5, slope = -0.087, adjusted $r^2$ =0.5, df = 18, $P < 0.001$, Fig. 2). Other variables did not affect the population growth of *Daphnia* and were therefore removed from the regression model according to Crawley (2005).

*Long-term effects of fenvalerate on Culex pipiens molestus*

Populations of *Culex* larvae exposed to fenvalerate showed similar but delayed development in comparison with the control populations (Fig. 1B). At the two highest concentrations of fenvalerate, the pesticide-induced delay of development resulted in higher levels of larval biomass than those in the control from day 21 to day 42.

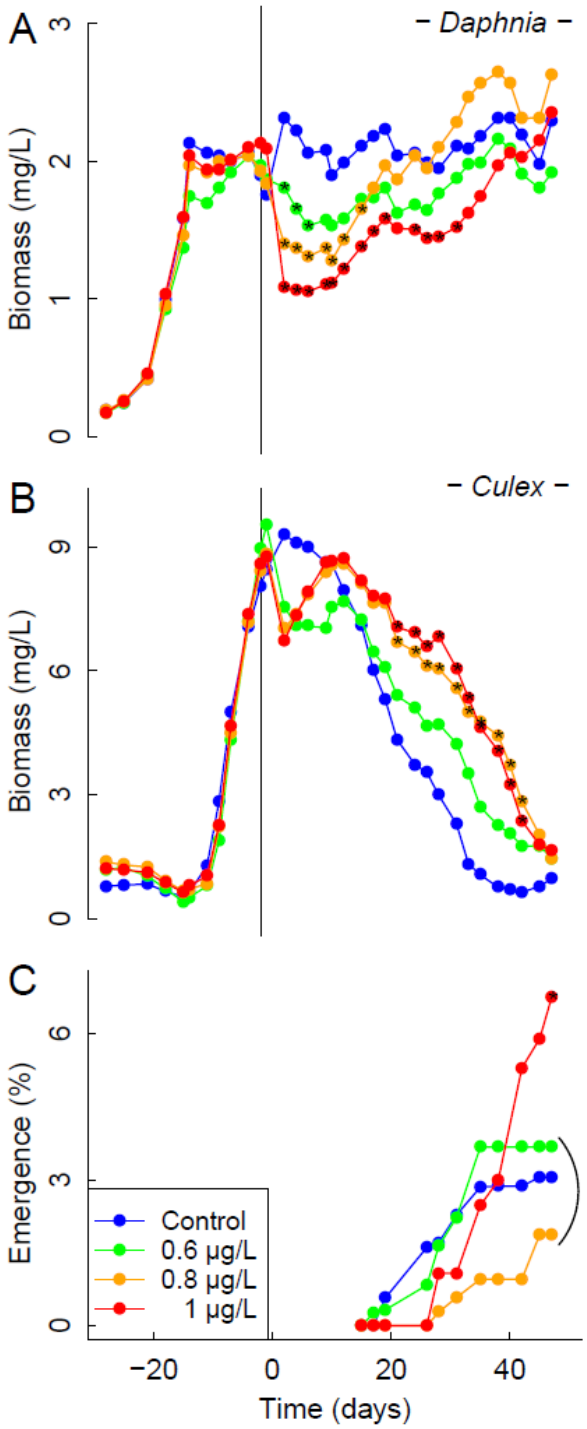


**Fig. 1.** Time courses for the biomass of *Daphnia* (A), biomass of *Culex* larvae (B), and emergence success of *Culex* larvae (C). Short-term exposure to fenvalerate at day 0. Asterisks indicate a significant difference from the control ($p < 0.05$, analysis of variance followed by Dunnett's *post hoc* multiple-comparison test). We compared the particularly high emergence success at 1 µg/L with the low emergence success for the other treatments by a non-parametric Wilcoxon test (one-sided, pooling of treatments is marked by a bracket). Confidence limits and error bars have been omitted for clarity.

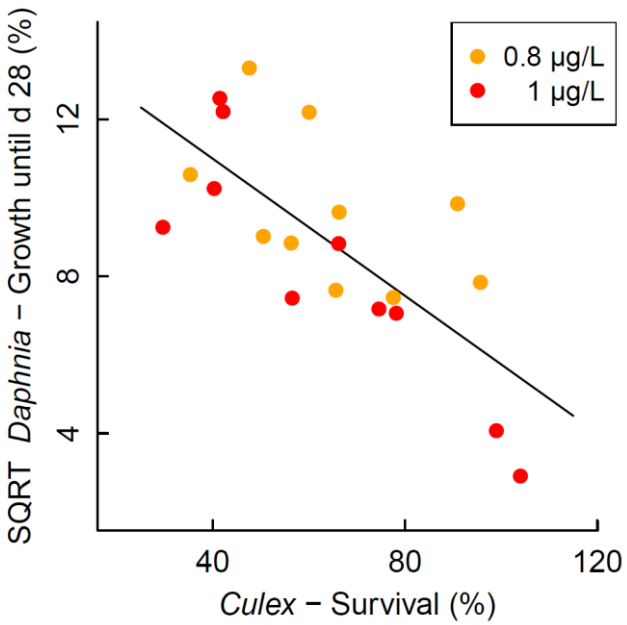


**Fig. 2.** Population growth of *Daphnia* until day 28 after the pesticide pulse as a function of the 48-h survival of *Culex* larvae (intercept = 14.5, slope = -0.087, adjusted $r^2$ =0.5, df = 18, $P < 0.001$). The population growth of *Daphnia* is given as percentage increase of population biomass from day 2 to day 28 after the pesticide pulse. Values of the population growth were square root-transformed to achieve normality and homoscedasticity of residuals after regression analysis.

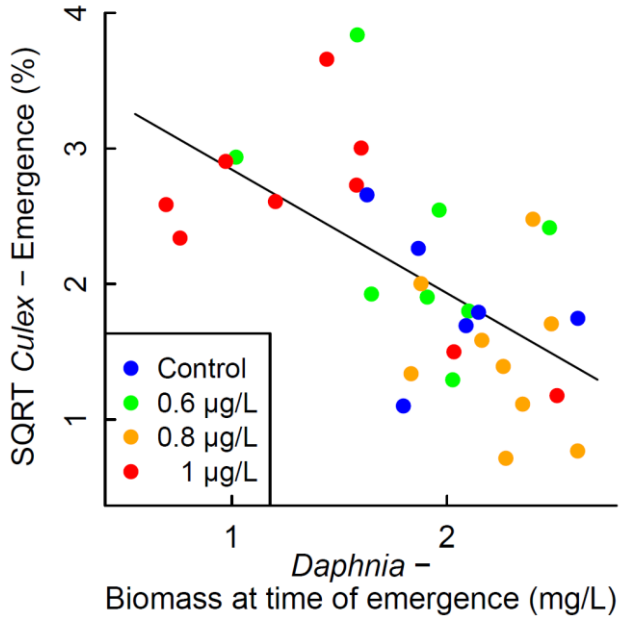


**Fig. 3.** The emergence success of *Culex* larvae at day 47 after the pesticide pulse as a function of the population biomass of *Daphnia* at time of mosquito emergence around day 28 (intercept = 3.76, slope = -0.9, adjusted $r^2$ =0.35, df = 30, $P < 0.001$). The emergence success of *Culex* larvae was square root-transformed to achieve normality and homoscedasticity of residuals after regression analysis.

The emergence success was generally low, with a common median of 2.9% in the control, 0.6 µg/L and 0.8 g/L treatments at the end of the experiment. The emergence success in the 1 µg/L treatment was higher, with a median of 6.7% (Wilcoxon rank sum test, one-sided; control, 0.6 and 0.8 µg/L vs. 1 µg/L, $P = 0.03$, Fig. 1C).

The emergence success of *Culex* larvae was alternatively explained by the mean biomass of *Daphnia* of week four (adjusted $r^2 = 0.24$, $P = 0.003$), five (adjusted $r^2 = 0.25$, $P = 0.002$) and six (adjusted $r^2 = 0.34$, $P < 0.001$) after pesticide pulse. These three weeks span the period of first emergence of *Culex* larvae around day 28. Accordingly, we calculated and regressed the biomass of *Daphnia* at the exact time of the first emergence of *Culex* populations; this variable was found to be the best predictor for the emergence success of *Culex* larvae (intercept = 3.76, slope = -0.9, adjusted $r^2 = 0.35$, df = 30, $P < 0.001$, Fig. 3). Other variables did not affect the emergence success of *Culex* larvae and were therefore removed from the regression model according to Crawley (2005).

## Discussion

*Properties of the Nanocosm test system*

The Nanocosm system allows the investigation of two populations under high stress conditions due to intra- and interspecific interactions. Especially directly after the oviposition of adult mosquitoes, high densities of *Culex* larvae compete for limited food resources. The resulting starvation conditions in the Nanocosm system strongly differ from the usual high food levels of standard test systems (e.g. OECD, 2008; OECD, 2010). Other authors have observed strongly reduced survival and emergence rates of mosquitoes due to intraspecific competition (Agnew et al., 2000; Agnew et al., 2002; Reisen et al., 1984) and interspecific interactions (Duquesne et al., 2011; Stav et al., 2005). In the field, low survival and pupation rates of mosquitoes of around 10% and less were frequently observed (Bradshaw and Holzapfel, 1992; Hawley, 1985; Sunish et al., 2006).

*Direct effects influenced by intraspecific interactions*

Shortly after exposure to fenvalerate, the 48-h $LC_{50}$ values and concentrations that caused total mortality were found to be similar for the two species. For the *Daphnia* populations, the observed 48-h $LC_{50}$ value of 0.9 µg/L corresponded to the published

24-h $LC_{50}$ values of 1.8 to 6.58 µg/L fenvalerate, which were obtained in tests on individuals of *Daphnia magna* of different ages (Day and Kaushik, 1987). The acute sensitivity of the *Culex* populations was slightly but insignificantly lower than that of the *Daphnia* populations with an observed 48-h $LC_{50}$ of 1.7 µg/L. The slightly lower sensitivity of *Culex* larvae was in agreement with the published 24-h $LC_{50}$ values ranging between 3 and 10 µg/L fenvalerate that were established for closely related *Culex* species (reviewed by Clark and Brooks, 1989).

The population biomass directly before exposure increased the acute mortality for both species. This relationship could be revealed due to the high variation of population densities and biomass directly before exposure. We explain this negative effect of population biomass on the sensitivity of species by intraspecific competition. Similar increases in toxicant sensitivity due to strong intraspecific interactions were also observed for populations of *Limnephilus lunatus* Curtis that were exposed to fenvalerate (Liess, 2002) and *Daphnia* spp. that were exposed to esfenvalerate (Knillmann et al., 2012). The negative influence of intraspecific competition on toxicant sensitivity can be explained by a reduced food supply and thus a higher sensitivity to fenvalerate, as determined by single-species tests on *Daphnia magna* (Pieters et al., 2005).

As an additional direct effect, we observed a delayed development of *Culex* larvae resulting in higher biomass values compared to the control. A similar delayed development was also observed for species like the grass shrimp *Palaemonetes pugio* (McKenney and Hamaker, 1984), the caddisfly *Limnephilus lunatus* (Liess and Schulz, 1996), and *Daphnia magna* (Pieters et al., 2005). This effect has been explained by a sustained inhibition of food uptake after exposure to fenvalerate (Day and Kaushik, 1987; Reynaldi et al., 2006).

*Indirect Effects based on interspecific interactions*

Several weeks after exposure, the slight differences in sensitivity between the two species were amplified to contrasting long-term effects: *Daphnia* populations recovered slowly whereas *Culex* larvae showed increased success of emergence. We explain the contrasting developments of the two species by interspecific interaction as follows. The recovery of the *Daphnia* population was delayed by the presence of competing *Culex* larvae. After pulse exposure to 1 µg/L fenvalerate, *Daphnia* populations took 31 days to recover. This recovery time was twice as long as observed for isolated populations of *Daphnia magna* in Nanocosm systems (Liess and Foit, 2010; Liess et al., 2006), or modelled by Barnthouse (2004). The reason for this discrepancy is that, in isolated

populations without interspecific competition, toxicant-induced mortality generally improves the food supply of survivors, which leads to a rapid recovery of populations. Such positive effects of toxicant-induced mortality on the subsequent development of populations have often been observed (Beketov and Liess, 2005; Forbes et al., 2003; Liess, 2002; Linke-Gamenick et al., 1999; Moe et al., 2002; Muturi et al., 2010; Postma et al., 1994). However, to the authors' knowledge, the recovery of populations in the context of permanent high competitive pressure has not yet been investigated. On the basis of the results from our two-species system, we argue that populations within a community context recover more slowly than isolated populations if the reduction in intraspecific competition is replaced by interspecific competition with other species that are less affected or recover more rapidly.

*Culex* larvae profited from the slow recovery of *Daphnia* and showed an increased success of emergence. At all concentrations of fenvalerate, the emergence success of *Culex* larvae increased with a decreasing biomass of *Daphnia* during the first week of emergence. The initial small difference in sensitivity to the toxicant developed into an unexpected long-term benefit for *Culex* larvae due to interspecific interactions.

In the field, survival and emergence rates are usually low (Bradshaw and Holzapfel, 1992; Hawley, 1985; Sunish et al., 2006). We therefore argue that a toxicant-induced increase of emergence rates by a factor of two is highly relevant for the development of populations in the field.

We conclude that competition influences toxicant sensitivity and population recovery within the context of a community. Given that, in nature, populations typically experience competition, we recommend the use of test systems that include competing species to predict natural recovery rates. This factor also needs to be considered in the emerging field of ecotoxicological modelling.

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# Chapter 4
# Short-term disturbance of a grazer has long-term effects on bacterial communities – Relevance of trophic interactions for recovery from pesticide effects

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## Abstract

Little is known about the transfer of pesticide effects from higher trophic levels to bacterial communities by grazing. We investigated the effects of pulse exposure to the pyrethroid fenvalerate on a grazer-prey system that comprised populations of *Daphnia magna* and bacterial communities. We observed the abundance and population size structure of *D. magna* by image analysis. Aquatic bacteria were monitored with regard to abundance (by cell staining) and community structure (by a 16S ribosomal RNA fingerprinting method). Shortly after exposure (2 days), the abundance of *D. magna* decreased. In contrast, the abundance of bacteria increased; in particular fast-growing bacteria proliferated, which changed the bacterial community structure. Long after pulse exposure (26 days), the size structure of *D. magna* was still affected and dominated by a cohort of small individuals. This cohort of small *D. magna* grazed actively on bacteria, which resulted in low bacterial abundance and low percentage of fast-growing bacteria. We identified grazing pressure as an important mediator for translating long-term pesticide effects from a grazer population on its prey. Hence, bacterial communities are potentially affected throughout the period that their grazers show pesticide effects concerning abundance or population size structure. Owing to interspecific interactions, the recovery of one species can only be assessed by considering its community context.

## Introduction

Pesticides are widely used in agriculture and can have negative effects on non-target organisms such as bacterial communities. Investigations of pesticide effects on bacteria have dealt mainly with the short-term disturbance of endpoints, such as growth, enzyme

activity, species diversity, and community structure (DeLorenzo et al., 2001; Widenfalk et al., 2008; Cycon and Piotrowska-Seget, 2009; Lew et al., 2009). However, little is known about the long-term effects of pesticides on bacteria, including those on trophic interactions in aquatic ecosystems.

Aquatic bacteria are known to serve as an important food source for a wide range of grazers (Hall and Meyer, 1998; Langenheder and Jurgens, 2001). Despite the importance of such grazer-prey relationships, indirect pesticide effects between grazers and bacterial communities have rarely been studied. Friberg-Jensen et al. (2003) have shown that bacteria proliferated when the abundance of grazing crustaceans was seriously reduced by an application of cypermethrin. In addition, low bacterial abundances were observed in association with high densities of ciliates and small flagellates 45 h after exposure to atrazine (DeLorenzo et al., 1999a). The aforementioned studies focussed on short-term effects of pesticides. Populations of grazers and prey were thereby monitored by integrating endpoints. Integrating endpoints are simple measures that ignore the structure of a system, such as the total abundance or biomass of a population system (Liess and Foit, 2010). These integrating endpoints were often found to recover within one or two generation times after exposure (Sherratt et al., 1999; Barnthouse, 2004). In contrast, longer recovery times were observed repeatedly for differentiating endpoints that focus on the structure of a system. For example, long recovery times were observed for the disturbed size structure of population systems (Driskell et al., 2001; Johnston and Keough, 2005) and species composition of community systems (Liess et al., 2008; Pesce et al., 2008).

We investigated the long-term effects of pesticides on bacterial communities in the presence of trophic interactions. For this investigation, we exposed a grazer-prey system, which comprised the cladoceran *Daphnia magna* and bacterial communities, to pulses of the pyrethroid fenvalerate. We monitored two differentiating endpoints, the population size structure of *D. magna* and the community structure of bacteria, in order to focus on the long-term effects of a pesticide pulse on the grazer and its prey.

## Methods

*Test system*

Populations of *D. magna* and bacterial communities were cultured in cylindrical glass vessels (Harzkristall, Derenburg, Germany) that contained 4.5 L of Elendt M7 medium

(OECD, 1997). The communities were initiated with 30 neonates of the grazer *D. magna* 37 days before disturbance. *D. magna*, clone B (Bayer, Monheim, Germany), were fed daily with a suspension of batch-cultured green algae (*Desmodesmus subspicatus*). The quantity of food given daily was $1.1 \times 10^5$ cells/mL (0.45 mg C/L). The bacterial cell density in this non-sterile open system accounted for $0.4 \times 10^6$ cells/L (standard error = $0.024 \times 10^6$ cells/L, n = 20; one day before disturbance). The base of each glass vessel contained 500 g of washed aquarium gravel (diameter, 1–2 mm) as a support for the bacteria to promote self-purification of the test system. A clear glass plate was placed on top of each vessel to prevent excessive evaporation. The studies were performed at 20°C. The photoperiod was controlled (16:8 h light:dark), and lighting was provided by a 70 W, cool-white fluorescent tube situated 10 cm above the test vessels. The water used was aerated three times a day for 15 min via silicone tubing (immersed 14 cm below the surface of the water; diameter, 4 mm; tapered ending, 0.5 mm). Every week, 75% of the water in the test vessels was replaced via silicone tubing capped with a 200 μm nylon mesh to prevent the loss of daphnids. To minimize a possible effect of water replacement, bacterial communities were in general monitored after a minimum recovery time of 4 days after water change. A detailed description of the test system can be found in Liess and Foit (2010).

*Short-term disturbance*

The grazer-prey systems of *D. magna* and bacterial communities were exposed to pulses of the pyrethroid fenvalerate at the following nominal concentrations: 0 μg/L (control; six replicates), 0.8 μg/L (five replicates), 1 μg/L (four replicates), and 3 μg/L (three replicates). The measured exposure concentrations were, on average, 22% less than the nominal concentrations and were given as nominal values. On day 7 after exposure, we replaced 80% of the water with fresh, uncontaminated Elendt M7 medium. A previous investigation with an identical test design demonstrated a temporary reduction of the nominal concentration of 3.2 μg/L fenvalerate to 77, 26, 18, 11, and 4% at 1, 24, 48, 96, and 144 h after exposure, respectively (Liess et al., 2006). An additional treatment (five replicates) did not involve exposure to fenvalerate but rather consisted of a mechanical removal of *D. magna* at the same time when fenvalerate was applied in the other treatments. This treatment allowed us to observe the effects caused by a reduction in the density of daphnids in the absence of any delayed effects of the pesticide fenvalerate. For this purpose, 50% of the population of *D. magna* were mechanically removed, killed by boiling in water, and then returned to the test systems to mimic conditions of

pesticide exposure. This treatment is referred to as mechanical$_{50\%}$. The treatments mechanical$_{50\%}$ and 1 µg/L fenvalerate had similar acute effects on the abundance of *D. magna*. This similarity in the short-term effects of the two treatments allowed a direct comparison of the following recovery processes. A detailed description of the short-term disturbance of *D. magna* can be found in Liess and Foit (2010).

*Monitoring of D. magna populations*

The abundance and size structure of *D. magna* were recorded three times a week by image analysis. Algorithms for automatic detection were implemented in the public domain software *ImageJ* (Rasband, 1997-2009). In contrast to traditional subsampling, image analysis is non-invasive, quick, and enables frequent recording of whole populations. Individuals of *D. magna* were divided into three classes in terms of body size: neonates and juveniles (size class I, 0.8–2.3 mm), small adults (size class II, 2.3–2.8 mm), and large adults (size class III, > 2.8 mm). Comparison of the results of the image analysis with manual measurements of abundance and body size showed a high degree of agreement between the methods. Correlations for abundance were determined as follows: log image analysis counts = 0.14 + 0.91 log manual counts ($r^2$ = 0.999, $p < 0.001$). Correlations for body size were determined as follows: log image surface area = 1.65 + 1.77 log manual length ($r^2$ = 0.991, $p < 0.001$) (Liess et al., 2006). The total biomass of populations and that of each of the different size classes were calculated as the sum of individual dry weights (W; µg), which were estimated on the basis of detected body lengths (L; mm). For *D. magna* we used the relationship $W = 1.5 \times 10^{-8} L^{2.84}$ (Dumont et al., 1975). A detailed description of the image analysis of *D. magna* can be found in Liess et al. (2006).

*Monitoring of bacterial abundance*

The total abundance of bacteria was monitored on days -1, 2, 5, 13, and 26 after disturbance by image analysis of stained bacteria cells. The water in all the test vessels was sampled. A volume of 10 mL was filtered through an isopore polycarbonate filter with pores that measured 0.2 µm in diameter (Millipore, Schwalbach, Germany). We used black filter paper as a dark background to improve the clarity of the images of bright cells. Samples were fixed with paraformaldehyde (4% final concentration) and stained with 4'-6-Diamidino-2-phenylindole (DAPI; 2 µg/mL for 5 min in the dark). We randomly photographed ten spots per filter paper. Photos were taken using a Sony 3 CCD camera coupled to an Axioskop fluorescence microscope (Zeiss, Göttingen,

Germany). Each image showed an area of the filter paper equivalent to 0.003 $mm^2$ (63 x 47 µm). The selected sample volume of 10 mL in combination with an average concentration of bacteria of 500–1000 cells/mL and an effective filter diameter of 41 mm minimised the overlap of cells in the images.

The bacterial cells were detected automatically using *ImageJ* (Rasband, 1997-2009). The bright bacterial cells were separated from the dark background of the filter paper by applying an adaptive threshold algorithm. The adaptive threshold was conducted by subtracting a smoothed version of the image (mean convolution filter, radius = 7) from the original image; relevant details are subsequently detected by setting a threshold (greyscale difference > 8). Objects that were in contact were separated by watershedding algorithms. The bacterial cells that were detected were counted automatically ($Cells_{Detected}$). Comparison of the results obtained by image analysis with manual counts of cell densities ($Cells_{Counted}$) for 50 randomly selected images showed a linear relationship and high degree of agreement between the two methods. The regression equation was $Cells_{Counted} = 1.01 \times Cells_{Detected}$ ($r^2 = 0.9998$, $p < 0.001$). To calculate the bacterial abundance of one sample, we averaged the detected cell densities of the ten photos that had been taken of each filter paper. In addition, we measured the length of the first five DAPI-stained cells in the upper left corner of each image and determined the mean cell size to be 0.6 µm in length (standard deviation ± 0.28 µm, n = 250).

*Monitoring of bacterial community composition*

The community structure was monitored by terminal restriction fragment length polymorphism (T-RFLP) analysis on days -1, 2, 5, 13, and 26 after disturbance. DNA was extracted as follows (Maher et al., 2001): filters were incubated overnight at 55°C with 3 mL of lysis buffer (150 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5% SDS, 0.4 mg/mL proteinase K), extracted subsequently with phenol–chloroform (1:1), and precipitated with ethanol, sodium-acetate (pH 5.2, 0.3% final concentration), and 6 µL of glycogene. Finally, DNA pellets were dissolved overnight in 30 µl of sterilized destilled water. Bacterial 16S rRNA genes were amplified by PCR using the bacteria-specific primers 27F and 1492R (Lane, 1991). The forward primer was labelled at the 5′ end with phosphoramidite fluorochrome 5-carboxyfluorescein. Purified PCR products (20 ng) were digested overnight with 10 U of MspI and loaded into an ABI3100 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany). T-RFLP profiles were analysed using GeneScan V 3.1 software (Applied Biosystems, Darmstadt, Germany).

Normalisation and definition of the signal threshold of the T-RFLP profiles, in addition to generation of a matrix with relative peak abundance for further statistical analysis, were performed as described in Wu et al. (2009) using the statistical software R (R Development Core Team, 2008). Functions that implement the procedure for identifying the "true" peaks and binning the different fragment lengths are available at http://www.webpages.uidaho.edu/~joyce/Lab%20page/TRFLP-STATS.html (Abdo et al., 2006).

*Statistical analysis*

All analyses and construction of plots were conducted with the statistical software R (R Development Core Team, 2008). Functions that were not implemented in the standard packages *base* and *stats* are given in parentheses in the following. A level of 0.05 was used to define significance for statistical analyses unless otherwise stated.

The biomass and abundance of *D. magna* is given as a moving average with the mean value of three successive observations. For the time courses of *D. magna* and bacteria (biomass and abundance), the differences between the means of the control and disturbance treatments were calculated using one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* multiple-comparison test (R-package *multcomp*, function *glht*). In the few cases where the conditions of data normality (Shapiro–Wilk test) and homoscedasticity (Levene's test) were violated, differences between means were calculated using the nonparametric Kruskal–Wallis test, followed by a non-parametric multiple-comparison test (R-package *pgirmess*, function *kruskalmc*; Siegel and Castellan, 1988).

We used multiple regression analysis to identify significant relationships between bacterial abundance and predicting variables related to the populations of *D. magna*. The bacterial abundance was square-rooted to achieve normality and homoscedasticity of residuals after regression analysis. As predictors we selected the total abundance and biomass of *D. magna* and the abundance of each size class (see the section entitled *Monitoring of D. magna populations*). As a further predictor, we calculated the daily loss of total biomass due to the mortality of *D. magna*. Multiple regression analysis was performed by stepwise model simplification (Crawley, 2005). The effect of pesticide exposure on bacterial abundance was investigated subsequently by analysis of covariance. This was done by adding a new factor to the regression model with the two levels of non-toxic (control and treatment $\text{Mechanical}_{50\%}$) and toxic disturbance

(exposure to 0.8 and 1 µg/L fenvalerate). The analysis of covariance was performed by stepwise model simplification (Crawley, 2005).

Information about the structure of bacterial communities was obtained from T-RFLP profiles. T-RFLP profiles consist of T-RFs (terminal restriction fragments; peaks). T-RFs are quantified in terms of their relative abundance and can be regarded as surrogates for the bacterial phylotypes present in a sample.

In an initial conventional analysis of the structures of bacterial communities, we ordinated the T-RFLP profiles by non-metric multidimensional scaling (NMS; R-package *vegan*, function *metaMDS*; Minchin, 1987). We thereby ordinated mean community profiles, that is, profiles with the averaged relative abundance of T-RFs, for a particular sampling day and treatment. By using the Bray-Curtis coefficient as dissimilarity index, we attained a two-dimensional configuration with a final Kruskal's stress of 13.8%. The NMS ordination allowed a graphical separation of T-RFLP profiles into distinct groups. To confirm the differences in community structures among the identified groups, we conducted pairwise comparisons by multi-response permutation procedures (MRPP; R-package *vegan*, function *mrpp*; Mielke et al., 1976; Mielke and Berry 2001). A level of 0.0083 was used to define significance (Bonferroni correction in case of four pairwise comparisons).

To identify long-term effects better, we developed a second analysis for the structures of bacterial communities. We grouped bacteria according to their response to disturbance. The first group consisted of those bacteria (i.e. T-RFs) that proliferated rapidly two days after mechanical disturbance (group $Bacteria_{Mechanical}$); the second group consisted of those bacteria (i.e. T-RFs) that proliferated rapidly after pesticide exposure (group $Bacteria_{Pesticide}$). Rapidly proliferating T-RFs were identified by comparing T-RFLP profiles of day -1 and day 2 after disturbance by indicator species analysis (R-package *labdsv*, function *duleg*; Dufrene and Legendre, 1997). We accepted T-RFs with significance values < 0.3 as rapidly proliferating bacteria to increase the number of T-RFs for grouping. $Bacteria_{Mechanical}$ and $Bacteria_{Pesticide}$ are given with the summed relative abundances of identified T-RFs. We compared the relative abundances of the groups $Bacteria_{Mechanical}$ and $Bacteria_{Pesticide}$ for different treatments by one-way ANOVA and stepwise model simplification. Model simplification was performed by aggregating factor levels that did not differ significantly from each other (Crawley, 2005). In cases where more than two factor levels remained after model simplification,

we performed *post hoc* multiple-comparison tests to assess differences from the control (see above: multiple-comparison test of bacterial abundance).

## Results

*Population dynamics of D. magna and bacterial abundance after disturbance*

Directly after disturbance, the biomass of *D. magna* decreased significantly for all treatments and recovered by day 14 (0.8 µg/L treatment) and day 17 (1 µg/L and mechanical$_{50\%}$ treatments), respectively (Fig. 1A). In contrast, the size structure of populations differed from that of the control for about one month (Fig. 1 B; Liess and Foit, 2010); in this study we only present the abundance of juvenile *D. magna* (neonates and juveniles; Fig. 1C). After initial mortality, the populations that underwent the 1 µg/L and mechanical$_{50\%}$ treatments were dominated by cohorts of juveniles – the abundance of juvenile *D. magna* increased to values significantly above the control level until day 32.

Bacterial abundance showed the opposite trend over time (Fig. 1D). The bacterial abundance in the 1 µg/L treatment had increased above the control level on day 2 and reached control level by day 5 after exposure. The bacterial abundance in the mechanical$_{50\%}$ treatment showed a similar but nonsignificant increase compared with the control level. Subsequently, the bacterial abundance for both treatments (1 µg/L and mechanical$_{50\%}$) decreased and reached levels below that of the control on day 26 after disturbance.

Multiple regression analysis showed a significant relationship (df = 97, adjusted $r^2 = 0.56$, $p < 0.001$) between bacterial abundance and two variables related to the populations of *D. magna*. The abundance of bacteria was affected most strongly by the abundance of juvenile *D. magna* (partial regression coefficient, $\beta = -0.15$; standard error, SE = 0.017; explained variance, EV = 43.8%, $p < 0.001$) and was also affected by the daily loss of total biomass due to the mortality of *D. magna* ($\beta = 0.006$, SE = 0.0024, EV = 12.1%, $p = 0.0086$). The abundance of bacteria was not affected by exposure to fenvalerate. This was analyzed by adding a factor of disturbance (toxic or non-toxic disturbance) to the regression model. This factor had no significant effect on bacterial abundance (difference of intercepts, $p = 0.22$; differences of slopes, $p > 0.15$).

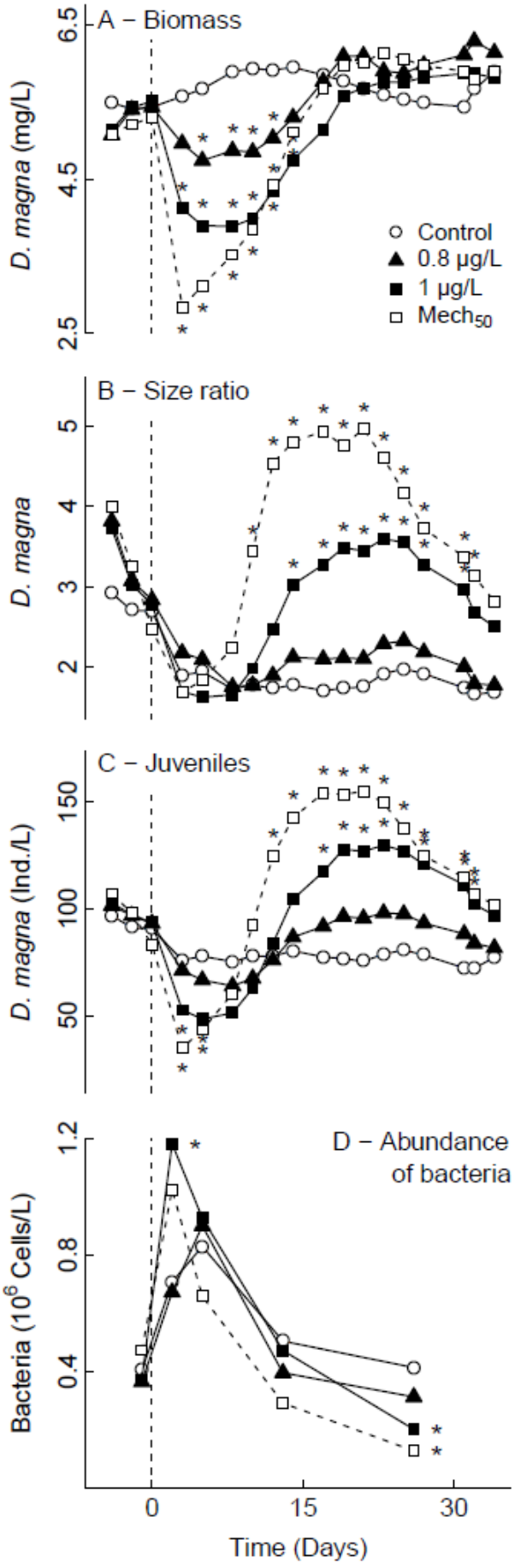


**Fig. 1.** Time courses of total biomass of *D. magna* (A), size ratio of juveniles /adults of *D. magna* (B), abundance of juvenile *D. magna* (C), and abundance of bacteria (D). Short-term disturbance on day 0 (dashed line). Asterisks indicate significant difference from the control ($p < 0.05$, analysis of variance followed by Dunnett's *post hoc* multiple-comparison test). Confidence intervals have been omitted for clarity.

*Community structure of bacteria after disturbance*

In an initial conventional analysis, we ordinated mean bacterial community profiles by non-metric multidimensional scaling (NMS; Fig. 2). The NMS ordination allowed us to identify four distinct and meaningful groups of community profiles (Table 1). We confirmed that the differences between the groups of community profiles were significant by pairwise comparisons: Group *Undisturbed* vs. *Pesticide* ($p = 0.001$), Group *Undisturbed* vs. *Mechanical* ($p = 0.002$), Group *Undisturbed* vs. *Recovery* ($p = 0.001$), and Group *Pesticide* vs. *Mechanical* ($p = 0.012$). No significant differences were found within groups with respect to different treatments.

**Table 1:** Grouping of bacterial community profiles. Roman numerals refer to the grouping shown in Figure 2; N = number of community profiles (T-RFLP profiles) for pairwise comparisons.

| | **Group** | **n** | **Description** |
|---|---|---|---|
| I | *Undisturbed* | 45 | Community profiles before disturbance and community profiles of the control two days after disturbance. |
| IIa | *Pesticide* | 18 | Community profiles of the 0.8 and 1 µg/L treatment two days after fenvalerate application. |
| IIb | *Mechanical* | 3 | Community profiles of the mechanical$_{50\%}$ treatment two days after mechanical removal. |
| III | *Recovery* | 12 | Community profiles 26 days after disturbance. |

In a second analysis of bacterial community structures, we grouped bacterial phylotypes (i.e. T-RFs) according to their response to disturbance. We identified and grouped nine T-RFs that proliferated rapidly after mechanical disturbance (Bacteria$_{Mechanical}$ group) and 13 T-RFs that proliferated rapidly after exposure to pesticide (Bacteria$_{Pesticide}$ group). From the 16 T-RFs identified in total, only three T-RFs were represented in both groups (Bacteria$_{Mechanical}$ and Bacteria$_{Pesticide}$).

Bacteria$_{Mechanical}$ and Bacteria$_{Pesticide}$ are presented with the summed relative abundance of identified and grouped T-RFs; these levels are calculated separately for individual sampling days and treatments (Fig 3). Standard errors (SE) and the p-values of

significant differences from the control level after model simplification are given in parentheses. The relative abundance of the $Bacteria_{Mechanical}$ group is shown in Fig. 3A. Two days after disturbance, the $Bacteria_{Mechanical}$ group reached a relative abundance of 23% in the control treatment (± SE, 4.4%), 38% in the pesticide treatments (± 2.7%, $p = 0.019$), and 55% in the $mechanical_{50\%}$ treatment (± 2.9%, $p < 0.001$). On day 26 after disturbance, the $Bacteria_{Mechanical}$ group reached a relative abundance of 43% in the control and 0.8 µg/L treatments (± 1.4%), and 34% in the 1 µg/L and $mechanical_{50\%}$ treatments (± 3.1%, $p = 0.029$). The relative abundance of the $Bacteria_{Pesticide}$ group is shown in Fig. 3B. Two days after disturbance, the $Bacteria_{Pesticide}$ group reached a relative abundance of 39% in the control (± 4.1%), 54% in the 0.8 µg/L treatment (± 2.8%, $p = 0.017$), 63% in the 1 µg/L treatment (± 3.1%, $p < 0.001$), and 23% in the $mechanical_{50\%}$ treatment (± 0.7%, $p = 0.01$). On day 26 after disturbance, the $Bacteria_{Pesticide}$ group reached a mean relative abundance of 29% in the four treatments (± 3.2%).

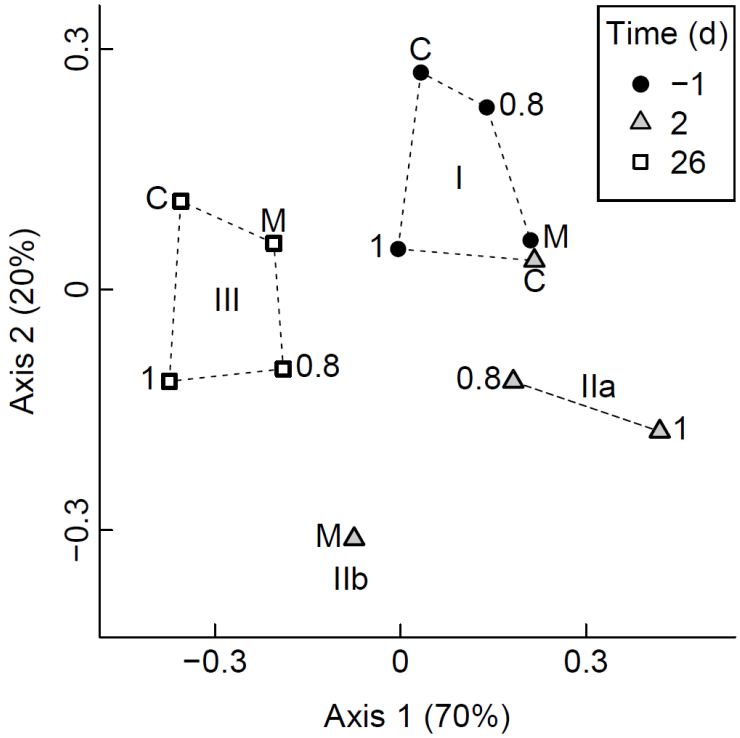


**Fig. 2.** Non-metric multidimensional scaling (NMS) of mean bacterial community profiles. Treatments are labelled as C (Control), 0.8 and 1 (exposure to fenvalerate in µg/L), and M (mechanical reduction in *D. magna* density by 50%). Community profiles were sampled one day before (black circles), 2 days after (grey triangles), and 26 days after (white squares) short-term disturbance. Broken lines and Roman numerals mark four distinct groups of community profiles (cf. Table 1). The variation represented by each axis (%) is shown in parentheses.

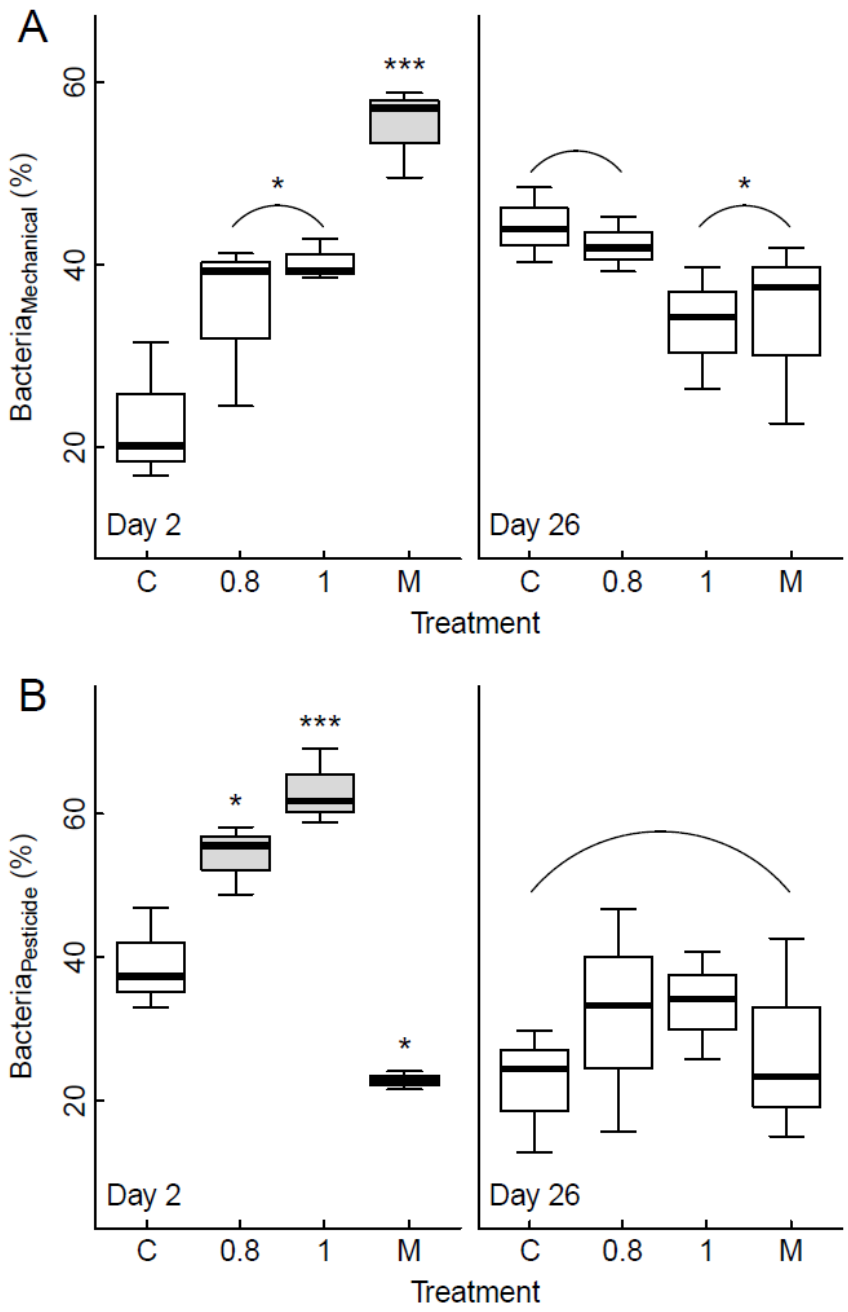


**Fig. 3.** Relative abundance of fast-growing bacteria that proliferated directly after mechanical disturbance ($Bacteria_{Mechanical}$, Fig. 3A) or exposure to pesticide ($Bacteria_{Pesticide}$, Fig. 3B) in the treatments C (Control), 0.8 and 1 (exposure to fenvalerate in µg/L), and M (mechanical reduction in *D. magna* density by 50%). Proliferating bacteria (i.e. T-RFs) in respective treatments (grey shaded boxes) were identified by indicator species analysis (Dufrene and Legendre, 1997). The horizontal line indicates the median, the box covers the 25–75% percentiles and the maximum length of each whisker is 1.5 times the interquartile range. Arcs indicate the pooling of factor levels for model simplification. Asterisks indicate significant differences from the control group (Significance codes: 0 < *** < 0.001 < ** < 0.01 < * < 0.05; analysis of variance followed by Dunnett's *post hoc* multiple-comparison test).

## Discussion

*Trophic interactions between bacteria and D. magna*

Multiple regression analysis revealed that juvenile *D. magna* were the most efficient grazers on bacteria compared to other size-classes. The bacteria in our test systems were small (0.6 µm in length). Active grazing of small-sized *D. magna* on small bacteria has also been identified in previous feeding experiments (Brendelberger, 1991). A significant effect of grazing pressure due to nanoplanktonic bacterivores (flagellates and ciliates) can be ruled out in this study. Nanoplanktonic predators were only rarely observed, which can be explained by the severe top-down control due to high densities of *D. magna* (Jürgens, 1994).

*Short-term disturbance of abundance and community structure of bacteria*

Two days after disturbance, high levels of mortality of *D. magna* were accompanied by a short-term proliferation of bacterial cells. According to the results of the multiple regression analysis, bacteria benefited from the mortality of *D. magna* by a reduced grazing pressure and decomposition of dead biomass. After exposure to fenvalerate, the proliferation of bacteria was even greater than that after mechanical disturbance. We explain this by an additional indirect effect of fenvalerate. Fenvalerate is known to inhibit the uptake of food by *D. magna* for several days (Day and Kaushik, 1987; Reynaldi et al., 2006). We propose that inhibition of the food uptake by surviving *D. magna* reduced the grazing pressure on bacteria further and was responsible for the comparatively higher bacterial abundance observed after pesticide exposure. Similar short-term effects of pesticides on bacterial abundance have been observed repeatedly in natural communities (DeLorenzo et al., 1999b; Friberg-Jensen et al., 2003).

The short-term proliferation of bacteria is associated with changes in community structure. According to the NMS ordination, community profiles obtained after exposure to pesticide differed significantly from profiles obtained after mechanical disturbance. The grouping of bacteria according to their response to disturbance (see Methods) revealed the following insights. Two days after disturbance, some bacteria (i.e. T-RFs) increased in relative abundance. These fast-growing bacteria clearly profited from the reduced grazing pressure. A similar proliferation of potential r-strategists after disturbance has been observed repeatedly in previous studies (Haybach et al., 2004; Pesce et al., 2008; Cycon and Piotrowska-Seget, 2009). Interestingly, the composition of bacteria (i.e. T-RFs) that grew rapidly after mechanical disturbance

($Bacteria_{Mechanical}$) differed from that of bacteria that grew rapidly after pesticide exposure ($Bacteria_{Pesticide}$). This can only be explained by direct effects of fenvalerate. Direct effects of fenvalerate on the cell functions and community structure of bacteria have been observed repeatedly (Das and Mukherjee, 1998; Luo et al., 2004; Pham et al., 2004).

*Long-term disturbance of abundance and community structure of bacteria*

At 26 days after disturbance, the abundance of bacteria in the 1 µg/L and $mechanical_{50\%}$ treatments was still below that in the control. This can be explained by the unrecovered size structure of *D. magna* - cohorts of juveniles that still dominated the disturbed populations and grazed actively on bacteria. For detailed information on the mechanisms of recovery of *D. magna,* see Liess and Foit (2010).

According to the NMS ordination, the structures of the bacterial communities had become similar by day 26. In contrast, the grouping of bacteria (i.e. T-RFs) on their response to disturbance revealed the presence of indirect long-term effects on the structures of the bacterial communities: in the 1 µg/L and $mechanical_{50\%}$ treatments, we observed a low relative abundance of the group $Bacteria_{Mechanical}$. Hence, the high abundance of juvenile *D. magna* reduced both the total bacterial abundance and the relative abundance of fast-growing bacteria. A similar long-term effect on the trait group $Bacteria_{Pesticide}$ was not detected.

*Conclusion*

The unrecovered size structure of grazing *D. magna* provoked a long-term disturbance of abundance and community structure of bacteria. This result highlights two important aspects of effect assessment. Firstly, we revealed that grazing pressure is an important mediator of pesticide effects between trophic levels. Secondly, it was not the total biomass of a grazer but rather its unrecovered size structure that gave rise to the observed long-term effects on bacterial communities. This result contributes to the general debate about assessment endpoints (Giddings et al., 2001; Liess et al., 2005). The recovery of a system is usually predicted by integrating endpoints, such as total abundance or biomass. We have demonstrated that such endpoints may be insufficient to describe recovery within our model system. The differentiating endpoint of the population structure of *D. magna* was most appropriate for explaining the long-term pesticide effects on bacterial communities.

**Aknowlegdements**

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# Lebenslauf

**Persönliche Daten**

| | |
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**Studium und Promotion**

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| seit 01/2005 | Doktorandenstelle am Helmholtz-Zentrum für Umweltforschung in Leipzig, Department System-Ökotoxikologie. |
| 10/1998 – 06/2004 | Diplomstudiengang Geoökologie an der Technischen Universität Braunschweig (Gesamtnote: 1,3).<br>Thema der Diplomarbeit: Mathematische Modellierung der Ausbreitung genetischer Informationen durch Pollentransport (Note: 1,3). |

**Tätigkeiten im Steuerfach**

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**Schule**

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| 07/1994 | Abitur am Gymnasium Lehrte |
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## Weitere Veröffentlichungen (peer-reviewed)

Kattwinkel, M., Kuehne, J.V., Foit, K., Liess, M., 2011. Climate change, agricultural insecticide exposure, and risk for freshwater communities. Ecol. Appl. 21, 2068–2081.

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# Erklärung

Hiermit erkläre ich an Eides statt, dass ich meine Arbeit selbstständig und ohne fremde Hilfe verfasst habe, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Ich erkläre ebenfalls an Eides statt, dass ich keine vergeblichen Promotionsversuche unternommen habe und die Dissertation weder in der gegenwärtigen noch in einer anderen Fassung bereits einer anderen Fakultät vorgelegen hat.

Kaarina Foit
Leipzig, 4.11.2012