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**The influence of the environmental context on the sensitivity
of freshwater zooplankton communities to pesticides**

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THE INFLUENCE OF ENVIRONMENTAL CONTEXT ON THE SENSITIVITY OF
FRESHWATER ZOOPLANKTON COMMUNITIES TO PESTICIDES

by

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"The first law of ecology is that everything is related to everything else." (Barry Commoner, American Scientist)

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List of Abbreviations

| | |
|------------|--|
| ANCOVA | Analysis of covariance |
| ANOVA | Analysis of variance |
| b_k | Species weight (PRC) |
| CAS RN | Chemical Abstract Service Registry Number |
| C_{dt} | Regression coefficient (PRC) |
| Chl a | Chlorophyll a |
| DMSO | Dimethylsulfoxide |
| DT50 | Half life |
| ECOLINK | Germany-Russian research initiative on understanding effects of environmental toxicants at population and community levels |
| EC $_{50}$ | Median effective concentration |
| GC-MS | Gas chromatography – mass spectrometry |
| IPCC | Intergovernmental Panel on Climate Change |
| K $_{oc}$ | Soil organic carbon-water partition coefficient |
| K $_{ow}$ | Octanol-water partition coefficient |
| LOEC | Lowest observed effect concentration |
| LC $_{50}$ | Median lethal concentration |
| NOEC | No observed effect concentration |
| NOEL | No observed effect level |
| OECD | Organisation for Economic Co-operation and Development |
| PEC | Predicted environmental concentration |
| PICT | Pollution-induced community tolerance |
| PNEC | Predicted no effect concentration |
| PRC | Principal response curve |
| RDA | Redundancy analysis |

Summary

Aquatic ecosystems worldwide are exposed to a variety of pesticides, and they are increasingly affected by global environmental change. Thus, aquatic ecosystems are threatened by a multitude of stressors that interact in complex ways. However, there is limited understanding or practical consideration of the effects of multiple stressors in ecological risk assessments of pesticides. This thesis contributes to the assessment of the effects of additional stressors on the impact of toxicants on aquatic communities. Two outdoor microcosm experiments with zooplankton communities were conducted at the Helmholtz Centre for Environmental Research in Leipzig in 2008 and 2009 for a period of 4 and 5 months, respectively. In both experiments, zooplankton communities were exposed to concentrations of the pyrethroid insecticide esfenvalerate (0.03, 0.3 and 3 $\mu\text{g/L}$). In addition, abiotic and/or biotic factors were changed. In the first experiment, solar radiation and community density were manipulated. In the second experiment, the hydrological regime was experimentally altered. Following a general introduction to the thesis (Chapter 1), the effects of altered solar radiation and community density (Chapters 2, 3) and water level fluctuations (Chapters 4, 5) on the sensitivity of zooplankton communities to esfenvalerate are presented and discussed. The results of each chapter are summarised and integrated in a general discussion (Chapter 6). In Chapter 2, the manipulation of solar radiation by shading and community density by harvesting organisms from the microcosms resulted in treatments with different degrees of competition for food. The lowest observed effect concentration (LOEC) of esfenvalerate in the treatment with the highest level of competition was 100 times smaller than in the treatment with the least competition. Thus, the experiment demonstrates that competition for food may considerably affect a community's sensitivity to pesticides. The next chapter (Chapter 3) compares the results of this experiment with a similar experiment conducted simultaneously in Karasuk, Russia. Similar to the results found in Leipzig, community sensitivity to esfenvalerate along an experimental competition gradient in Russia differed up to a factor of 100. Likewise, the treatment representing the highest level of competition was the most sensitive to the toxicant. This study reveals that the direction and magnitude of alterations in community sensitivity observed in outdoor microcosm studies may be reproducible and consistent across different biogeographic regions. Chapter 4 is devoted to the study of the effects of fluctuations in water level achieved by removing and adding water, on the impact of esfenvalerate on the zooplankton community. The results show that the zooplankton community, including the dominant taxon of *Daphnia* spp., is more sensitive to esfenvalerate when the water level fluctuates. Thus, the study demonstrates that the effects of a toxicant on a community may be greater in the presence of stressors such as water level fluctuations. Although esfenvalerate exposure and water level fluctuations were expected to affect the organisms through different mechanisms, both

stressors caused a strong reduction in the abundance of the dominant taxon, *Daphnia* spp. Based on this observation, the hypothesis that zooplankton communities exposed to repeated esfenvalerate contamination develop tolerance and that this development is enhanced under fluctuating water levels was tested. The results of this study support this hypothesis and are presented and discussed in Chapter 5. Thus, this study demonstrates that non-chemical stressors may facilitate the development of a community's tolerance to a toxicant through community-level mechanisms. In conclusion, the collective work of the thesis adds to the increasing evidence that community sensitivity to pesticides is affected by additional biotic and abiotic stressors. The results can be applied to the improvement of current ecological risk assessments of pesticides.

Zusammenfassung

Aquatische Ökosysteme sind weltweit einer Vielzahl von Pestiziden ausgesetzt. Darüber hinaus werden sie vermehrt durch globale Umweltveränderungen beeinträchtigt. Demnach sind aquatische Ökosysteme durch eine Vielzahl von Stressoren bedroht, welche auf komplexe Weise miteinander in Beziehung stehen. Trotzdem sind das Verständnis und die praktische Berücksichtigung von Multistressoren-Effekten in der ökologischen Risikobewertung von Pestiziden limitiert. Die vorliegende Arbeit leistet einen Beitrag zur Beurteilung von Effekten zusätzlicher Stressoren auf die Auswirkungen von Pestiziden auf aquatische Artgemeinschaften. Zwei Freiland-Mikrokosmenexperimente mit Zooplanktongemeinschaften wurden in den Jahren 2008 und 2009 über eine Periode von jeweils 4 und 5 Monaten am Helmholtz Zentrum für Umweltforschung in Leipzig durchgeführt. In beiden Experimenten wurden die Zooplanktongemeinschaften mit dem Pyrethroid-Insektizid Esfenvalerate (0.03, 0.3 und 3 $\mu\text{g/L}$) kontaminiert. Zusätzlich wurden abiotische und/oder biotische Faktoren verändert. Im ersten Experiment wurden die Sonneneinstrahlung und die Dichte der Zooplanktongemeinschaft manipuliert. Im zweiten Experiment wurde der Wasserhaushalt der Mikrokosmen experimentell verändert. Nach einer allgemeinen Einführung (Kapitel 1) werden die Effekte von veränderter Sonneneinstrahlung und Dichte der Artgemeinschaft (Kapitel 2, 3) und Wasserpegelschwankungen (Kapitel 4, 5) auf die Sensitivität von Zooplanktongemeinschaften gegenüber Esfenvalerat präsentiert und diskutiert. Schließlich werden die Resultate der einzelnen Kapitel zusammengefasst und diskutiert (Kapitel 6). Im Kapitel 2 führten die Veränderungen der Sonneneinstrahlung und der Dichte der Artgemeinschaften durch Beschattung der Mikrokosmen respektive Entnahme von Organismen zu Experimentalbedingungen (Treatments) mit verschiedenen Intensitäten der Nahrungskonkurrenz. Die niedrigste Konzentration mit beobachteter Wirkung (LOEC) im Treatment mit der stärksten Konkurrenz lag 100 Mal unter derjenigen im Treatment mit tiefster Konkurrenz. Dieses Experiment zeigt demnach, dass Konkurrenz um Nahrung die Sensitivität von Artgemeinschaften gegenüber Pestiziden erheblich beeinflussen kann. Das nachfolgende Kapitel (Kapitel 3) vergleicht die Resultate dieses Experiments mit einem Experiment gleicher Art, welches zeitgleich in Karasuk, Russland, durchgeführt wurde. Ähnlich wie in Leipzig unterschied sich in Russland die Sensitivität der Zooplanktongemeinschaften gegenüber Esfenvalerat zwischen den verschiedenen Treatments, welche einen Konkurrenzgradienten darstellten, um einen Faktor von 100 basierend auf dem LOEC. Ebenso waren die Artgemeinschaften im Treatment mit der stärksten Konkurrenz am empfindlichsten. Diese Studie zeigt auf, dass sowohl die Richtung als auch die Stärke der Änderungen in der Sensitivität von Artgemeinschaften, die aus Freiland-Mikrokosmenexperimenten abgeleitet wurde, über verschiedene biogeographische Regionen reproduzierbar und konsistent sind. Kapitel 4 widmet sich der Studie

der Effekte von Wasserpegelschwankungen, welche durch Entnahme und Zugabe von Wasser simuliert wurden, auf die Auswirkung von Esfenvalerat auf Zooplanktongemeinschaften. Die Resultate zeigen, dass die Zooplanktongemeinschaft und die dominante Gattung *Daphnia* spp. in Anwesenheit von Wasserpegelschwankungen sensibler auf Esfenvalerat reagieren. Die Studie zeigt demnach, dass die Effekte von Schadstoffen auf eine Artgemeinschaft in der Anwesenheit von Stressoren wie Wasserpegelschwankungen grösser sein können. Obwohl erwartet wurde, dass Esfenvalerat und die Wasserpegelschwankungen die Organismen über unterschiedliche Mechanismen beeinflussen, führten beide Stressoren zu einer starken Abnahme in der Abundanz der dominanten Gattung *Daphnia* spp. Basierend auf dieser Tatsache wurde die Hypothese getestet, dass Zooplanktongemeinschaften, welche wiederholten Kontaminationen mit Esfenvalerat ausgesetzt werden, Toleranz entwickeln und sich diese unter Wasserpegelschwankungen schneller einstellt. Die Resultate dieser Studie, welche diese Hypothese bestätigen, werden in Kapitel 5 präsentiert und diskutiert. Diese Studie zeigt demnach, dass nicht-chemische Stressoren eine wichtige Rolle in der Toleranzbildung von Artgemeinschaften gegenüber Pestiziden über Mechanismen auf Gemeinschaftsebene spielen können, und somit die Entwicklung von Gemeinschaftstoleranz erleichtern. Zusammenfassend weist diese Arbeit nach, dass zusätzliche abiotische und biotische Stressoren die Sensitivität von Artgemeinschaften gegenüber Pestiziden beeinflussen können. Die Ergebnisse können dafür verwendet werden, um die derzeitige ökologische Risikobewertung von Pestiziden zu ergänzen.

Chapter 1

Introduction

1.1 Overview

Aquatic ecosystems around the world are threatened by various anthropogenic activities. One of the major threats to aquatic ecosystems is contamination by a multitude of chemical contaminants (Millennium Ecosystem Assessment, 2005). In addition to this chemical pollution as well as land-use change and water diversion, global climate change increasingly places pressure on aquatic ecosystems (Poff et al., 2002).

In natural systems, multiple stressors exist and often do not act independently (i.e., additively) but instead affect organisms in a complex and interactive manner that results in a combination of effects that are greater (i.e. synergistic effects) or lesser (antagonistic effects) than the sum of the effects of each individual stressor (e.g., Breitburg et al., 1998; Crain et al., 2008). As a consequence, the combined effects of multiple stressors cannot be adequately predicted by simply considering the effects of single stressors (Christensen et al., 2006).

While many studies have assessed the combined effects of pesticides and abiotic and biotic stressors on individual organisms (e.g., the review by Heugens et al., 2001), surprisingly few studies have considered these combined effects on entire communities. Hence, there is limited understanding or practical consideration of the effects of multiple stressors in the ecological risk assessments of pesticides that are required for the market launch of these products. The uncertainty created by this lack of understanding can result in the implementation of either over- or underprotective standards and, as a consequence, economic and management inefficiency or environmental hazards, respectively (Suter, 2007).

1.2 Background

1.2.1 Direct and indirect effects of pesticides

Pesticides enter aquatic ecosystems via run-off from treated areas and agricultural land, aerial spraying and spray drift, washing from the atmosphere by precipitation, erosion, discharge of effluent from factories, and sewage (Edwards, 1977). In rare cases, pesticides are also applied directly to water surfaces, such as for mosquito control. Water quality data from various monitoring programs show that pesticides are ubiquitous in aquatic

ecosystems. For instance, a comprehensive set of studies conducted by the U.S. Geological Survey on major river basins across the U.S. in the early to mid- 1990s showed that more than 90 per cent of water and fish samples from all streams in the U.S. contained pesticides, and more than 50 per cent contained five or more pesticides (Gilliom et al., 2006). In northern Germany, a total of 257 organic pollutants were detected in the four largest rivers in concentrations high enough to likely have acute toxic effects on river organisms (Schäfer et al., 2011). In fact, several field investigations have shown that pesticides may have pronounced impacts on the sensitive components of the biological communities in aquatic systems (Beketov et al., 2009; Liess and von der Ohe, 2005; Rasmussen et al., 2011; Schäfer et al., 2007, 2012). These local effects on biodiversity have been shown to scale up to the regional level (Beketov et al., 2013). Thus, these investigations show that despite the risk assessments currently being implemented, pesticides remain an important environmental stressor for aquatic systems. In view of the fact that pesticide use has not decreased in the last decade (e.g., Eurostat Database; <http://epp.eurostat.ec.europa.eu>) and is even predicted to increase in coming decades due to climate change (Kattwinkel et al., 2011), there is an urgent need for revisions to the current practice of risk assessment.

The most obvious impacts of pesticides on the biota are the direct effects of acute poisoning. Ecological risk assessments of pesticides usually focus on the direct effects of a contamination on individual organisms (Box 1). However, pesticides may also affect organisms indirectly, such as by interfering with their food supply or by altering the aquatic habitat (see review by Fleeger et al., 2003). For instance, in zooplankton communities, the reduction or elimination of *Daphnia* populations by pesticides has often resulted in rotifer blooms (Day et al., 1987; Hurlbert et al., 1972). Other studies have shown that pesticide contamination can influence the swimming behaviour of prey organisms, which results in an alteration of the predator-prey relationships within the community (Dodson et al., 1995; Preston et al., 1999).

To summarise, toxicants are capable of causing a variety of indirect ecological effects that may be equally or even more important than their direct toxic effects (Feldman et al., 2000). Therefore, it is important to perform experiments to help understand the mechanisms underlying these observed effects on the biota to better predict the risks to ecosystems.

Box 1: Pesticide Risk Assessment

Pesticide Risk Assessment is based on the concept that potential contamination risks can be determined by comparing pesticide concentrations that elicit known effects on organisms to concentrations that are predicted to occur in the environment following a specific pattern of use. Risk assessment procedures for plant protection products are reported in the Annex IV of the EC Directive 91/414/EC. These risk assessment procedures comprise three steps: I) exposure assessment, II) characterisation of the effects, and III) characterisation of the risk. Step I) can be assessed through either direct measurements (a posteriori) or model application (a priori). Exposure is generally expressed as the predicted environmental concentration (PEC) in an environmental compartment. The calculation for pesticide concentration in surface water is based on the recommended dose for pest control and the percentage of expected drift. In step II, laboratory derived ecotoxicologi-

cal endpoints, such as the median effective concentration (EC_{50}) at which 50% of the population is affected and the no observed effect concentration (NOEC), are computed for test species such as daphnids or fish. To extrapolate the results to entire communities, the predicted no effect concentration (PNEC) is calculated by dividing the experimentally determined NOEC by a safety factor and/or assessment factor. This safety factor is selected according to the quantity and/or quality of available data. The safety factor takes into account the uncertainty in the estimations of risk assessment and the actual conditions in the field. It may vary from 1000 for acute toxicity data from more than one species (and be applied to the lowest LC_{50}), to 50 for chronic toxicity data that are not necessarily from the most sensitive species (applied to the lowest NOEC), to 10 for chronic toxicity data based on data from the most sensitive species (applied to the lowest NOEC), to an even lower value for toxicity data based on tests with communities in semi-realistic environments, i.e., micro- or mesocosm studies. In step III, both exposure and effect data are compared. According to Directive 91/414/EC, risk is quantified through the calculation of the toxicity to exposure ratio, i.e., the ratio between an indicator of the effect (e.g., NOEC) and an exposure indicator (e.g., PEC). If $PEC > PNEC$, adverse effects may occur and further information may be required to refine both the PEC and PNEC.

1.2.2 Combined effects of pesticides and abiotic environmental factors and species interactions

Abiotic environmental factors and interactions with other organisms play a major role in determining the distribution and abundance of aquatic organisms (Gannon and Stemberger, 1978). Important abiotic environmental factors in aquatic ecosystems include temperature, salinity, dissolved oxygen level, pH and nutrient concentrations. Biotic factors include inter- and intraspecific competition, predation, herbivory, mutualism, disease and parasitism (Krebs, 1994, Chapter 12-15) and they are highly dependent on the abiotic factors present in an ecosystem. Even slight changes to abiotic factors may affect biotic interactions by decreasing or enhancing the suitability of the habitat for certain species and hence reducing or allowing the appearance of certain species.

Abiotic and biotic factors can alter the effects of pesticides and other toxicants on organisms, populations, and communities. The influence of various abiotic conditions on the sensitivity of organisms to toxicants has been assessed for several factors, including water temperature (see reviews by Cairns et al., 1978; Heugens et al., 2001) and salinity (see reviews by Hall and Anderson, 1995; Heugens et al., 2001) which have received the greatest attention. However, factors such as pH (Fisher and Lohner, 1986; Wilde et al., 2006), UV radiation (see review by Blaustein et al., 2003; Duquesne and Liess, 2003), dissolved oxygen (van Der Geest et al., 2002) and the combinations of two or more of these factors (Hanazato and Dodson, 1995; Lohner and Warwick Fisher, 1990) have also been studied. Generally, toxicity has been found to be positively correlated with temperature and UV radiation, and negatively correlated with pH. The influence of salinity is less clear; while the toxicity of metals and organophosphate insecticides have generally increased

with decreasing and increasing salinity, respectively, no clear relationship between the two stressors has been observed for other chemicals. The interactions have been explained by several physical and physiological processes, including mechanisms such as bioavailability, toxicokinetics and organism sensitivity (Heugens et al., 2001). In fact, the effects of abiotic factors on the impact of a toxicant impact depend on its identity. For instance, metal toxicity generally increases with decreasing salinity while the toxicity of organophosphate insecticides generally increases with higher salinity.

Similar to abiotic factors, the influence of biotic factors, particularly predation and competition, on the effects of toxicants to organisms have been well recognized. For example, predator cues have been observed to increase the effects of the insecticide carbaryl on amphibian larvae (Relyea, 2003; Relyea and Mills, 2001). A simulated predation study showed that the combination of predation and short-term exposure to the pyrethroid esfenvalerate may lead to the local extinction of brine shrimp populations (Beketov and Liess, 2006). In contrast, another study with the predatory phantom midge and its prey, *Daphnia* sp., showed that predation may result in lower sensitivity to the toxicant, an antagonistic effect (Coors and De Meester, 2008).

As with predation, competition has also been shown to alter an organism's sensitivity to toxicants. The intensity of competition mainly depends on population density and food availability. For instance, food limitation has been shown to increase the effect of chlorpyrifos on *Ceriodaphnia cf. dubia* (Rose et al., 2002). Increased pesticide effects have also been observed in mayfly larvae, *Clocon dipterum*, exposed to the pyrethroid esfenvalerate under conditions of food limitation (Beketov and Liess, 2005). Similarly, increased population densities have enhanced the short-term effects of fenvalerate in cadisfly larvae (Liess, 2002), as well as on the interacting populations of *Daphnia* and *Culex* larvae (Foit et al., 2012).

A few studies have also assessed the effect of the combination of abiotic environmental factors and biotic factors on pesticide sensitivity (e.g., Barry, 1997; Liess et al., 2001; Relyea, 2006). For example, exposure to copper and UV-B in combination with a higher level of competition induced by food shortage was shown to affect the Antarctic amphipod *Paramoera walkeri* at concentrations 15 times lower than normal (Liess et al., 2001). Another study showed that predatory stress and low pH did not increase the lethality of the insecticide carbaryl, i.e. green frogs and larval bullfrogs were affected additively (Relyea, 2006).

As the above-mentioned examples show, the combined effects of pesticides and abiotic and/or biotic factors on aquatic ecosystems are very complex and can result in reduced or enhanced sensitivity to toxicants.

1.2.3 Individual vs. community level effects

While studies at the lower levels of biological organisation (e.g., the individual) are used to determine the mechanisms or modes of toxic action for pesticides, interpretations of adverse responses to toxicological events for the purpose of making risk-management decisions are typically associated with higher levels of biological organisation (e.g., the community or ecosystem) (Bradbury et al., 2004). The majority of existing studies on the combined effects of pesticides and abiotic or biotic factors have been conducted at the individual or population level (see the examples above). It follows that community

level studies, i.e., studies on an assemblage or association of populations of two or more different species occupying the same geographical range, have been rather scarce. Table 1.1 compiles community-level studies that have experimentally assessed the effects of abiotic environmental or biotic stressors combined with a toxicant on aquatic communities. The compilation is based on an online literature search on aquatic communities. Only community studies that analysed a community endpoint, i.e., an endpoint including two or more populations, were taken into account.

The majority of the studies listed in Table 1.1 did not assess the statistical significance of the interaction between the two stressors. Furthermore, only a few studies reported whether the additional stressor resulted in different pesticide effects than those in the absence of an additional stressor. However, knowledge of both interactions between stressors and alterations in effect concentration are important for ecological risk assessments of multiple stressors.

Table 1.1: Literature review on the combined effects of toxicants and abiotic and biotic factors on communities. Only studies that analysed a community endpoint were included (i.e., an endpoint including two or more populations).

| No. | Community | Endpoints | Stressors | | Effects | | Reference |
|-----|---|---|---------------------------------|------------------------|-----------------|-----------------|--------------------------------|
| | | | A: toxicant | B: (a)biotic | A × B | Diff. LOEC | |
| 1 | Zooplankton, gastropoda, chydoridae, cyclopoida, harpacticoida | Community structure, density | Endosulfan ¹ | Predator | sig. | yes | Barry and Davies (2004) |
| 2 | Zooplankton, cladocera, copepoda, phytoplankton, periphyton, snails, tadpoles | Species richness, density, biomass | Malathion | Predator | sig. | yes | Relyea and Hoverman (2008) |
| 3 | Cladocera, isopoda | Density | Chlorpyrifos | Presence of Macrophyte | nr | na ² | Brock et al. (1992a) |
| 4 | Rotatoria, tubellaria, mollusca, hirudinea, oligochaeta | Density | Chlorpyrifos | Presence of Macrophyte | nr | na ² | Brock et al. (1992b) |
| 5 | Macroinvertebrate, zooplankton | Community structure | Lambda-cyhalothrin ¹ | Nutrient level | sig. | yes | Roessink et al. (2005) |
| 6 | Zooplankton, phytoplankton | Community structure | Chlorpyrifos | Nutrient level | nr | yes | van Wijngaarden et al. (2005a) |
| 7 | Zooplankton, phytoplankton | Chla, abundance | Chlorpyrifos | Nutrient level | na ² | na | van Donk et al. (1995) |
| 8 | Rotifera, cladocera | Density | Carbaryl | Predator | ns | na ² | Chang et al. (2005) |
| 9 | Microorganisms | Various functional and structural variables | Copper pyrrithion | Nutrient level | nr | na ² | Sundbäck et al. (2007) |

Table 1.1: Literature review on the combined effects of toxicants and abiotic and biotic factors on communities. Only studies that analysed a community endpoint were included (i.e., an endpoint including two or more populations). (continued)

| | | | | | | | |
|----|--|--|---------------------------------------|----------------|------|-----------------|-------------------------|
| 10 | Phytoplankton, bacterioplankton, heterotrophic nanoflagellates, copepoda, oyster, macoma, sea anemone, entire system | Production, in vivo fluorescence, Chl <i>a</i> , density, respiration | Increased trace element loadings | Nutrient level | sig. | na ³ | Breitburg et al. (1999) |
| 11 | Phytoplankton, bacteria | Abundance, growth rate, cell size, activity | Water soluble fraction of crude oil | UVB radiation | ns | na ³ | Sargian et al. (2005a) |
| 12 | Plankton, phytoplankton | Chl <i>a</i> , in vivo fluorescence, abundance, cellular characteristics, growth rates, production | Tributyltin | UVB radiation | sig. | na ³ | Sargian et al. (2005b) |
| 13 | Phytoplankton, nanophytoplankton, picophytoplankton, bacteria | Total abundance, forward scatter signal, photochemical yield, abundance, growth rate, Chl <i>a</i> , SSC | Water soluble fraction of crude oil | UVB radiation | sig. | na ³ | Sargian et al. (2007) |
| 14 | Zooplankton (Copepoda, rotifera, cladocera) | Density | Carbaryl ¹ | Predator | nr | nr | Hanazato (1991) |
| 15 | Phytoplankton | Photosynthetic electron transport | Anthracene/1,2-dihydroxyanthraquinone | Light exposure | nr | nr | Marwood et al. (1999) |

Abbreviations: A × B = interaction stressor A and B, ns = not significant, nr = not reported, na = not applicable, Chl*a* = Chlorophyll-*a*, SSC = Sideward light scatter, Diff. LOEC = significant difference in LOEC

¹ multiple contaminations ² not full factorial experiment ³ only 1 concentration tested

1.2.4 Use of outdoor microcosms in pesticide risk assessment to assess community-level effects of pesticides

European regulations require risk assessments to protect biological populations under natural conditions (European Parliament, Council, 2009, Article 4.3). However, risk assessment procedures generally employ laboratory toxicity tests in which individual organisms are exposed to pesticides under constant and favourable conditions. In fact, laboratory tests allow for a high degree of control over external variables, high replicability and, therefore, high reliability. However, due to their simplistic environmental conditions, these studies lack ecological realism. Thus, the question remains as to how accurately these controlled laboratory tests may predict the effects of toxicants in natural aquatic ecosystems, which are characterised by variable and suboptimal environmental conditions. In contrast to laboratory experiments, field studies offer a high degree of ecological validity, but they are hampered by their complexity, limited reproducibility and, hence, limited reliability (Fig. 1.1).

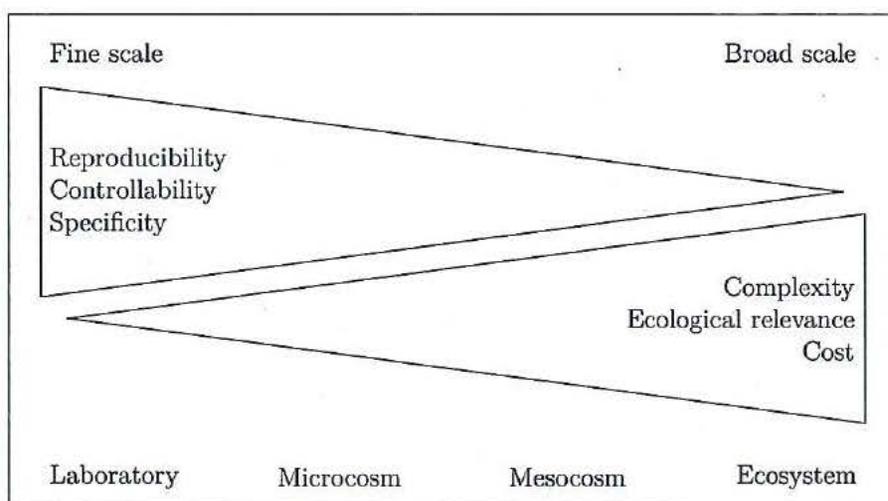


Figure 1.1: Laboratory vs. ecosystem testing. Advantages and disadvantages of various test systems (modified from Brock et al., 2000; Legendre and Legendre, 1983)

The artificial outdoor aquatic system, microcosm or mesocosm, is a frequently used approach that is intermediate to laboratory tests and field studies. Microcosms can be defined as experimental systems with water volumes of less than 15 m³ or experimental streams that are less than 15 m in length; and mesocosms are experimental systems that have a volume of 15 m³ or more or experimental streams greater than 15 m in length (Crossland et al., 1992). In aquatic risk assessment, micro- and mesocosms are used within the so-called tiered approach, in which they represent high-tier test systems and are used to refine the risk assessment outcomes of lower tier standard laboratory tests that indicate ecological concerns (de Jong et al., 2008; Giddings et al., 2002). Although smaller and less complex than real-world aquatic ecosystems, micro- and mesocosms provide the opportunity to perform ecosystem level research in replicated test systems with a high degree of control over environmental conditions in a way that is manageable in terms of

costs and logistics (Gearing, 1989; Giddings, 1983; Landner et al., 1989). In fact, they allow for the integration of realistic exposure regimes with the assessment of endpoints at higher levels of organisation (e.g., indirect effects) and recovery. As a consequence, endpoints examined in microcosm and mesocosm studies are not restricted to simple estimates such as mortality and growth but generally include an array of structural and functional measures (Newman and Clements, 2008). For each endpoint, no observed effect concentrations (NOECs) and lowest observed effects concentrations (LOECs), two major statistical endpoints used in toxicity testing (Box 2) can be identified.

However, the realistic identification of community level effects in outdoor experimental systems such as micro- or mesocosms is impeded by high variation between replicates and a scattered, low-abundance distribution of the majority of the taxa (Beketov and Liess, 2012; Beketov et al., 2008; Knauer et al., 2005; Liess and Beketov, 2011; Liess and Beketov, 2012; Sanderson et al., 2009; Wang and Riffel, 2011). Furthermore, micro- and mesocosm experiments are characterised by a low number of replicates (Knillmann et al., 2012a) and samples (Campbell et al., 1999; de Jong et al., 2008).

These circumstances affect repeatability, reproducibility and, eventually, the predictive ability of results from micro- or mesocosm studies. Consequently, ecological risk assessment of pesticides is mostly based on temperate toxicity data even in other climatic or geographic regions (Abdullah et al., 1997; Bourdeau et al., 1989; Lacher and Goldstein, 1997). Thus, it is very important to validate whether toxicity data and ecological risk assessment approaches from temperate studies are applicable to other parts of the world. Until now, only a few studies have compared the effects of pesticides on aquatic communities in different climatic or biogeographic regions. However, Daam et al. (2009) and López-Mancisidor et al. (2008b) showed that measurements of the sensitivity of aquatic communities to pesticides derived from outdoor micro- or mesocosms may be comparable between different climatic regions.

Box 2: NOEC and LOEC

The NOEC is the highest concentration in a test with a mean response not statistically significantly different from the mean response of the control. Analogously, the LOEC is the lowest test concentration having a mean response that differs significantly from that of the control. NOEC and LOEC are generated by post-analysis (ANOVA) multiple comparison tests (Newman, 1995). NOEC and LOEC are major summary statistics in ecotoxicological testing. In environmental risk assessment, NOEC is often defined as the concentration of a pollutant that will not harm the species being studied. However, NOEC has been strongly criticised as a poor estimator of a “safe” pesticide concentration, partly because a lack of statistical significance does not mean that there is no effect. Therefore, several authors have called for banning its use in environmental guidelines and publications in favour of the $x\%$ effects concentration (EC_x) in environmental guidelines and publications (Jager, 2012; Landis and Chapman, 2011). However, a recently published article noted that most experimental designs and the resulting data are often unsuitable for EC_x determination and that the use of EC_x also has shortcomings; it concluded that the two measures should be complementary (Green et al., 2013).

1.2.5 Impact of climate change on aquatic ecosystems

Temperature and precipitation are important abiotic factors for aquatic ecosystems as they directly and indirectly regulate many ecological processes in these systems (Poff et al., 2002). Both factors are increasingly projected to change and thereby alter ecological processes in aquatic ecosystems. For example, the average global air temperature has been projected to rise 0.2°C per decade (Pachauri and Reisinger, 2007). The most evident ecological impact of global warming is a poleward shift in species' ranges (see review by Parmesan, 2006). In addition to the shift in species' distribution, climate change is also an important driver of phenological changes (see review by Visser and Both, 2005). Such changes may disrupt the dynamics of trophic linkages (e.g., Durant et al., 2007; Winder and Schindler, 2004). Similar to temperature, the frequency and intensity of precipitation events are also predicted to change. While there may be significant differences between seasons and regions, it is agreed that the amount of rain falling in a given precipitation event may increase in most regions of the world (Pachauri and Reisinger, 2007). Changes in precipitation are generally amplified in runoff, and models predict an increase in runoff (10-14%) in the high latitudes of North America and Eurasia, in parts of South America, in eastern equatorial Africa and in some major islands of the equatorial eastern Pacific Ocean with a decrease (10-30%) in southern Europe, the Middle East, mid-latitude western North America, and southern Africa by 2050 (Milly et al., 2005). These changes in patterns of precipitation and runoff will alter the hydrological regimes of ecosystems, which in turn will significantly affect species composition and ecosystem productivity (see review by Bunn and Arthington, 2002).

Changes in temperature and precipitation are also projected to influence the use of pesticides in agriculture. Mean annual temperatures have been shown to positively correlate with the rate of insecticide application (Kattwinkel et al., 2011). Together with the predicted increase in the intensity and frequency of rain and storm events (Pachauri and Reisinger, 2007), runoff and thus exposure to pesticides in aquatic systems is expected to increase. Indeed, it has already been demonstrated that flood events increase pesticide pollution in water bodies (e.g., Donald et al., 2005). Thus, pesticide concentrations in water bodies are likely to increase as climate change progresses and further affects aquatic ecosystems.

Climate change is projected to alter atmospheric circulation, which will increase the flux of ozone from the upper to the lower atmosphere and shift the distribution of ozone within the upper atmosphere (Hegglin and Shepherd, 2009). As a result, the amount of UV radiation reaching Earth's surface will significantly change, and variations between regions will be pronounced (Hegglin and Shepherd, 2009). Exposure to solar UV radiation can alter productivity (review by Vincent and Roy, 1993), reproduction (e.g., Day et al., 1999), development (e.g., Huovinen et al., 2000) and mutation rates (review by Sinha and Häder, 2002) in aquatic ecosystems.

1.3 Aim of the thesis

Considering the various forecasts for changes to key abiotic factors, it is important to understand how those factors may interact with pesticide impacts and whether these effects may be similar between regions to anticipate and alleviate the combined effects of

global change and contaminants.

Zooplankton plays an important role in controlling the phytoplankton community and as a direct or indirect food source for other aquatic organisms at higher trophic levels, e.g., fish. Due to zooplankton's important position in the food web, understanding and predicting the impacts of the combined effects of global change and contaminants on zooplankton is essential to manage aquatic resources and counteract the impacts of climate change and other anthropogenic stressors on aquatic ecosystems. Thus, the aim of this thesis is to evaluate the influence of abiotic and biotic factors on the effects of pesticides on zooplankton communities and thus contribute to the effective risk assessment of pesticides under global climate change. Three specific aims were addressed by this thesis:

The first aim was to assess the effects of different environmental factors on the impacts of a pesticide on a community. Rather than being based on a specific climate change scenario, the factors examined were models for broad potential environmental changes. We hypothesised that:

- (1) Increased competition due to reduced solar radiation and high community density increases the sensitivity of zooplankton communities to a single pulse of esfenvalerate (Chapter 2).
- (2) Water level fluctuations increase the sensitivity of zooplankton communities to a single pulse of esfenvalerate (Chapter 4).

The second aim was to evaluate the predictive ability of outdoor microcosm experiments used to study the effects of multiple stressors by comparing the outcomes of outdoor microcosm studies conducted in two different biogeographical regions. We hypothesised that, independent of the biogeographical region, high competition due to reduced solar radiation and high organism density increases the sensitivity of zooplankton communities to a single pulse of esfenvalerate, i.e., the sensitivity of zooplankton communities to esfenvalerate are similar in Leipzig and Karasuk, (Russia) (Chapter 3).

The third aim was to study the response of the zooplankton community to multiple applications of a pesticide in the presence of an additional environmental factor and to compare them with responses to a single application (Chapter 5). We hypothesised that multiple esfenvalerate contamination events induce the development of community tolerance to the insecticide and that this development is enhanced in communities exposed to water level fluctuations. This hypothesis was based on the findings of the study on the effects of a single pulse contamination with esfenvalerate under fluctuating water levels (Chapter 4) that showed that the toxicant and hydrological changes affected the same taxa. The results of each chapter are summarised and integrated in a general discussion that outlines the implications for the risk assessment of pesticides (Chapter 6).

1.3.1 Test system and substance used in the present studies

To address the aims of the thesis, outdoor microcosm experiments were conducted. These microcosms (height: 38 cm, radius: 25 cm, volume: 80 L) were established at the Helmholtz Centre for Environmental Research (UFZ) in Leipzig, Germany (51°21'13 N, 12°25'55 E) (Fig. 1.2). They were designed to mimic small and shallow ponds dominated by zooplankton. Zooplankton species play a crucial role in the aquatic food webs through

their consumption of phytoplankton and as a direct or indirect food source for higher trophic level consumers. Thus, it forms an important link in the transformation of energy from producers to consumers. Furthermore, due to its high density, relatively short life span, high species diversity and variability in tolerance to stressors, such as eutrophication, acidification or toxic contamination, zooplankton is a good indicator of the health of aquatic ecosystems.



Figure 1.2: Outdoor microcosms test system at the Helmholtz Centre for Environmental Research – UFZ in Leipzig, Germany (51°21'13 N, 12°25'55 E). The microcosms (height: 38 cm, radius: 25 cm, volume: 80 L) mainly contained zooplankton.

Table 1.2: Selected physicochemical characteristics of the pyrethroid insecticide, esfenvalerate, used as model substance in the microcosm experiments

| Compound | Esfenvalerate |
|---------------------------------|--|
| Chemical abstract name (IUPAC) | (α S)- α -cyano-3-phenoxybenzyl (2S)-2-(4-chlorophenyl)-3-methylbutyrate |
| CAS RN | 66230-04-4 |
| Log Kow | > 6 |
| Koc | 215 |
| Solubility (mg/L, Water, 25 °C) | < 0.3 |
| DT50 (days) | 10 |
| Mode of action | Voltage-dependent sodium-channel agonist |
| Pest organisms | Moths, flies, beetles, and other insects |

In the present studies, the insecticide esfenvalerate was used as a model substance. Esfenvalerate is a voltage-dependent, sodium-channel agonist, i.e., it causes sodium channels to stay open longer than normal, which leads to repetitive firing of neurons (Vijverberg and van den Bercken, 1990). As a result, arthropods exposed to esfenvalerate may exhibit symptoms such as hyperexcitation, tremors and convulsions followed by lethargy and paralysis which eventually lead to death¹. Esfenvalerate is primarily used in the

¹http://www2.dupont.com/Production_Agriculture/en_US/assets/downloads/pdfs/K-09355.pdf

Table 1.3: Summary of relevant toxicity data for standard test organisms most susceptible to esfenvalerate. Note that different endpoints and the time period to the endpoint were considered

| Organism | Concentration ($\mu\text{g/L}$) | Endpoint | Reference |
|--|--------------------------------------|-------------------------|-------------------------------|
| <i>Daphnia magna</i> | 0.029 | LC ₅₀ (96 h) | Beketov (2004) |
| <i>Caenis milaria</i> | 0.0147 | LC ₅₀ (96 h) | Beketov (2004) |
| <i>Ceriodaphnia dubia</i> | 0.28 | LC ₅₀ (96 h) | Werner et al. (2002) |
| <i>Chironomus riparius</i> (1st instar) | 0.13 | LC ₅₀ (96 h) | Samsøe-Petersen et al. (2001) |
| <i>Artemia</i> spp. | 0.01 | NOEL | Beketov and Liess (2006) |
| <i>Hyazella azteca</i> | 0.09 | LC ₅₀ (24 h) | Lozano et al. (1989) |
| <i>Danio rerio</i> | 0.24 | LC ₅₀ (96 h) | Ma et al. (2009) |
| <i>Leucorrhinia</i> spp. | 2.08 | EC ₅₀ (24 h) | Lozano et al. (1989) |
| <i>Pimephales promelas</i> (Fathead Minnow) | 0.13 | EC ₅₀ (96 h) | Lozano et al. (1989) |

cultivation of corn and potatoes in Germany. The physicochemical characteristics of this substance are shown in Table 1.2. Esfenvalerate is highly hydrophobic and has a strong tendency to adhere to soil particles (Table 1.2).

Esfenvalerate has been shown to be highly toxic to non-target freshwater invertebrates in both the laboratory (Lozano et al., 1992) and in outdoor experiments (Beketov, 2004). Table 1.3 shows toxicity data relevant to standard aquatic test organisms. *Daphnia magna* is among the species most sensitive to esfenvalerate (Table 1.3). The mayfly *Caenis milaria* was also reported to be highly sensitive to this insecticide (Table 1.3). In the present studies, both *Daphnia* spp. and mayflies were present, so a significant part of the community was composed of species sensitive to the tested compound.

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Chapter 2

Environmental context determines community sensitivity of freshwater zooplankton to a pesticide

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2.1 Abstract

The environment is currently changing worldwide, and ecosystems are being exposed to multiple anthropogenic pressures. Understanding and consideration of such environmental conditions is required in ecological risk assessment of toxicants, but remains basically limited. In the present study, we aimed to determine how and to what extent alterations in the abiotic and biotic environmental conditions can alter the sensitivity of a community to an insecticide, as well as its recovery after contamination. We conducted an outdoor microcosm experiment in which zooplankton communities were exposed to the insecticide esfenvalerate (0.03, 0.3, and 3 $\mu\text{g/L}$) under different regimes of solar radiation and community density, which represented different levels of food availability and competition. We focused on the sensitivity of the entire community and analysed it using multivariate statistical methods, such as principal response curves and redundancy analysis. The results showed that community sensitivity varied markedly between the treatments. In the experimental series with the lowest availability of food and strongest competition significant effects of the insecticide were found at the concentration of 0.03 $\mu\text{g/L}$. In contrast, in the series with relatively higher food availability and weak competition such effects were detected at 3 $\mu\text{g/L}$ only. However, we did not find significant differences in the community recovery rates between the experimental treatments. These findings indicate that environmental context is more important for ecotoxicological evaluation than assumed previously.

Keywords: Abiotic factors; Biotic interaction; Mesocosm; Risk assessment; Freshwater zooplankton

2.2 Introduction

Environmental conditions are currently changing worldwide. For example, the Millennium Ecosystem Assessment (2005) revealed that freshwater ecosystems are threatened by multitudes of stressors, such as climate change, habitat loss, invasive species, and pollution by chemical toxicants. Such multiple and variable pressures must be understood and considered thoroughly with respect to ecological practices such as the ecological risk assessment of toxicants (Cairns, 2010). However, currently there is limited practical consideration and understanding of the effects of environmental context in such risk assessment. The uncertainty that this lack of understanding creates can result in the implementation of either over- or under-protective standards and, as a consequence, economic/management inefficiency and environmental hazards, respectively (Suter, 2007).

It is well known that environmental factors can affect the sensitivity of individual organisms to toxicants (e.g. review of Heugens et al., 2001). Such effects have been documented in test systems for abiotic (Brecken-Folse et al., 1994; Lydy et al., 1999; Munkegaard et al., 2008; Preston et al., 1999) and biotic stressors (Beketov and Liess, 2006; Coors and De Meester, 2008; Maul et al., 2006), as well as for combinations of the two (Barry, 1997; Hanazato and Dodson, 1995; Liess et al., 2001; Relyea, 2006). Such effects have also been shown in the field within the ecosystem context (Duquesne and Liess, 2003).

However, knowledge about the effects of environmental factors on the sensitivity of entire communities and ecosystems remains scarce. Although there have been many studies that have focused on the combined effects of various environmental factors and chemical toxicants in semi-natural experimental ecosystems (mesocosms), most of these investigations did not provide information about the magnitude of changes in the sensitivity of the entire community as a single and integral entity (hereinafter referred to as community sensitivity). The reasons for this include the following (which frequently occur in combination): 1) the specific experimental design was not aimed at understanding the magnitude of such changes, 2) the research focused on single-taxon endpoints and disregarded the sensitivity of the community as a whole, and 3) the investigated factors and ranges of toxicant concentration chosen were inappropriate for this purpose (e.g. Barry and Davies, 2004; Chang et al., 2005; Hanazato, 1991; Relyea and Hoverman, 2006). Furthermore, the possible adaptation of a community to the environmental context complicates the assessment of the changes in community sensitivity that are caused by environmental factors.

To our knowledge, there are only two studies that have quantitatively analysed differences in community sensitivity via the experimental manipulation of environmental factors. The first is a study by van Wijngaarden et al. (2005a), which showed that, depending on the environmental conditions, a no-observed-effect concentration (NOEC) of $0.1 \mu\text{g/L}$ and $\geq 1 \mu\text{g/L}$ for chlorpyrifos was found in the "warm Mediterranean" and "cool temperate" scenario, respectively. Similarly, a study by Roessink et al. (2005) revealed occasionally NOECs of 100 ng/L and $> 250 \text{ ng/L}$ for the insecticide lambda-cyhalothrin in mesotrophic (macrophyte-dominated) and eutrophic (phytoplankton-dominated) ditch microcosms, respectively. However, in both of these studies, the differences in the sensitivity of the communities to the treatments were attributed to different recovery rates, rather than to community sensitivity itself, because the differences were observed at a considerable time after contamination and at concentrations of toxicants that were higher than those initially causing effects. Thus, the ultimate limits of the sensitivity of communities were not affected by the environmental context. Therefore, the influence of the environmental context on community sensitivity appeared to be rather negligible in terms of risk assessment.

The results of these two studies, together with recent comparisons of community sensitivity in different regions (Daam et al., 2009; López-Mancisidor et al., 2008a, 2008b; Schäfer et al., 2007), indicate that environmental context might have little importance as a factor that determines the sensitivity of a community (although it might modulate the dynamics of recovery). However, recent comparisons of the effect-concentration thresholds for modern nonpersistent insecticides in mesocosm studies, as well as across laboratory, mesocosm, and field studies, suggest that environmental context might modulate community sensitivity by a factor of up to 100 or higher and thus be of crucial importance (Beketov et al., 2008; van Wijngaarden et al., 2005b). This is also supported by numerous laboratory investigations that have shown pronounced changes in the sensitivity of individual organisms to toxicants in response to environmental factors (Heugens et al., 2001) and by population-level studies that have demonstrated the mechanisms by which sensitivity is altered by biotic or abiotic factors in experimental populations (Beketov and Liess, 2005, 2006; Friberg-Jensen et al., 2003; Hanazato, 1998; Hanazato and Hirokawa, 2004; Liess, 2002; Wendt-Rasch et al., 2003). An important potential reason for the

differences in sensitivity mentioned above is that the communities might be adapted to the environmental factors to different degrees. Adapted communities are not expected to exhibit changes in sensitivity because environmental factors do not act as stressors for such communities. In contrast, communities that are exposed to a "new" stressor, to which they have not adapted, are expected to exhibit higher (or lower) levels of sensitivity due to the combined action of the stressor and a toxicant. The investigations mentioned above provide important insights regarding the influence of environmental context on the sensitivities of communities and populations to toxicants. At the same time, these studies show explicitly that it remains unclear to what extent environmental factors can change the sensitivity of communities to toxicants (e.g. community-structure NOEC), and how such alterations in sensitivity should be considered in terms of ecotoxicological risk assessment. However, taking into account the ubiquity of multiple stressors and ongoing fundamental global changes in environmental conditions (e.g. climate), understanding of such changes in sensitivity is indispensable for improving the realism of ecotoxicological risk assessment.

In the present study, we aimed to understand how and to what extent alterations in the abiotic and biotic environmental conditions can alter the sensitivity of a community to an insecticide and its recovery after contamination. We conducted an outdoor microcosm experiment with freshwater zooplankton communities. The communities were exposed to a single pulse of contamination with the insecticide esfenvalerate under different regimes of solar radiation and community density, which represented different levels of food availability and competition. The magnitudes of the changes in these environmental parameters were designed to cause no major changes in the structure of the plankton community in the absence of the toxicant, but potentially to affect the sensitivity of the community to the toxicant. In addition, the environmental parameters were manipulated only shortly before contamination to prevent preliminary adaptation and stabilisation of the communities.

2.3 Materials and methods

2.3.1 Experimental design

To investigate the influence of environmental context on the sensitivity of a community to a pesticide, outdoor microcosm experiments were conducted under different regimes of solar radiation and community density, which represented different levels of food availability and competition between the organisms. Solar radiation was modified with an awning and community density was manipulated by the regular harvesting of approximately 30% of the zooplankton community. Light and temperature are well-known factors that regulate algal growth (Andersson et al., 1994) and, in turn, the availability of food to support the development of zooplankton communities (Ingle et al., 1937). Changes in community density as a result of direct harvesting influenced both food availability and competition.

To represent conditions of (i) high food/low competition, (ii) medium food/medium competition, and (iii) low food/high competition, we established three treatments: (i) "No Shadow - Harvesting", (ii) "No Shadow - No Harvesting", and (iii) "Shadow - No Harvesting", respectively. The microcosms were assigned randomly to these three

treatments and each of the treatment groups was exposed to 0, 0.03, 0.3, and 3 $\mu\text{g/L}$ of the insecticide esfenvalerate. For each treatment and concentration, six replicate microcosms were established, which resulted in a total of 72 microcosms ($3 \times 4 \times 6 = 72$).

2.3.2 Artificial outdoor pond system

A set of artificial outdoor ponds (microcosms) was established at the UFZ – Helmholtz Centre for Environmental Research (Leipzig, Germany). Microcosms as model ecosystems are useful in risk assessment of chemicals when lower-tier models and higher-tier laboratory studies indicate potential hazards (e.g. Sanderson et al., 2009). Each pond was made of a tank with the following characteristics: height of 38 cm, radius of 24.75 cm, and total volume of 80 L. The microcosms were filled with 60 L of tap water. A substrate was added to cover the bottom of each microcosm with a 1-cm layer of sediment. The substrate was a 1/1 mixture of sediment collected from a nearby natural permanent pond and sand. In addition, approximately 10 g of dried shredded fallen leaves (mainly from *Populus* sp.) were added to all microcosms. To colonise the microcosms, macroinvertebrates were collected from five small natural permanent ponds at the end of May/beginning of June and subsequently distributed equally among all the microcosms. No fish were introduced into the system.

Solar radiation was regulated by using an awning that was mounted at an angle of approximately 45° to the earth's surface to shield the microcosms during the most light-intensive time of the day (12–4 p.m., Fig. 2.1 1F). At the beginning of the experiment, all the microcosms were shaded to allow the communities to develop to a similar extent. One month after the last introduction of macroinvertebrates, four days before contamination, the awning was removed from the microcosms that had been allocated to the treatments “No Shadow – Harvesting” and “No Shadow – No Harvesting”.

Harvesting was performed using a plankton net (10 × 12 cm, 250- μm mesh size), which was placed at the base of the microcosm and lifted diagonally through the water column to sieve a water volume of approximately 6.8 L (10% of the entire community) per movement. Harvesting was conducted twice a week with first one, and then two, acts of sieving, to remove approximately 30% of the macroinvertebrate community per week in total. Before each harvesting event, the water was mixed gently to ensure a uniform spatial distribution of the organisms. Harvesting was started 24 days after the last introduction of macroinvertebrates and 10 days before contamination, that is, six days before the awning was removed. The reason for this asynchronicity is that we assumed that it would take longer for harvesting to have an effect on food availability and competition than the sudden increase in sunlight due to the removal of the awning. Altogether, these changes were aimed at modulating the environmental conditions in the microcosms shortly before contamination to prevent preliminary adaptation and stabilisation of the communities, which in turn was expected to change the sensitivity of the communities.

2.3.3 Application of esfenvalerate and monitoring

All treatment groups were exposed to the pyrethroid insecticide Sumicidin Alpha (BASF, Limburgerhof, Germany), an emulsified concentrate that contains 50 g/L of the active substance esfenvalerate ((αS)- α -cyano-3-phenoxybenzyl (2S)-2-(4-chlorophenyl)-3-methylbut-

2. Environmental context determines community sensitivity of freshwater zooplankton to a pesticide

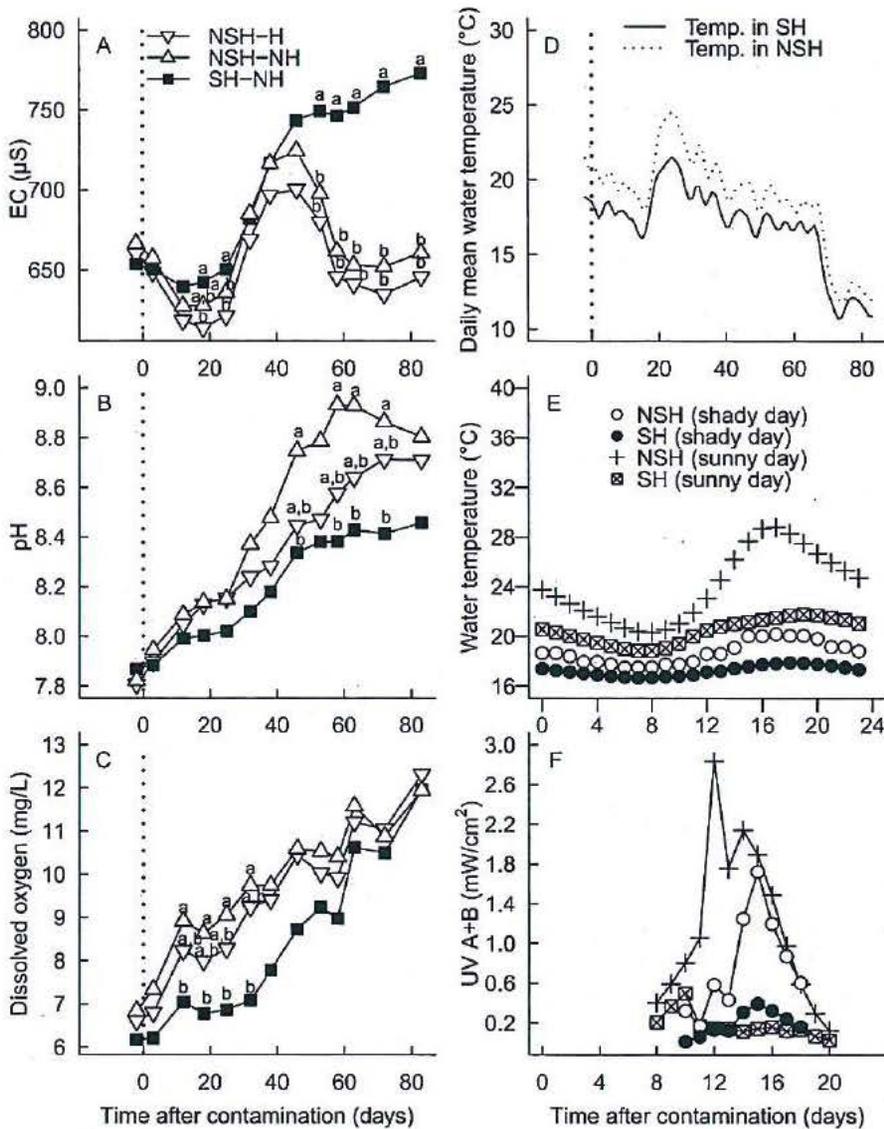


Figure 2.1: Environmental parameters: A – electrical conductivity ($\mu\text{S}/\text{cm}$); B – pH; C – dissolved oxygen concentration (mg/L); D – daily average water temperature ($^{\circ}\text{C}$); E – water temperature ($^{\circ}\text{C}$) on a shady day (18.07.2008) and sunny day (28.07.2008); F – ultraviolet A+B radiation (mW/cm^2) on a shady day and a sunny day; Data in A to D are presented as three-point moving averages. Different letters in A to C indicate significant differences ($P < 0.05$, pairwise t -test with untransformed data). The vertical dashed line indicates the time of contamination. The abbreviations NSH-H, NSH-NH, and SH-NH stand for the treatments “No Shadow – Harvesting”, “No Shadow – No Harvesting”, and “Shadow – No Harvesting”, respectively.

urate), which is an enriched isomer of fenvalerate. Esfenvalerate is a broad-spectrum non-selective pyrethroid insecticide that is used primarily in the cultivation of corn and potatoes in Germany. It is very hydrophobic ($K_{ow} > 6$) and has a strong tendency to sorb to soil particles ($K_{oc} = 215,000$) (Kelley, 2004). It is highly toxic to non-target freshwater invertebrates in both laboratory (Beketov, 2004) and outdoor test systems (Lozano et al., 1992).

The concentrations of esfenvalerate for the experiment were chosen on the basis of the results of standard 48-h acute toxicity tests with *Daphnia magna* (OECD, 2004). Specifically, three concentrations were selected. The medium concentration was approximately equal to the 48-h median lethal concentration (LC_{50}) for *D. magna*, whereas the low and high concentrations were an order of magnitude lower and higher than the medium concentration, respectively. A preliminary test with *D. magna* resulted in a 48-h LC_{50} value of $0.37 \mu\text{g/L}$ esfenvalerate (95% confidence interval: 0.08–1.78), which was consistent with the literature (Fairchild et al., 1992). Thus, the nominal concentrations in the present study were 0.03, 0.3, and $3 \mu\text{g/L}$. The solutions were prepared by diluting Sumicidin Alpha in dimethylsulfoxide (DMSO); the final volume of DMSO in the microcosms was far below the level recommended by OECD (2000). The concentrations applied in the study reflect concentrations in natural waterbodies, which range from trace concentrations to $0.1 \mu\text{g/L}$ esfenvalerate (Bacey et al., 2005; Brady et al., 2006).

Esfenvalerate can be degraded photolytically. As a consequence, to ensure equal exposure in shaded and unshaded microcosms, contamination was carried out after sunset on 7 July, 2008. To measure the actual concentrations of esfenvalerate, water samples were collected in 1-L brown glass bottles at 2, 9, 16, 24, 48, and 168 hours after contamination. For each concentration, 12 samples were taken. The samples were subjected to solid-phase extraction using Chromabond C18 Hydra columns (Machery-Nagel, Düren, Germany), followed by gas chromatography/mass spectrometry (VARIAN CP-3800 gas chromatograph/VARIAN 2100T mass spectrometer, columns: RTX5 RESTEK) with single ion monitoring. The limit of detection was $0.01 \mu\text{g/L}$.

2.3.4 Macroinvertebrates and environmental parameters

Macroinvertebrate sampling was conducted on a weekly basis starting one week after the last introduction of macroinvertebrates, on 11 June, 2008. The last sampling was performed on 17 September, 2008. Macroinvertebrates were sampled using a PVC tube (length: 31.7 cm; radius: 3.55 cm) with a lid. The tube was lowered quickly through the water column and closed with the lid which was positioned in the centre of the bottom of the microcosm. Before sampling, the water column was mixed carefully to distribute the organisms evenly. The content of the tube was filtered through a sieve ($180\text{-}\mu\text{m}$ mesh size) and the organisms were preserved directly in 70% ethanol. The cladocerans, copepods, ostracods, and insects in the samples were counted. Organisms were identified to the level of class (Ostracoda, Arachnida), order (Odonata, Copepoda), or genus (Cladocera, Chaoboridae, Culicidae, Baetidae).

Electrical conductivity (EC) (HI-98312; Hanna Instruments, Woonsocket, RI, USA), pH (HI-98127; Hanna Instruments, Woonsocket, RI, USA), and dissolved oxygen (DO) concentration (WTW Multi 340i Meter; WTW Instruments, Weilheim, Germany) were measured on a weekly basis. The measurements were carried out in the morning (between

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8 a.m. and 11 a.m.) in the centre of the microcosms at a depth of approximately 5 cm. Water temperature was recorded every hour using Handylog DK501-PL data loggers (Driesen & Kern, Bad Bramstedt, Germany). Total UV [A + B] intensity was determined by measuring the solar radiation just above the water surface on two different days using a UV-VIS radiometer RM-21 (Dr. Gröbel UV-Elektronik GmbH, Ettlingen, Germany). All measurements were carried out in a subsample of 32 microcosms, except for temperature, which was measured in six microcosms, and UV radiation, which was measured above the water surface of a random microcosm.

2.3.5 Data analyses

The effects of esfenvalerate on the structure of macroinvertebrate communities were analysed by the principal response curve (PRC) method followed by a set of redundancy analyses (RDA). The PRC method (van den Brink and Ter Braak, 1999) is a multivariate technique that is based on the RDA ordination technique. This technique was developed especially for the analysis of experimental communities in studies that involve repeated sampling over time, and currently is considered to be a standard method for mesocosm studies (de Jong et al., 2008). The statistical significance of the PRC models (first and second principal components), in terms of displayed treatment variance, was tested by Monte Carlo permutation tests that were performed for the entire time series, using an F-type test statistic based on the eigenvalue of the components (Lepš and Šmilauer, 2003; van den Brink and Ter Braak, 1999). Not all the PRCs that were based on the second component were significant and the variance explained by the second component was marginal compared with that explained by the first component. As a consequence, we only considered PRC models based on the first principal component.

RDAs with nominal toxicant concentration ($\log_{10}(x+1)$ -transformed) as the only explanatory variable were applied, and then Monte Carlo permutations for each sampling date and toxicant concentration were performed to test the statistical significance of toxicant effects at different concentrations of toxicant and different time points. This was carried out to deduce the lowest-observed-effect concentration (LOEC) and no-observed-effect concentration (NOEC) (Beketov et al., 2008). In the present study, we considered the NOEC to be the highest tested concentration at which no significant negative effects on the community structure were observed. The LOEC is the lowest tested concentration at which significant negative effects occur. Additional PRC analysis was conducted with a dataset for macroinvertebrates that had not been exposed to contamination (i.e. controls only) and "No Shadow – No Harvesting" as a control treatment to examine the influence of the different environmental conditions on the macroinvertebrate community structure. Before all multivariate analyses, abundance data were ($\log_{10}(4x+1)$)-transformed to avoid false discrepancies between zero abundance values and low abundance values (for rationale, see van den Brink et al., 2000).

To understand the nonmonotonicity of the dose–response relationship that appeared in the PRC analysis of the treatment "No Shadow – No Harvesting" (stronger effect at a low concentration than at a medium concentration), the taxa delineated as the organisms most affected by this PRC were analysed and the taxa that were mainly responsible for this anomaly were identified. For graphic illustration, the abundance data were centred by the division of the $\log_{10}(x+1)$ -transformed abundance data for the contaminated microcosms

at each time point and concentration by the transformed data for the uncontaminated microcosms at the corresponding time point and by the subtraction of 1 from this value. To test for differences in taxon abundance between control and contaminated microcosms, the Kruskal–Wallis multiple comparison test was conducted for each time point with log-transformed abundance data. The environmental parameters such as EC, pH, DO concentration, and daily average water temperature are presented as three-point moving averages. To test for significant differences pairwise *t*-tests were conducted for each time point using untransformed data. Multivariate analyses were performed with CANOCO 4.5 for Windows (Wageningen, Netherlands). The other analyses were carried out with the free software R, version 2.10.1 for MAC OS X (<http://www.r-project.org/>).

2.4 Results

2.4.1 Esfenvalerate exposure dynamic

The actual concentrations of esfenvalerate measured in the microcosms were approximately within the range of the nominal concentrations (Table 2.1). The lowest concentration could only be detected at the 2-hour time point and had dropped below the detection limit at 9 hours after contamination. The high and medium concentrations declined rapidly over the first 16 hours after contamination, on average to 14% and 57% of the concentration measured 2 hours after contamination, respectively. In the microcosms exposed to either the medium or high concentration, esfenvalerate was not detected one week after contamination (Table 2.1). The concentrations showed no significant differences between the three treatments ($P > 0.05$, ANOVA).

2.4.2 Environmental parameters

The shaded and unshaded microcosms differed with respect to environmental characteristics (Fig. 2.1). The awning reduced the daily average water temperature (Fig. 2.1 D), as well as the amplitude of diurnal variations in temperature (Fig. 2.1 E). The daily average water temperature was significantly lower in the shaded microcosms than in the unshaded ones ($P < 0.001$). Similarly, the intensity of UV radiation differed greatly both among sunny days and among cloudy days between the two types of microcosm (Fig. 2.1 F). The shaded microcosms exhibited significantly lower pH and DO concentration (Fig. 2.1 B and C, respectively) and higher EC (Fig. 2.1 A) than the unshaded microcosms, which suggested lower photosynthetic activity and algal density in the shaded microcosms (for mechanisms, see Falkowski, Raven, et al., 2007; Kirk, 1994). In addition, chlorophyll *a* concentrations were measured as a measure of algal density. However, these measurements failed to reflect the productivity of the algae owing to the interdependence between the production of algae and their consumption by phytophagous organisms.

2.4.3 Effects on the structure of the macroinvertebrate community

To analyse the response of the macroinvertebrate community to esfenvalerate, we performed PRC analyses for each treatment: "No Shadow - Harvesting", "No Shadow - No Harvesting", and "Shadow - No Harvesting". The graphs of the first PRCs (Fig. 2.2) show little variation before contamination and clear concentration-dependent deviations from the control after contamination. Statistical significance of the first PRCs was confirmed by Monte Carlo permutation tests ($P < 0.05$). In all treatments, the highest concentration had the strongest effect on the structure of the community, with no recovery during the observation period. Most of the taxa present exhibited positive taxon scores (b_k), which indicated that the insecticide had a negative effect on their abundance. According to the PRC analyses, the most affected species were *Daphnia* sp. and *Ceriodaphnia* sp.

The strength of the effect in terms of the concentration level that was found to cause statistically significant alterations in the community structure differed considerably between the three treatments. The strongest effect of the toxicant was observed with the treatment "Shadow - No Harvesting". With this treatment, the low and medium concentrations of esfenvalerate had significant effects ($P < 0.05$) on community structure until 11 and 16 days after contamination, respectively (Fig. 2.2 C). The weakest effect of esfenvalerate was found with the "No Shadow - Harvesting" treatment. In this treatment, only the highest concentration of insecticide resulted in significant differences ($P < 0.05$) in community structure as compared with the control series (Fig. 2.2 A). The treatment "No Shadow - No Harvesting" (Fig. 2.2 B) exhibited intermediate sensitivity as compared with the other two treatments. In this treatment, effects of the toxicant on community structure were detected at the lowest concentration, as for the treatment "Shadow - No Harvesting", but in contrast to the latter treatment, no significant effect was detected with the medium concentration (for possible mechanisms, see section 2.4.4).

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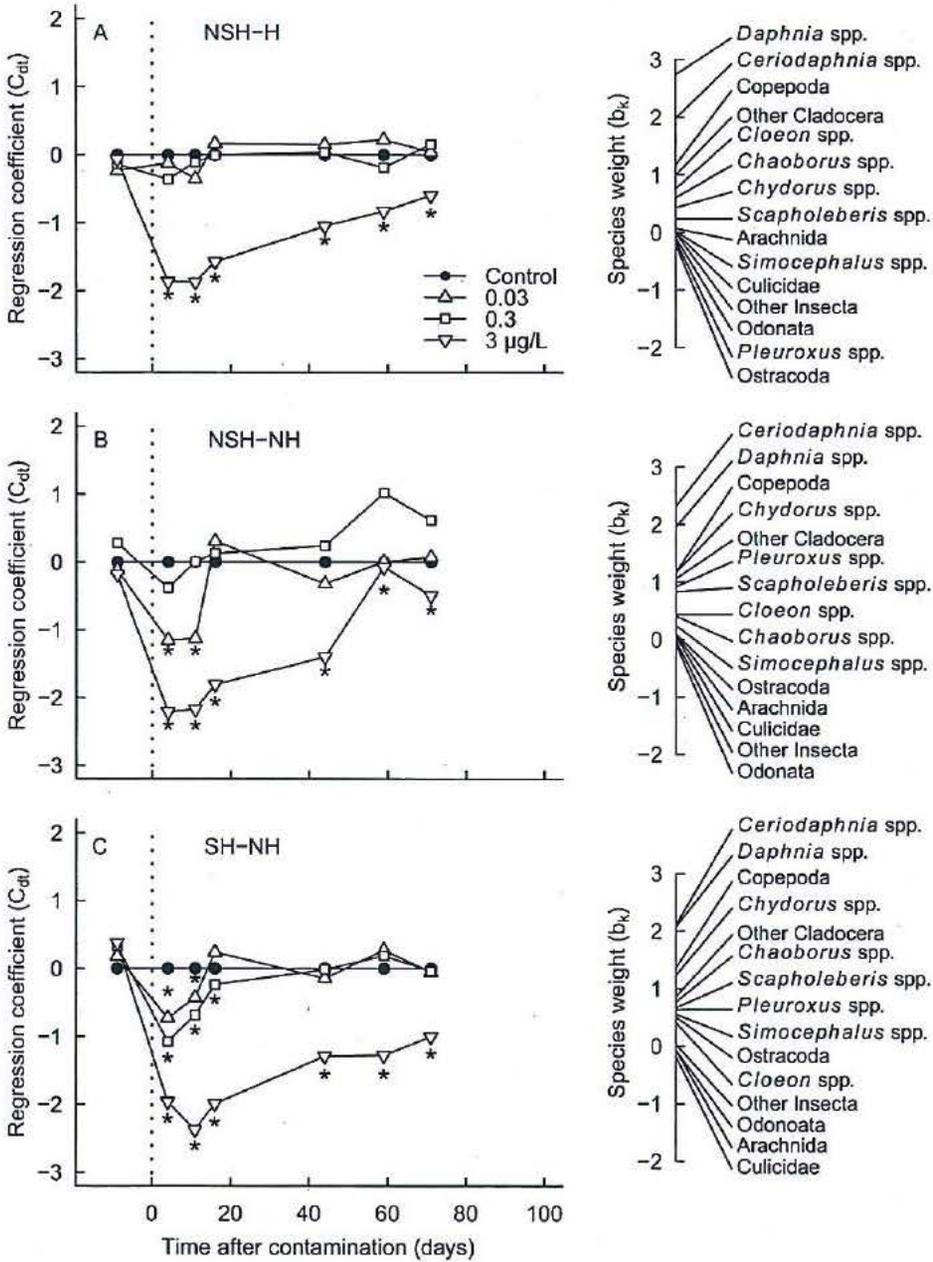


Figure 2.2: Principal response curves (PRC) that indicate the effect of the insecticide esfenvalerate on the macroinvertebrate community under the three experimental treatments (A-C). Asterisks indicate significant effects of the toxicant at particular concentrations ($P < 0.05$, Monte Carlo permutation test following RDA). The vertical dashed line indicates the time of contamination. The abbreviations are the same as in Figure 2.1.

Table 2.1: Residue analysis of esfenvalerate

| Time after contamination (h) | Mean measured concentrations of esfenvalerate ± standard deviation (n=3) at different time points after contamination | | | | | | | | |
|------------------------------|---|-----------|-------|-----------|-----------|-----------|-----------|-----------|-----------|
| | Nominal concentration (µg/L) | | | | | | | | |
| | 0.03 | | | 0.3 | | | 3 | | |
| | NSH-H | NSH-NH | SH-NH | NSH-H | NSH-NH | SH-NH | NSH-H | NSH-NH | SH-NH |
| 2 h | 0.01±0.02 | 0.01±0.01 | 0±0 | 0.09±0.06 | 0.11±0.13 | 0.06±0.03 | 2.28±1.19 | 1.21±0.44 | 1.77±0.83 |
| 9 h | ND | ND | ND | 0.10±0.10 | 0.12±0.02 | 0.10±0.12 | 1.42±0.68 | 1.62±0.79 | 1.40±0.84 |
| 16 h | ND | ND | ND | 0.05±0.02 | 0.02±0.03 | 0.04±0.06 | 0.22±0.11 | 0.21±0.07 | 0.33±0.12 |
| 24 h | ND | ND | ND | 0.02±0.02 | 0±0 | 0.01±0.01 | 0.18±0.07 | 0.16±0.05 | 0.20±0.02 |
| 48 h | - | - | - | 0±0 | 0±0 | 0.01±0.01 | 0.15±0.08 | 0.16±0.05 | 0.18±0.04 |
| 168 h | - | - | - | ND | ND | ND | ND | ND | ND |

ND - Not detected (values below detection limit of 0.01 µg/L)
 NSH-H - No Shadow - Harvesting
 NSH-NH - No Shadow - No Harvesting
 SH-NH - Shadow - No Harvesting

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Table 2.2: Lowest-observed-effect concentrations (LOEC) and no-observed-effect concentrations (NOEC) for different sampling dates, derived by Monte Carlo permutation tests following redundancy analyses. The abbreviations are the same as in Table 2.1.

| Time after contamination (days) | LOEC ($\mu\text{g/L}$) | | | NOEC ($\mu\text{g/L}$) | | |
|---------------------------------------|--------------------------|--------|-------|--------------------------|--------|--------|
| | NSH-H | NSH-NH | SH-NH | NSH-H | NSH-NH | SH-NH |
| -9 | NA | NA | NA | NA | NA | NA |
| 4 | 3 | 0.03 | 0.03 | 0.3 | < 0.03 | < 0.03 |
| 11 | 3 | 0.03 | 0.03 | 0.3 | < 0.03 | < 0.03 |
| 16 | 3 | 3 | 0.3 | 0.3 | 0.3 | 0.03 |
| 44 | 3 | 3 | 3 | 0.3 | 0.3 | 0.3 |
| 59 | 3 | 3 | 3 | 0.3 | 0.3 | 0.3 |
| 71 | 3 | 3 | 3 | 0.3 | 0.3 | 0.3 |

NA – not applicable

The LOECs and NOECs were calculated for each of the observation time points and are summarized in Table 2.2. The maximum differences between the calculated values of NOECs and LOECs for the different treatments were more than a factor of 10 and 100, respectively Table 2.2. Interpretation of these values, however, should take into account the distances between the concentrations tested, and therefore the ranges between the values rather than precise numbers should be considered (e.g. the LOEC of 3 $\mu\text{g/L}$ means that the actual LOEC could be found in the range from > 0.3 to 3 $\mu\text{g/L}$, as the concentrations tested are 0.03, 0.3, and 3 $\mu\text{g/L}$). PRC analyses performed with data for microcosms that were not subjected to contamination (controls) showed no significant differences ($P > 0.05$) in community structure between the treatments over the entire experimental period. Hence, manipulation of the environmental parameters did not cause significant alterations in the structure of the plankton community in the absence of the toxicant.

2.4.4 Limitations of the community approach

Analyses of the structure of the entire community by PRC revealed basic patterns in the reaction of the entire community to the pesticide. However, in the treatment “No Shadow – No Harvesting” (Fig. 2.2 B), surprisingly the PRC analysis indicated that the toxicant had no significant effects at the medium concentration, whereas the effects at the low and high concentrations were significant. To reveal the causes of this lack of effect, we analysed the dynamics of individual taxa that showed highly (≥ 1) positive species weights (b_k) in this PRC, and therefore were considered to be the taxa affected most strongly by this treatment. *Daphnia* sp. and *Ceriodaphnia* sp. exhibited the highest species weights (Fig. 2.2), which were distinctly different from those of the other taxa. When analysed individually, these two taxa showed markedly different dynamics. *Daphnia* sp. showed a clear dose-response relationship in all the treatments. In contrast, in the treatment

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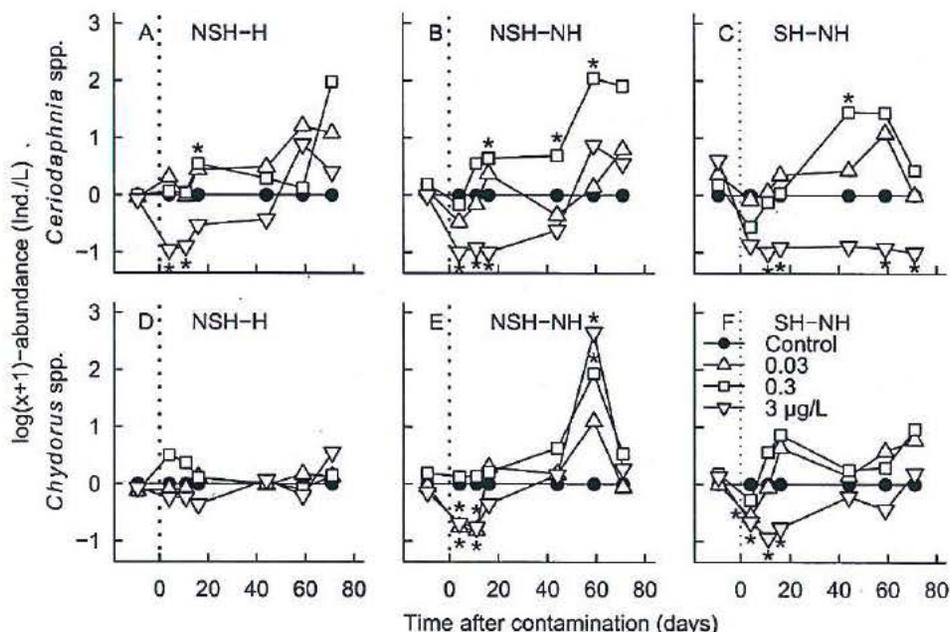


Figure 2.3: Abundance of *Ceriodaphnia* sp. (A–C) and *Chydorus* sp. (D–F) in the three experimental treatments. Abundance was $\log_{10}(x+1)$ -transformed and standardised as a deviation from the control. Asterisks indicate significant effects of the toxicant at particular concentrations ($P < 0.05$, Kruskal–Wallis multiple comparison test with $\log_{10}(x+1)$ -transformed abundance data). The vertical dashed line indicates the time of contamination. The abbreviations are the same as in Figure 2.1.

“No Shadow – No Harvesting”, the populations of *Ceriodaphnia* sp. showed pronounced positive, and obviously indirect, effects shortly after the initial negative effect, as well as rapid recovery, at all of the concentrations tested except $3 \mu\text{g/L}$ (Fig. 2.3 A–C). Similar, but less significant, positive effects were also found for *Chydorus* sp., which was also assigned a highly positive species weight in the PRC analysis for the treatment “No Shadow – No Harvesting”. Clearly, such positive effects on *Ceriodaphnia* sp. and *Chydorus* sp. at the medium concentration of esfenvalerate under the treatment “No Shadow – No Harvesting” (Fig. 2.3 D–F) can explain, at least in part, the absence of significant effects at the medium concentration derived by the PRC-RDA analysis and the presence of significant effects at the high and low concentrations (Fig. 2.2 B). In general, the dynamics of *Ceriodaphnia* sp. and *Chydorus* sp. also reveal that these two taxa tend to be less sensitive than assumed by the PRC analysis. As a consequence, the single-taxon sensitivity and dynamics derived from the observed PRC patterns should be interpreted carefully.

2.5 Discussion

The present study shows that environmental context can strongly modulate the sensitivity of a biological community to a pesticide. Significant effects of the insecticide on the community structure were observed at different levels of the tested concentrations. Thus, in the experimental series with the lowest availability of food and strongest competition significant effects of the insecticide were found at the concentration of 0.03 $\mu\text{g/L}$. In contrast, in the series with relatively higher food availability and weak competition such effects were detected at 3 $\mu\text{g/L}$ only. Remarkably, these differences were observed immediately after the contamination. To our knowledge, such a difference in community sensitivity has not been shown previously by studies that focused on the combined effects of environmental factors and toxicants.

The effects of environmental context on sensitivity to toxicants have been investigated actively for individual species, but rarely for communities. It is well known that both abiotic factors, such as temperature, salinity, and UV radiation (Brecken-Folse et al., 1994; Duquesne and Liess, 2003; Lydy et al., 1999; Preston et al., 1999), and biotic factors, such as food supply, competition, predation, and parasitism (Beketov and Liess, 2006; Coors and De Meester, 2008; Hanazato, 1991), can significantly increase the sensitivity of individuals and populations to toxicants. Furthermore, it was found that a combination of abiotic and biotic stressors can cause a particularly strong increase in the sensitivity of individuals to toxicants (Barry, 1997; Hanazato and Dodson, 1995; Liess et al., 2001; Relyea, 2006).

Despite the large number of diverse investigations that have focused on different environmental factors and toxicants, there have been only two studies that have quantitatively analysed changes in community sensitivity due to the experimental manipulation of the environmental context (Roessink et al., 2005; van Wijngaarden et al., 2005a). As mentioned in the introduction, these studies provided valuable insights into alterations in community sensitivity by environmental conditions. However, in both of these studies, the ultimate limits of community sensitivity were not altered and the differences in sensitivity were transient and based on different recovery rates. This stands in contrast to the present study that revealed differences in the immediate effect rather than recovery. Remarkably, in the study by Roessink et al. (2005), community structure differed between the treatments on all sampling days. Therefore, the communities had clearly already adapted to the different environmental conditions. The results of these two studies indicated that environmental context does not have an important role in determining community sensitivity. These findings were supported further by comparisons of community sensitivity among different geographical regions that are characterised by different environmental conditions (Daam et al., 2009; López-Mancisidor et al., 2008a, 2008b; Schäfer et al., 2007); community sensitivity varied little between the regions considered.

In contrast to these earlier investigations, the results of the present study reveal strong and consistent alterations in community sensitivity as a consequence of environmental context. In the present study, the communities had not adapted to the environmental conditions. They were exposed to the toxicant shortly after the manipulation of the environmental factors was started. This lack of adaptation might have contributed to the differences in the changes in sensitivity between the present study and the previous experiments mentioned above. Such results are in line with previous studies that highlighted

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the importance of population dynamics (e.g. stages and trends in the dynamics, development stages, presence/absence of predators), as a factor that may alter sensitivity and recovery dynamics of the populations affected by toxicants (e.g. reviews by Fleeger et al., 2003; Hanazato, 1998).

The magnitude of the alteration due to environmental factors that was observed in the present study was comparable to the results of long-term (in terms of the life cycles of the test organisms) studies on population levels. The latter studies showed that sensitivity to a given toxicant was changed by up to a factor of 100 (chronic survival of caddisflies, Liess, 2002) or even 1000 (fecundity of mayflies, Beketov and Liess, 2005) by food limitation and intraspecific competition. Strong effects of food limitation and competition on the sensitivity of populations were also shown for *Daphnia pulex* and the insecticide carbaryl (Hanazato and Hirokawa, 2004). Furthermore, exacerbating effects of these parameters were also found for the dynamics of recovery of *Daphnia magna* (Hanazato, 1998; Liess and Foit, 2010).

The present study is similar to the experiments mentioned above in terms of the factors that affect the sensitivity. Thus, in the present study, the different treatments were designed to set up different levels of food availability and competition for food between the phytophagous zooplankton organisms, and thus to induce subsequent differences in the entire community at all trophic levels present. The treatment "Shadow - No Harvesting", which was designed to produce the lowest level of available food and the strongest competition, resulted in the highest level of sensitivity. In contrast, the treatment "No Shadow - Harvesting", which was designed to produce the highest level of available food and the weakest competition, resulted in the lowest level of sensitivity. Analysis of the DO concentration and pH indicated that photosynthetic activity, and thus productivity, was higher in unshaded microcosms than in shaded microcosms, which confirmed that the design resulted in the expected differences in food availability between treatments. Such relationships of light and temperature with algal photosynthesis and productivity, as well as between photosynthesis, DO concentration, and pH, are described well in the literature (Falkowski, Raven, et al., 2007).

An alternative mechanism that could explain the observed differences in sensitivity between treatments is the occurrence of different exposure dynamics due to differences in water temperature and algal concentrations. Under conditions of increased water temperature, the compound might be degraded more rapidly (Müller et al., 1998), whereas the amount of esfenvalerate sorbed to algae might increase with increased algal concentration (Day and Kaushik, 1987). However, as mentioned in section 2.4.1, the measurement of esfenvalerate concentrations in the water revealed no statistically significant differences between the treatments. Therefore, even if there were some slight differences in concentration that were not detected, these differences evidently cannot explain the observed pronounced changes in community sensitivity. Taking all this into account, we can conclude that the present results support those of the previous studies that focused on food availability and competition, and confirm that these biotic factors might be of crucial importance as determinants of the sensitivity of populations and communities to toxicants. Importantly, the present study also demonstrates a link from the abiotic (sunlight) to the biotic environment (food availability), and finally to the sensitivity of the zooplankton community.

Regarding the duration of the effects on the community that were observed in the

present study, the effects lasted until the end of the experiment (71 days) for the high concentration of esfenvalerate, and up to 11 and 16 days for the low and medium concentrations, respectively (the duration varied between treatments, Fig. 2.2). Although the duration of the effects observed at the medium concentration (which corresponds approximately to the 48-h LC_{50} value of *Daphnia magna*, as mentioned in section 2.3.3) was not long, it corresponds approximately to the average generation time of all the crustaceans (except copepods and ostracods) present in the microcosms studied (for the mechanisms that underlie these long-term effects, see Knillmann et al., submitted). Therefore, similar effects on organisms with longer generation times might cause alterations of much longer duration (Beketov et al., 2008; Sherratt et al., 1999). For example, aquatic insects that have uni- and semivoltine life cycles, such as stoneflies and mayflies, might be affected for periods of time that are comparable to the intervals between the annual application of pesticides. As a consequence, they could easily become extinct in the contaminated areas (Beketov et al., 2008; Liess et al., 2008).

The present findings highlight the importance of environmental context in determining the sensitivity of communities to toxicants, and thus reinforce the numerous studies on single organisms and populations of single species. As a consequence, they highlight the fundamental role of environmental context in ecotoxicological evaluation. With respect to the risk assessment of pesticides and other toxicants, the findings reported herein have two important implications. First, the design and standardisation of higher-tier risk assessment studies (i.e. mesocosms, de Jong et al., 2008) should take into account the abiotic and biotic environmental context, and especially food availability and competition, which can be affected by abiotic parameters. The mechanisms that underlie the alterations in community sensitivity should be understood and taken into account in higher-tier risk assessment studies. Second, the safety factors that are applied to derive ecologically safe concentrations on the basis of both standard laboratory toxicity tests and mesocosm experiments should take into account the differences in community sensitivity between different habitats, climates, and regions. Important factors that should be considered in this respect are habitat stability, the frequency of disturbances, and the "novelty" of the stressors (i.e. whether the community has been exposed previously to the stressor), which determine the adaptation of the community. In turn, the degree to which the community has adapted might crucially determine the extent of the changes in sensitivity that are caused by the environmental factors. The practical realisation of these implications requires extensive research, which should include not only experiments with model systems, but also field monitoring studies aimed at understanding parameters and sensitivities that are specific to systems or regions (Liess et al., 2008). At the same time, it will help us to improve profoundly the ecological realism of risk assessment and to explain existing discrepancies between the outcomes of toxicological evaluations performed in laboratory, mesocosm, and field studies (Beketov et al., 2008).

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(HIGRADE).

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Chapter 3

Community sensitivity to a xenobiotic can be predictable – An evidence from a cross-Eurasia experiment

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in preparation

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3.1 Abstract

Community ecology has the reputation as science with limited predictive potential. Responses of biological communities to stressors are frequently considered as unpredictable and irreproducible. To challenge these assumptions we performed an experiment with artificial ponds located in Central Europe (Germany) and Northern Asia (Southwestern Siberia, Russia). In both regions the zooplankton communities were similarly exposed to an insecticide under different regimes of solar radiation and periodical harvesting, which represented different regimes of food availability and competition. The results showed unprecedented consistency of the community sensitivity modulations between the regions, with the lowest-observed-effect concentration differing by a factor of up to 100. Furthermore, the results evidenced that integrative parameters such as community sensitivity can be predictable, while detailed changes in taxonomic community structure cannot.

One sentence summary: Overall community sensitivity to a xenobiotic as an integrative endpoint can be predictable and consistent even between continents.

3.2 Main text

The primary aim of basic and applied ecology is to elucidate predictable patterns and regularities in reaction of complex ecological systems, such as communities or ecosystems, to their environment. Predictive potential of ecology, particularly community ecology, has been frequently questioned, as most of the community-level phenomena are specific to the taxa involved and the environmental conditions present at the time/place of the experiment or observation (Ghilarov, 2001; Lawton, 1999; Murray, 2000). This makes the ecological results irreproducible and limits their implications beyond the system in which they were found (van Straalen, 2003).

In ecology there are two main points of view explaining the low predictability of the ecological phenomena. The first one states that ecological systems are too complex, have too many interacting components, are very sensitive to external conditions, and are therefore intrinsically unpredictable (Egler, 1970; Lawton, 1999; Simberloff, 2004). The second one suggests that unpredictability of the ecological systems originates from the too complex questions asked, i.e. the complexity is not in the object, but rather in the question, and ecologists tend to not simplify their study objects and the questions asked (McGill et al., 2006; Turchin, 2001). Examples supporting the latter point of view include approaches aimed to describe biological communities using general and widely comparable parameters, i.e. traits and trait-based approaches (e.g. functional traits research program McGill et al., 2006) or functional and integrative parameters based on large-scale observations (Brown, 1995). Thus, the questions “what should be the trait characteristics of the vegetation?” or “what should be the nitrification rate?” have considerably higher chances to yield predictable patterns in comparison to the question “what should be the detailed taxonomic structure of a community?” (Fukami et al., 2005).

Despite the growing number of evidences supporting the latter of these two points of views (Kerr et al., 2007; McGill et al., 2006), the reputation of ecology as a predictive science remains weak. The main reason is that approaches that are specifically focused

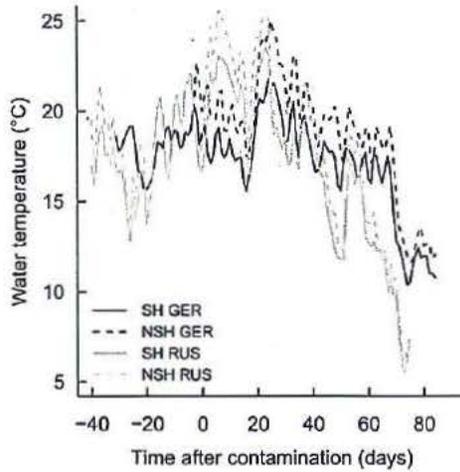


Figure 3.1: Daily average water temperature (°C) in the microcosms in Europe and Siberia and the treatments with (SH) and without shadow (NSH).

on reproducible and predictable parameters are still new and need further conceptual development and empirical support. Such parameters remain to be identified in various ecological sub-disciplines, such as for example sensitivity endpoints in ecotoxicology.

This problem results in disregard of ecological processes at community and ecosystem levels in such applied ecological discipline as ecological risk assessment of toxicants (Beke- tov and Liess, 2012). Currently, the risk assessment is mainly based on laboratory toxicity tests carried out at the (sub)organism level and also, to a lesser degree, experiments on simple artificial ecosystems. Results of such tests are used to predict the effects of con- taminants on the real-world ecosystems and entire landscapes, and the population-, and community-level processes are simply considered as unpredictable and unknown. To pre- vent unexpected effects of the toxicants due to these unknown processes risk assessment employ arbitrary taken coefficients called safety factors (Suter, 2007). This approach hav- ing poor empirical basis was repeatedly questioned for its low ecological realism, due to discrepancies between the predicted and field-observed effects (Beke- tov et al., accepted; Schäfer et al., 2012).

Contrary to the current practice-based paradigm in the ecological risk assessment, a theoretical point of view rather suggests that the overall community sensitivity can be more predictable than detailed taxonomic endpoints, as it is a univariate integrative parameter based on multiple species responses (i.e. similar to the functional and macro- ecological endpoints mentioned above). However, despite clear practical and fundamental scientific relevance, predictability and reproducibility of community sensitivity to xenobi- otics have not been investigated so far.

The aim of the present study was to investigate how the environmental context can modulate the overall community sensitivity to a xenobiotic in two regions that are located remotely from each other. To achieve this aim we performed a cross-continental experi- ment with series of artificial ponds located in Germany, (Central Europe) and Southwest- ern Siberia, Russia (Northern Asia). To measure the overall community sensitivity we

3. Community sensitivity to a xenobiotic can be predictable – An evidence from a cross-Eurasia experiment

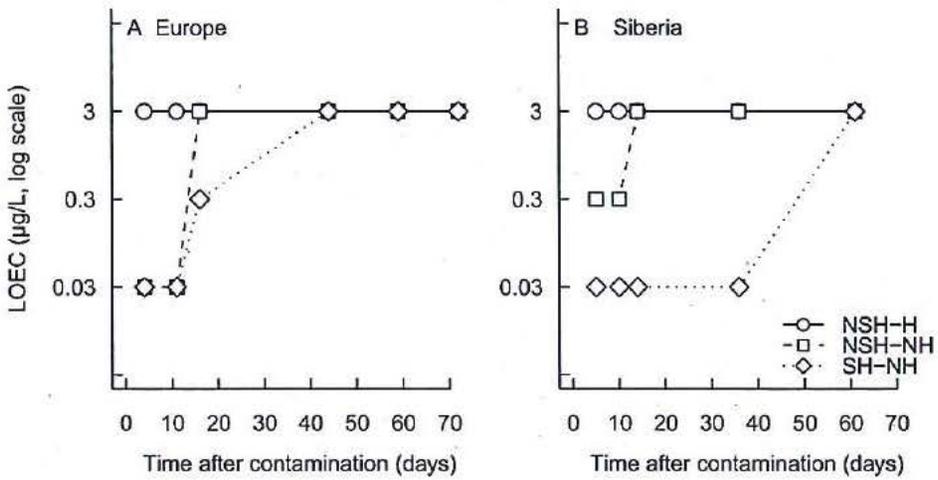


Figure 3.2: Community sensitivity to the insecticide esfenvalerate in the two regions (A – Europe and B – Siberia) and the three experimental treatments (NSH-H, NSH-NH, and SH-NH stand for the treatments “No Shadow – Harvesting”, “No Shadow – No Harvesting”, and “Shadow – No Harvesting”, respectively). The sensitivity expressed as Lowest-Observable-Effect Concentrations (LOEC) based on the nominal applied concentrations and derived by Monte Carlo permutation tests following the multivariate Redundancy analyses of the community structure.

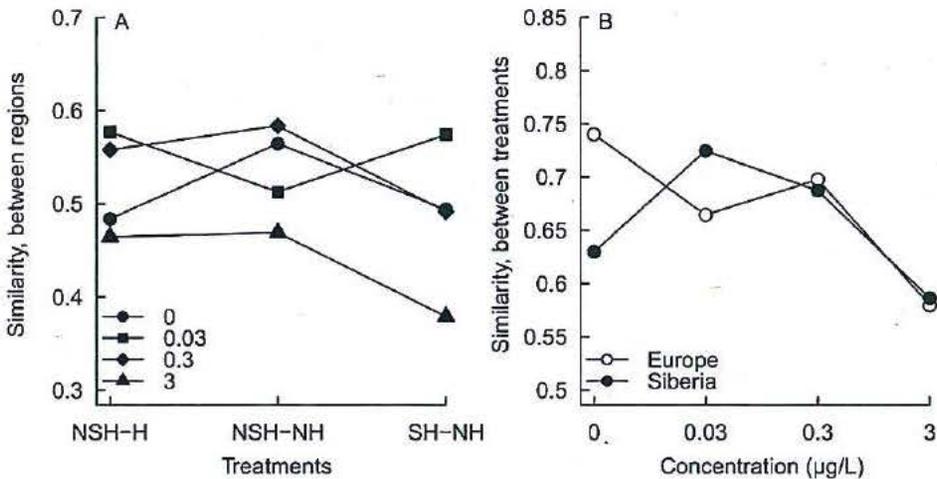


Figure 3.3: Similarities (Bray-Curtis Index) in the zooplankton communities between the two regions (A) calculated for the three studied treatments (NSH-H, NSH-NH, and SH-NH, for explanations see Fig. 3.2) and four esfenvalerate concentrations (µg/L), and between the three treatments (B) calculated for the each region.

used the lowest-observed-effect concentration (LOEC) based on multivariate Redundancy

Analysis (RDA). In both regions the zooplankton communities were exposed to the insecticide esfenvalerate under different regimes of solar radiation and periodical harvesting, which represented different regimes of food availability and competition. The treatments were No Shadow – Harvesting (NSH-H), No Shadow – No Harvesting (NSH-NH), and Shadow – No Harvesting (SH-NH). Manipulation of these parameters was started shortly before contamination to prevent adaptation of the communities, and the strength of the manipulations was selected to cause only subtle alteration in the structure of the plankton community (Stampfli et al., 2011). The two regions selected for the study do not differ drastically from each other in terms of the summer weather conditions (Fig. 3.1) and taxonomic composition of the zooplankton (Table S 3.3, Supplementary materials), and the moderate dissimilarity between the regions was particularly appropriate for the comparison performed.

Analysis of the overall community sensitivity detected pronounced differences between the treatments. Remarkably, these differences were consistent between the two study regions, with the LOECs similarly differing by a factor of up to 100 (0.03 – 3 $\mu\text{g/L}$, Fig. 3.2). Thus, in both the regions in the SH-NH treatment RDAs detected significant ($P < 0.05$) effects at concentrations down to 3 $\mu\text{g/L}$. In contrast, in the NSH-H significant effects were only identified at a concentration of 3 $\mu\text{g/L}$ in both the regions. The NSH-NH treatment was characterized by intermediate sensitivity in comparison to the other two treatments (Fig. 3.2). Despite the differences in the duration of the effects (recovery was slower in Siberia, Fig. 3.2), these outcomes show that both in Europe and Siberia the communities occurring in the most unfavorable food/competition regime (i.e. SH-NH) were the most sensitive. Similarly, the communities in the most favorable conditions (i.e. NSH-H) exhibited the lowest sensitivity in both the regions (Fig. 3.2).

Analyses of the structural similarity between the communities showed that the increased sensitivity in the SH-NH treatment being the same in both regions (LOEC = 0.3 $\mu\text{g/L}$) was based on different regional-specific alterations in the community structure. Thus, the between-regions similarity was lowest in the most affected and most sensitive treatment SH-NH (Fig. 3.3). Besides, the lowest similarity was also detected in the series exposed to the highest toxicant concentration (Fig. 3.3). These results indicate that, when detailed community structure is considered, the communities in the two regions showed different reactions to the toxicant (e.g. see acute effects on the community structures outlined by the RDA ordination plots, Fig. S 3.1, Supplementary materials). Furthermore, dissimilarity in community structure between the regions increased with increasing pesticide impact (Fig. 3.3).

The treatment-specific regimes of shadow and harvesting did not cause any alterations in the community structure when the toxicant was not present. Thus, the RDAs performed with the data from uncontaminated replicates only and the treatments used as the only predictors revealed no statistically significant effects in any region and sampling date ($P > 0.05$).

The present results show that the direction and magnitude of modulation in the sensitivity was stable and reproducible across the regions. At the same time the details of the taxonomic structure of communities were different between the regions, and the communities in the two regions had their "own way" to the observed increased sensitivity. These outcomes represent, to our knowledge, the first experimental evidence that the overall community sensitivity to a stressor can be a highly variable (up to a factor of 100)

yet predictable parameter.

Regarding the discussion of the predictive potential of community ecology, the present results, although cannot provide a final conclusion, yet give a clear example supporting the opinion that unpredictability of the ecological systems originates from the too complex questions asked, rather than from the complexity of the ecological systems (McGill et al., 2006; Turchin, 2001). Thus, our study shows that predictability of the community reaction to a stressor depends on the endpoint considered. It shows unprecedented cross-continental consistence of the LOECs and lack of the consistence in terms of the community structure (i.e. decreased similarity). It also indicates that the overall community sensitivity can be considered as a fairly predicable parameter that is in this respect comparable to the functional and macroecological endpoints that integrate multitudes of small effects into a single parameter (Brown, 1995; Kerr et al., 2007).

The present findings have far-reaching implications for ecotoxicology and ecological risk assessment of toxicants. As mentioned above, the current risk assessment framework is almost exclusively based on the bottom-up approach (Beketov and Liess, 2012). In this scheme the population-, community-, and ecosystem-level processes are simply considered as unpredictable and unknown. This “unknown” is considered in the risk assessment by applying the safety factors, i.e. the arbitrary taken coefficients that are used to decrease the laboratory-derived safe concentrations and therefore to preclude unexpected effects in the natural environment.

Our findings challenge this paradigm, as we show that (i) community sensitivity can vary to the very pronounced magnitude, up to a factor of 100 (see also Stampfli et al., 2011), (ii) this variability can be reproduced and predicted, as it depends on the basic environmental factors. Therefore community-level alterations in the sensitivity cannot be considered as an intrinsically unpredictable noise. The magnitude, direction, basic factors and mechanism that determine the alterations in sensitivity require further investigations. This implies that a transition is needed in risk assessment from the arbitrarily-defined safety factors towards empirically based standards. Such a transition has the potential to significantly increase realism of this ecological practice, which was repeatedly disqualified due to discrepancies between the field-observed effects and predictions based on the laboratory studies (Beketov et al., accepted; Schäfer et al., 2012).

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Supplementary Material

Materials and Methods

Test systems. A set of artificial microcosms were established at the Helmholtz Centre for Environmental Research (UFZ) Leipzig, Germany (51°21'13 N, 12°25'55 E) and the field research station of the Institute for Systematics and Ecology of Animals in Karasuk, Novosibirsk Oblast, Russia (53°44'00 N, 78°02'00 E). Each pond was made of a tank inserted in the soil and with the following characteristics: height of 38 cm, radius of 25 cm, and a total volume of 80 L in Germany and height of 29 cm, sides 36 and 56 cm, and a total volume of 60 L in Siberia. The microcosms were filled with 60 L of tap water. A substrate (1/1 mixture of sediment collected from a natural permanent pond and sand) was added to cover the bottom of each microcosm with a 1-cm layer of sediment. In addition, approximately 10 g of dried shredded fallen leaves were added. To colonize the microcosms, zooplankton from different natural permanent ponds were collected and distributed equally among all the microcosms. For details about the test system in Germany see Stampfli et al. (2011).

Environmental conditions. Solar radiation was regulated by using an awning that was mounted at an angle of approximately 45° to the earth's surface to shield the microcosms mainly during mid-day. At the beginning of the experiment, all the microcosms were shaded to allow the communities to develop in similar conditions. One month after the introduction of zooplankton, the awning was removed from the microcosms that had been allocated to the treatments without shadow (i.e. NSH).

Harvesting was performed using a plankton net (10 × 12 cm, 250- μ m mesh size) which was placed at the bottom of the microcosms and lifted diagonally through the previously mixed water column, thereby sieving a water volume of approximately 6.8 L (corresponding to approximately 10% of the entire community) per movement. Harvesting was conducted twice a week with one and two acts of sieving, to remove approximately 30% of the zooplankton community per week. Harvesting was started 24 days after the introduction of zooplankton.

Contamination. All treatment groups were exposed to the pyrethroid insecticide Sumicidin Alpha (BASF, Limburgerhof, Germany), an emulsified concentrate that contains 50 g/L of the active substance esfenvalerate ((α S)- α -cyano-3-phenoxybenzyl (2S)-2-(4-chlorophenyl)-3-methylbutyrate). Esfenvalerate is very hydrophobic (Kow > 6) and has a strong tendency to sorb to soil particles (Koc = 215.000). It has been proven to be highly toxic to non-target freshwater invertebrates in both laboratory (Beketov, 2004) and outdoor test systems (Lozano et al., 1992).

The concentrations of esfenvalerate for the experiment were chosen on the basis of a preliminary standard 48-h acute toxicity test with *Daphnia magna* (OECD, 2004), revealing a 48-h median lethal concentrations (LC₅₀) value of 0.37 μ g/L. Specifically, three concentrations were selected, the medium concentration being approximately equal to the 48-h LC₅₀ value and the lowest and highest concentrations being an order lower and higher than the medium concentration, respectively. Thus, the nominal concentrations in the present study were: 0.03, 0.3 and 3 μ g/L.

Contamination was carried out 4 and 10 days after the awning was removed and harvesting was started, respectively. Esfenvalerate can be degraded photolitically. To ensure

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equal exposure in shaded and unshaded microcosms, contamination was performed after sunset. The measured concentrations of esfenvalerate applied at high and medium nominal concentrations are given in Fig. S2. For the measurements water samples were collected in 1-L brown glass bottles at 2, 9, 16, 24, 48, and 168 hours after contamination. For each concentration, 12 samples were taken. The samples were subjected to solid-phase extraction using Chromabond C18 Hydra columns (Machery-Nagel, Düren, Germany), followed by gas chromatography/mass spectrometry (VARIAN CP-3800 gas chromatograph/VARIAN 2100T mass spectrometer, columns: RTX5 RESTEK) with single ion monitoring. The limit of detection was 0.01 $\mu\text{g/L}$.

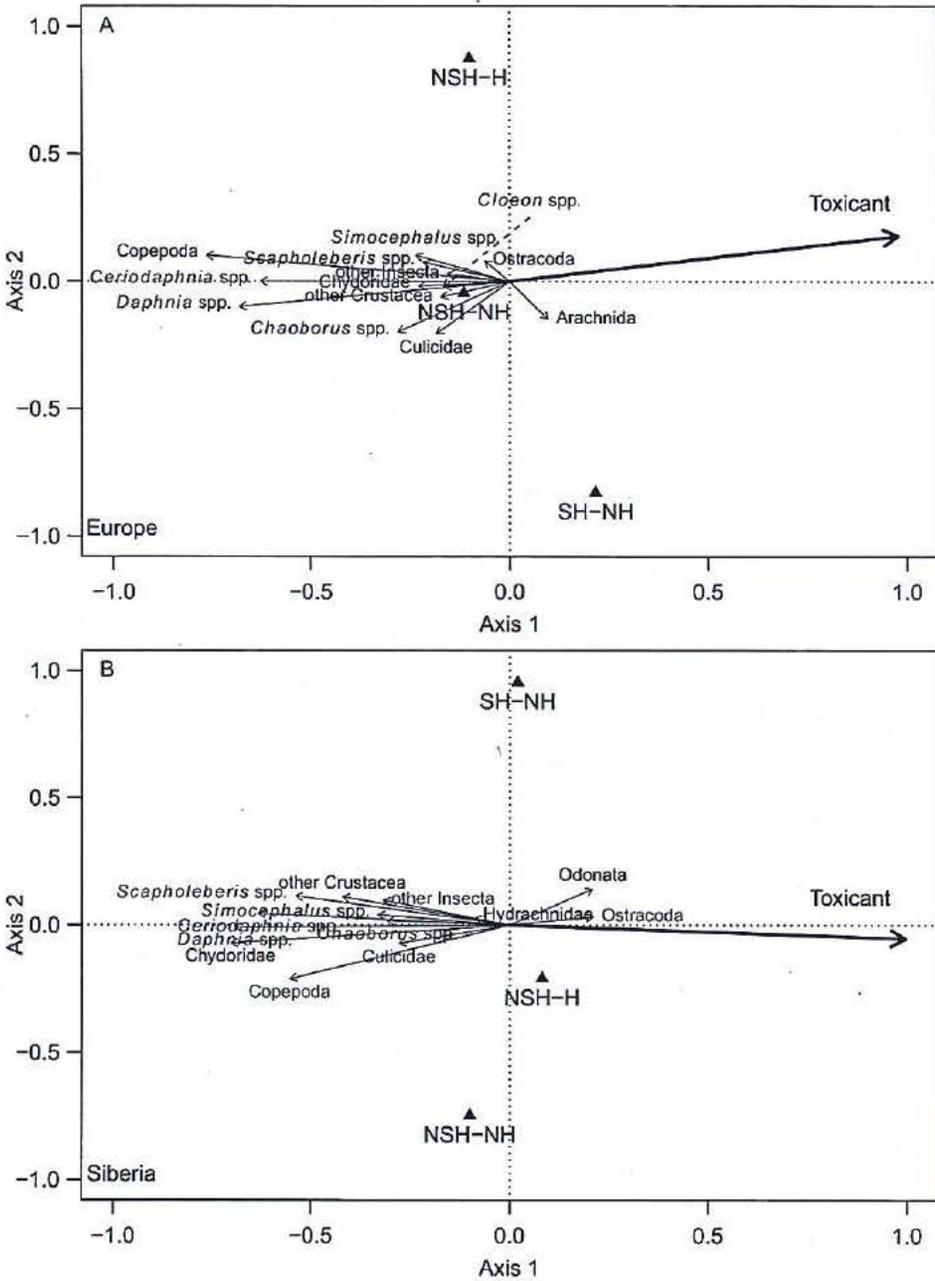
Sampling. Zooplankton was sampled once a week, starting one week after the introduction of zooplankton. Zooplankton was sampled using a PCV tube (length: 31.7 cm, radius: 3.55 cm) with a lid. The tube was lowered quickly through the previously gently mixed water column and closed with the lid, which was positioned in the centre of the bottom of the microcosms. The content of the tube was filtered through a sieve with 180- μm mesh size and the organisms were preserved directly in 70% ethanol.

Data analyses. Prior to analysis, species data were $\ln(4x+1)$ -transformed (for rationale, see van den Brink et al., 2000). The effects of esfenvalerate on the community structure were analyzed using the Redundancy Analysis (RDA). The statistical significance of effects of the toxicant at different toxicant concentrations and time points was tested by RDAs with nominal toxicant concentrations as an explanatory variable, followed by Monte Carlo permutations for each sampling date and toxicant concentration. This approach was used to infer the lowest-observed-effect concentration (LOEC) (Beketov et al., 2008; Stampfli et al., 2011).

Similarities between zooplankton communities were calculated using the Bray–Curtis similarity index. Similarities were calculated between (i) the corresponding treatments in the two regions (e.g. NSH–H Europe vs. NSH–H Siberia), and (ii) the different treatments within each region (e.g. NSH–H vs. NSH–NH in Europe and Siberia). All similarities were calculated for each toxicant concentration and sampling date. Then, similarities were averaged over time.

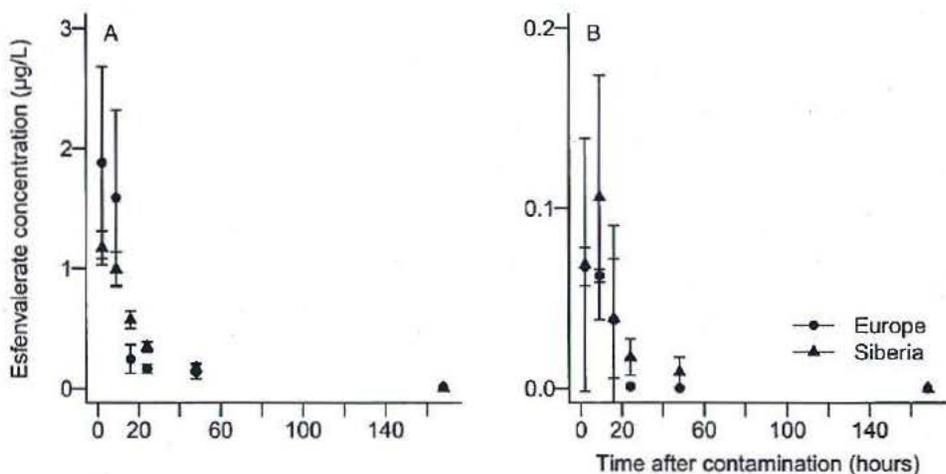
The statistical computations were performed using the open-source software package R, version 2.10.1 for MAC OS X (<http://www.r-project.org/>), CANOCO 4.5 for Windows (Wageningen, Netherlands) and Prism 5.0b for Mac OS X (GraphPad Software, Inc., La Jolla, CA, USA).

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S 3.1: Zooplankton community structure at first sampling date after contamination shown as ordination diagrams derived by Redundancy Analyses. The toxicant concentration and the three experimental treatments are used as predictors (NSH-H, NSH-NH, and SH-NH stand for the treatments “No Shadow – Harvesting”, “No Shadow – No Harvesting”, and “Shadow – No Harvesting”, respectively); species abundances as dependent variables.

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S 3.2: Measured concentration of esfenvalerate (mean \pm standard deviation, $\mu\text{g/L}$) in the series contaminated at nominal concentrations of 3 (A) and 0.3 $\mu\text{g/L}$ (B).

S 3.3: Full species taxa of the invertebrates collected in the pond microcosm systems in Europe and Siberia.

| Europe | Siberia |
|--------------------------|--------------------------|
| <i>Daphnia</i> sp. | <i>Daphnia</i> sp. |
| <i>Ceriodaphnia</i> sp. | <i>Ceriodaphnia</i> sp. |
| <i>Simocephalus</i> sp. | <i>Simocephalus</i> sp. |
| <i>Scapholeberis</i> sp. | <i>Scapholeberis</i> sp. |
| <i>Chydorus</i> sp. | Chydoridae |
| Daphnidae | Cladocera |
| <i>Cyclops</i> sp. | Cyclopoidae |
| <i>Pleuroxus</i> sp. | <i>Bosmina</i> sp. |
| <i>Alona</i> sp. | <i>Polyphemus</i> sp. |
| <i>Eurycercus</i> sp. | <i>Diaphanosoma</i> sp. |
| Diaptomidae | <i>Macrotrix</i> sp. |
| Ostracoda | Ostracoda |
| <i>Stylaria</i> sp. | Calanoidae |
| Hydracarina | Conchostraca |
| <i>Notonecta</i> sp. | <i>Gammarus</i> sp. |
| Coleoptera | Coleoptera |
| <i>Hydra</i> sp. | Hemiptera |
| Odonata | Odonata |
| <i>Cloeon dipterum</i> | Ephemeroptera |
| Culicidae | <i>Anopheles</i> sp. |
| Chironomidae | Chironomidae |
| <i>Chaoborus</i> sp. | Chaoboridae |
| Collembola | Diptera |
| Gastropoda | Lymneidae |
| | Planorbidae |
| | Hydrachnidia |
| | Oligocheta |
| | Hirudinea |

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Chapter 4

Two stressors and a community – Effects of hydrological disturbance and a toxicant on freshwater zooplankton

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4.1 Abstract

Climate change models predict an increase in the frequency and intensity of extreme fluctuations in water level in aquatic habitats. Therefore, it is necessary to understand the combined effects of hydrological fluctuations and toxicants on aquatic biological communities. We investigated the individual and combined effects of the insecticide esfenvalerate and recurring fluctuations in water level on zooplankton communities in a system of 55 outdoor pond microcosms. The communities were exposed to esfenvalerate contamination as a single pulse (at 0.03, 0.3, or 3 $\mu\text{g/L}$) and gradual removal of water and its subsequent replacement over three cycles and monitored until 84 days after contamination. The results showed that the sensitivities of the community and its constituent populations to the toxicant were increased by the hydrological stress. Specifically, for both the community structure and abundance of *Daphnia* spp. the lowest-observed-effect concentrations (LOEC) were 0.03 and 0.3 $\mu\text{g/L}$ for the series with fluctuating and constant water levels, respectively. Despite these differences in sensitivity, the interactive effects of the two stressors were found to be additive for both the community structure and the abundance of the most affected species. Presumably, it was not possible to detect synergism due to the strong individual effects of the water level fluctuations. Recovery times in the series exposed to the highest pesticide concentration were 64 and 55 days under fluctuating and constant water level regimes, respectively. Competition and water quality are suggested to be the major factors that underlie the observed effects of fluctuations in the water level. For the ecological risk assessment of toxicants, the present results suggest that (i) community sensitivity may vary substantially, depending on the environmental context, and (ii) this variability can be assessed experimentally to derive safety factors (coefficients used to avoid unexpected effects and define safe concentrations of toxicants) based on empirical findings. This contrasts with the current approach where such factors are usually defined arbitrarily.

Keywords: Combined effects; Community-level effects; Ecological Risk Assessment; Mesocosm; Multiple stressors; Pyrethroid pesticide

4.2 Introduction

Multiple anthropogenic pressures pose an increasing threat to aquatic ecosystems. In addition to the threats posed by habitat loss, invasive species, and toxicants, such ecosystems are affected by global climate change (Millennium Ecosystem Assessment, 2005). The importance of understanding and predicting the effects of multiple pressures on biological communities and ecosystems is well recognized, but remains a central challenge for ecology (Rohr et al., 2008). Fluctuations in the water level are important environmental perturbations for many aquatic communities. They may influence both productivity and biodiversity (Wantzen et al., 2002). The effects of fluctuations in the water level have been investigated for various taxonomic groups (e.g. review by Leira and Cantónati, 2008). According to the Intergovernmental Panel on Climate Change (Pachauri and Reisinger, 2007), both the frequency and the intensity of extreme weather events, such as heavy precipitation and severe drought, are projected to increase worldwide. Such

climatic changes are expected to cause extreme fluctuations in water levels that might exceed the levels with which many organisms can cope through physiological and behavioural adaptations (Coops et al., 2003). In addition to the direct effects that arise from climate change, aquatic ecosystems are threatened increasingly by toxicants. For example, exposure of aquatic organisms to agricultural pesticides and the related ecological effects may increase dramatically owing to increased pest pressure in many agricultural regions (Kattwinkel et al., 2011). Despite the frequency of hydrological disturbances and their potential effects on aquatic biota, relatively few studies have investigated the combined effects of toxicants and hydrological stress (fluctuations in water level) in aquatic ecosystems, although these two pressures may frequently affect aquatic systems simultaneously. Previous investigations of hydrological stress and pesticides can be divided into two categories: (1) studies on pesticides applied to systems that naturally undergo fluctuations in water level (Angeler and Moreno, 2007; Morrill and Neal, 1990) and (2) controlled factorial experiments, which analysed the effects of two stressors individually and in combination (Boone and Semlitsch, 2002; Rohr et al., 2004). To our knowledge, studies of the second type, which include controls for both pesticide exposure and changes in the hydrological regime, are particularly important for understanding the combined effects of these two stressors. Nonetheless, these have been limited to amphibians and did not consider community development based on interactions between species over several generations. These investigations on amphibians have shown that hydrological stress and toxicants affect endpoints at population level, such as time to, biomass at, and survival to metamorphosis of different amphibians, in an interactive manner. However, the type of interaction can differ depending on the species, endpoint, and toxicant investigated (Boone and Semlitsch, 2002; Rohr et al., 2004). Hence, chemicals can interact with hydrological stress, but the mode of action and magnitude of these effects, as well as the underlying mechanism, need further investigation. In the present study, we aimed to investigate the effects of fluctuations in water level and exposure to a pesticide on communities of zooplankton. In contrast to previous factorial experiments, we aimed to assess the effect of repeated reductions in water level on a community that develops as a self-regulated system and is shaped by interactions between species over several generations. For this purpose, we exposed freshwater zooplankton communities in outdoor microcosms to a single pulse of the insecticide esfenvalerate under conditions in which the water level remained constant or decreased periodically. We focused on the structure of the entire community by using multivariate statistical methods, as well as on the particular species affected most by the investigated stressors.

4.3 Materials and methods

4.3.1 Experimental design

Fifty-five outdoor ponds (height: 38 cm, radius: 25 cm, volume: 80 L) situated at the Helmholtz Centre for Environmental Research in Leipzig, Germany (51°21'13 N, 12°25'55 E), were inoculated with randomly sampled invertebrates (mainly zooplankton) and approximately 1.5 L of water from three permanent natural ponds located within a radius

of 15 km from the experimental site in mid-May 2009¹. To each microcosm we added 60 L of tap water, a 1 cm layer of substrate that consisted of a 1:1 mixture of sediment (from a nearby permanent natural pond) and sand, and approximately 10 g of shredded fallen leaves (*Populus* sp.). Microcosms were not covered to allow natural colonization. We manipulated two factors experimentally in a fully crossed design: (1) hydrological regime (constant or fluctuating water level) and (2) concentration of esfenvalerate (control, 0.03, 0.3, and 3 $\mu\text{g/L}$). The amount of water in the constant water level (CWL) treatment was left unchanged (60 L), whereas for the water level fluctuation (WLF) treatment, the amount of water was reduced gradually, maintained at a low level, and subsequently increased to the initial volume. This procedure was performed three times. The microcosms were contaminated by a single pulse of esfenvalerate during the first period with the minimum water level. The experimental set-up was designed to allow detection of distinct combined effects. For this, contamination was conducted at a time point at which the organisms were expected to experience greatest stress. Although the present set-up represents a model scenario, it may reflect a run-off driven pesticide input due to an intense but short rainfall event during dry conditions. For the CWL treatment, six and twelve replicate microcosms were established for each concentration of esfenvalerate and the control. For the WLF treatment, five and ten replicate microcosms were assigned for each concentration of pesticide and the control, respectively.

4.3.2 Water level fluctuations

After an initial acclimation period of 20 days, the water level in the microcosms assigned to the WLF treatment was reduced gradually over 14 days from 60 L to 10 L, kept at this level for 10 days, and subsequently increased to 60 L within three days. This procedure was repeated three times (Fig. 4.1). During the 14-day water removal period, the water level was reduced by careful daily removal of surface water to a predefined level using a glass beaker, which involved removal of 5 L on the days 1-6, 3 L on the days 7-11, 2 L on the days 12-13, and 1 L on day 14. At low water levels, the removal of water was performed by several small steps to avoid strong movements of the water and minimise the capture of organisms. The water was then filtered through a sieve (55 μm mesh size) and the few organisms that had been retained were returned to the microcosms. During periods of heavy precipitation, the microcosms were covered temporarily. At the end of the periods with a low level of water, the microcosms were refilled with tap water that had been adjusted to the ambient temperature. In the present study, the amount of water was reduced from 60 to 10 L, corresponding to a decrease in water level of 21.9 cm (from 28.2 cm to 6.3 cm) within 14 days. This reduction is approximately within the range of fluctuations in the water level observed in ponds in the floodplains of the River Elbe, Germany (unpublished data), which was the site of the study. Furthermore, with the progression of climate change, such conditions may worsen and be more widespread.

¹Min., max., and average air temperature: 8.7, 21.7, and 14.8°C, respectively. Precipitation: 0 mm, air pressure: 1010.4 hPa. Source: <http://www.uni-leipzig.de/~meteo/en/wetterdaten/index.php>

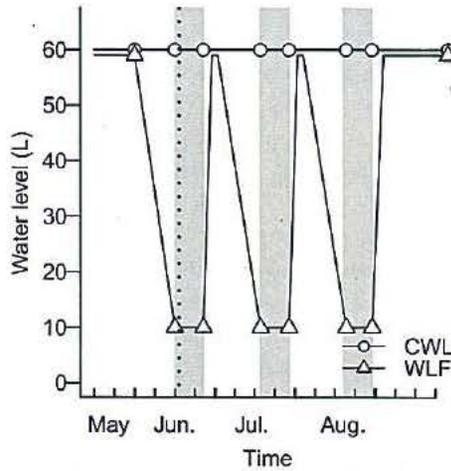


Figure 4.1: Scheme for fluctuations in the water level. The rhombus-shaped symbols indicate the sampling time points analysed in the present study. The vertical dashed line indicates the time of contamination. The periods of minimal water levels in the water level fluctuation treatment (WLF) are indicated by the grey-shaded columns.

4.3.3 Application and monitoring of esfenvalerate

Microcosms were exposed to Somicidin Alpha (BASF, Limburgerhof, Germany), an emulsified concentrate containing 62 mg/l of the active substance esfenvalerate [(α S)- α -cyano-3-phenoxybenzyl (2S)-2-(4-chlorophenyl)-3-methylbutyrate]. Esfenvalerate is a broad-spectrum non-selective pyrethroid insecticide. It is very hydrophobic ($\log K_{ow} > 6$) and has a strong tendency to sorb to soil particles ($K_{oc} = 215000$) (Kelley, 2004). It is highly toxic to non-target freshwater invertebrates (Lozano et al., 1992). The microcosms were contaminated with three different final concentrations (0.03, 0.3, and 3 $\mu\text{g/L}$ esfenvalerate) of the pesticide, which was diluted in dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) (for details, see Table 4.1). The DMSO was used to ensure homogeneous mixing of the high concentration of esfenvalerate. The final maximal volume of DMSO in the microcosms assigned to both the CWL and the WLF treatments was 0.00096% [vol/vol] or 10.6 mg/L, which is far below the solvent limit suggested by the OECD test guidelines (OECD, 2000) and the 48-h LC_{50} value for *Daphnia magna* (25000 mg/L, Goto and Hiromi, 2003). This amount was expected to neither simulate nor inhibit microbial activity (Griebler and Slezak, 2001), thus almost certainly eliminating the possibility of direct or indirect effects of DMSO on the pond community. Nevertheless, no solvent was added to the controls to ensure fully undisturbed conditions. In fact, this is supported by the dynamics of DO concentration in the CWL and WLF treatment (see Fig. S 4.1 in Supplementary Material) which do not show significant deviations in DO levels in the treated compared to the control systems (except for the highest concentration in the CWL treatment which, however, shows both positive and negative changes relative to the control). To introduce the pesticide into the microcosms, the solution was mixed in 0.5 L (CWL) and 1 L (WLF) of tap water, and immediately poured over the

surface of each microcosm. The esfenvalerate concentrations applied in the study ranged around the 48-h LC₅₀ value of 0.37 µg/L, which was determined for *Daphnia magna* in a preliminary laboratory study. They reflect concentrations in natural waterbodies, which range from trace concentrations to 0.166 µg/L (Bacey et al., 2005), or even 0.76 µg/L (Cooper et al., 2003). The microcosms were contaminated after sunset on the 18th of June, 2009, to prevent photodegradation. This date was the start of the first period with the minimum water level of 10 L (Fig. 4.1). After contamination, the water level was not manipulated for 10 days (i.e. no water was removed or added). During this period, microcosms were covered temporarily during heavy rains. Subsequent to this period, the microcosms were filled up to the level of 60 L. At the time of refilling, esfenvalerate was assumed to have disappeared completely from the water column (for details see Stampfli et al., 2011). Owing to a technical failure, esfenvalerate concentrations were not measured in the present experiment. However, the actual concentrations were measured in a comparable experiment that was conducted in an identical microcosm system. In this parallel experiment, the zooplankton communities in the CWL and WLF treatments were contaminated three times, with an interval of one month between each pulse. In both experiments, the water reduction and the first contamination occurred at the same time, and the environmental conditions were the same. The actual concentrations of esfenvalerate were measured for the third contamination, which took place in August 2009. Before the contamination, water samples were taken and analysed to ensure that esfenvalerate had not accumulated in the water column. Additional information on the dissipation of esfenvalerate under constant and fluctuating water levels was obtained from a supplementary experiment conducted at the end of August 2010. In this experiment, three microcosms for each hydrological regime were filled as described in section 2.1, except for the invertebrates, which were not introduced. After an acclimation period of six days, the water level was reduced, and the microcosms were contaminated the following day with the highest nominal concentration (for details on contamination, see above). Esfenvalerate concentrations were assessed after 2, 14, and 48 hours. The esfenvalerate concentrations presented in Table 4.1 were analysed by Eurofins Umwelt Ost GmbH (Jena, Germany). The water samples, which were filtered through a sieve with a mesh size of 180 µm and collected in 1-L brown glass bottles, were subjected to solid-phase extraction, followed by gas chromatography–mass spectrometry (GC-MS; Agilent Technologies Inc., Palo Alto, USA). The limit of detection was 0.01 µg/L.

4.3.4 Zooplankton and environmental parameters

Invertebrates were sampled every second week using a PVC tube (length: 31.7 cm, radius: 3.55 cm) with a lid. The tube was lowered quickly through the water column that had been mixed gently, and closed using the lid that had been positioned in the bottom centre of the microcosm. The content of the tube was filtered through a sieve (180 µm mesh size), and organisms were preserved in 70% ethanol (for details on the water volume removed using the tube sampler and the water level at each sampling date, see Table S 4.2 in Supplementary Material). The cladocerans, copepods, ostracods, and insects in the samples were counted. Organisms were identified to the taxonomic level of class (Ostracoda, Arachnida), order (Odonata, Copepoda), or genus (Cladocera, Chaoboridae, Culicidae, Baetidae). However, as rare species of random occurrence can give rise to

spurious correlations (Field et al., 1982), species with an overall frequency of < 1% were grouped before the analyses (see Table S 4.4 in Supplementary Material). The sampling method used was particularly adept at capturing zooplankton (DeVries and Stein, 1991), so all the invertebrates are hereafter referred to as zooplankton. Although rotifers are an important component of zooplankton communities, they were not considered in the present study, as the mesh size of the sieve used to filter the sampled water was too large and thus rotifers were not captured at a level that reflected their actual frequency. By comparison, Hanazato and Yasuno (1990) and López-Mancisidor et al. (2008a) used nets with mesh sizes of 40 μm and 55 μm , respectively. Abundance data from the WLF treatment were corrected for differences in sampled water volume; all abundances were given as individuals per sampling unit (Ind./S.U.), namely, 1.8% of the water level at the time of sampling. This unit was chosen to avoid the difficulty caused by extrapolation of the numbers of organisms to 1 L for organisms with zero abundance on one hand, and to compare equivalent proportions of the community (i.e. sampling one litre in a system with a volume of 10 L (WLF treatment) and 60 L (CWL treatment) equates to 10% and 1.66% of the present community, respectively) on the other. Unless stated otherwise, all abundances are given as Ind./S.U. Environmental parameters, such as electrical conductivity (EC), pH, dissolved oxygen (DO) concentration, water temperature, and turbidity were measured. According to a redundancy analysis, EC and DO concentration were the quantitative environmental parameters that explained most of the variance in the species data. We therefore limited information on environmental parameters to these two parameters. Measurements of EC ($\mu\text{S}/\text{cm}$; HI-98312, Hanna Instruments, Woonsocket, RI, USA) and DO concentration (mg/L; WTW Multi 340i Meter, WTW Instruments, Weilheim, Germany) were taken weekly. The measurements were carried out between 7 and 9 AM in a subsample of microcosms (six replicates per treatment level in both WLF and CWL treatments).

4.3.5 Data analyses

Esfenvalerate and environmental data

To test for significant differences in actual concentrations of esfenvalerate and environmental data (i.e. EC, DO concentration) between the hydrological and/or insecticide treatments, we conducted ANOVA followed by pairwise t-tests with pooled and unpooled variances for data with equal and unequal variances, respectively. Holm's correction was used for multiple comparisons. The Kruskal–Wallis test, followed by a nonparametric multiple comparison test (R-package `pgirmess`, function `kruskalmc` Giraudoux, 2011), was conducted for non-normally distributed data. Given that the EC and DO concentration were not significantly different among insecticide levels (including the control treatment), we pooled these data when testing for differences between the hydrological treatments. Redundancy analysis (RDA), using the forward and backward model selection technique, was conducted to assess the influence of the measured environmental parameters and the manipulated experimental factors on community structure. Before RDA, species data were $\ln(4x+1)$ -transformed to avoid false discrepancies between zero abundance values and low abundance values (for rationale, van den Brink et al., 2000). Pesticide concentrations were $\ln(x+1)$ -transformed, while the measured environmental parameters were left

untransformed. Hydrological treatment was used as a categorical variable, and time as a conditioning variable.

Biological data

The effects of esfenvalerate and/or hydrological stress on the community structure were analysed using the principal response curve (PRC) method. The statistical significance of each of the first PRC curves was tested by Monte Carlo permutation tests, which were performed for the entire time series using an F-type test statistic based on the eigenvalue of the components (van den Brink and Ter Braak, 1998). The statistical significance of effects of the toxicant at different toxicant concentrations and time points was tested by RDA with nominal toxicant concentrations as an explanatory variable, followed by Monte Carlo permutations for each sampling date and toxicant concentration. This approach was used to infer the lowest-observed-effect concentration (LOEC). Similarly, the statistical significance of the effects of fluctuations in water level (categorical variable: CWL, WLF) at different time points was tested. Before these multivariate analyses, abundance data and pesticide concentrations were transformed as described above for the RDA. For each sampling date, the statistical significance of the interactions between the pesticide and hydrological treatment at the community level was assessed by RDA followed by Monte Carlo permutation tests. The interaction between the pesticide treatment and hydrological treatment was used as an explanatory variable, and the two stressors were used as conditioning variables (for details, see van Wijngaarden et al., 2006). To analyse the interactive effects of the pesticide and fluctuations in water level on the most affected taxa according to the PRC, namely, *Daphnia* spp. and *Simocephalus* spp., we conducted an analysis of covariance (ANCOVA) based on the combined zooplankton data sets of the CWL and the WLF treatments as follows: $Y = xA + yB + z(A \times B) + \text{error}$ where Y is the response variable 'abundance' (continuous; $\ln(x+1)$ -transformed), A is the variable 'hydrological regime' (categorical: CWL or WLF), B is the variable 'pesticide concentration' (continuous; $\ln(x+1)$ -transformed), and (A \times B) is the interaction term. A lack of a significant interaction is called additivity, while a significant interaction term implies either an amplified effect (synergism) or a reduced effect (antagonism). To reveal the type of interaction, the signs of the parameter estimates for the interaction and main effects were compared. An interaction parameter with the same algebraic sign as the main effect parameters was interpreted as synergism, and an opposite sign was interpreted as antagonism. Interaction plots were used to visualise the interactions (see Fig. S 4.3 in Supplementary Material). Interactive effects of the pesticide and fluctuations in water level on the total zooplankton density were also analysed. However, owing to the difficulty in interpreting cross-over interactions, results are not discussed here. Statistical models were checked for error assumptions (constant variance, noncorrelation, and normality of residuals) and unusual observations (leverage and outliers) (Sheather, 2009). Differences in taxon abundance (including total zooplankton density) at the different time points among contaminated and uncontaminated series, as well as among the control series of the CWL and WLF treatments, were tested analogously to the differences in esfenvalerate concentration and environmental variables described in section 4.3.5. Taxon abundance was $\ln(x+1)$ -transformed before analysis. The PRC analyses and RDA were performed using CANOCO 4.5 for Windows (Wageningen, the Nether-

lands). The other analyses were carried out with the free software R, version 2.10.1 for Mac OS X (<http://www.r-project.org/>). In the present study, LOEC is defined as the lowest toxicant concentration at which a significant difference in community structure from the control is detected. Sensitivity is considered here in absolute terms, that is, as the ability of a system to respond to a stressor, and is quantified by the LOEC. As a consequence, sensitivity changes are independent of the type of interactions between the stressors, and higher sensitivity to one stressor caused by another stressor does not indicate the interaction type (i.e. synergism or additivity).

4.4 Results

4.4.1 Dynamics of esfenvalerate exposure

The measured esfenvalerate concentrations are presented in Table 4.1. In the CWL treatment, the concentrations that were measured two hours after contamination were within the range of the nominal concentrations (Table 4.1 A). In contrast, the concentrations measured in the WLF treatment were markedly lower than the nominal concentrations (except for the lowest concentration). Comparison of the actual concentrations between the two hydrological treatments showed significant differences in terms of the highest concentrations of esfenvalerate ($n = 3$, $P < 0.001$), with the concentration in the WLF treatment corresponding on average to 54% of the concentration in the CWL treatment. An additional exposure experiment with the highest nominal concentration of esfenvalerate showed that esfenvalerate dissipated more rapidly in the WLF treatment than in the CWL treatment (Table 4.1 B). Significant differences in measured concentrations were found between the treatments two and fourteen hours after contamination ($n = 3$, $P = 0.006$ and < 0.001 , respectively). The results of both exposure experiments indicated that the exposure via the water column was considerably lower in microcosms subjected to the WLF treatment.

4.4.2 Abiotic parameters and total density of zooplankton

For the RDA, we first included all environmental variables and performed forward and backward selection of environmental variables, leaving a set of variables that were significant according to the Monte Carlo permutation test ($P = 0.005$, 199 permutations). These were the variables of pesticide concentration, DO concentration, EC, and the hydrological regime. Together, they explained 20.7% of the variance in the species data (d.f. = 4, $P = 0.005$). Water levels and EC were negatively correlated, whereas water levels and DO concentrations were positively correlated, which suggested a possible key role for these environmental parameters in microcosms exposed to fluctuations in water level (for details, see Table S 4.5 in Supplementary Material). In fact, the decline in water level resulted in a significantly lower DO concentration (Fig. 4.2 A). The EC was significantly higher at a low water level than at a constant water level (Fig. 4.2 B). Fluctuations in water level were characterised by strong declines in water volume and hence increased densities. In fact, during the periods of low water level, the overall density of zooplankton was considerably higher for the WLF than for the CWL treatment (Fig. 4.2 C). In the former,

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Table 4.1: Residue analysis of esfenvalerate. A – Concentrations of esfenvalerate ($\mu\text{g/L}$) measured two hours after contamination in the microcosms assigned to the constant water level (CWL) and water level fluctuation (WLF) treatments. B – Dissipation of esfenvalerate at the nominal concentration of $3 \mu\text{g/L}$ over 48 hours.

| | Time after contamination in hours | Nominal concentration in $\mu\text{g/L}$ (μg per total volume ^a) | | Actual concentration in $\mu\text{g/L}$ | |
|---|-----------------------------------|--|--------------|---|------------------|
| | | CWL | WLF | CWL (n = 3) | WLF (n = 3) |
| A | | | | | |
| | 2 | 0.03 (1.8) | 0.03 (0.261) | 0.03 ± 0.001 | 0.05 ± 0.034 |
| | 2 | 0.3 (18) | 0.3 (2.61) | 0.28 ± 0.092 | 0.16 ± 0.015 |
| | 2 | 3 (180) | 3 (26.1) | 2.10 ± 0.10 | 1.13 ± 0.153 |
| B | | | | | |
| | 2 | 3 (180) | 3 (28.8) | 3.47 ± 0.532 | 2.09 ± 0.370 |
| | 14 | 3 (180) | 3 (28.8) | 2.72 ± 0.653 | 0.63 ± 0.157 |
| | 48 | 3 (180) | 3 (28.8) | 0.71 ± 0.083 | 0.20 ± 0.078 |

^a The nominal concentrations per total volume were calculated on the basis of the actual water level: 60 L (CWL) and 8.7 L (WLF) in experiment A, 60 L (CWL) and 9.6 L (WLF) in experiment B.

the density increased markedly with decreasing water volume, with maximum densities in the control series that ranged from 630 to 1104 Ind./L. By comparison, densities in the control series of the CWL treatment reached a maximum of 201 Ind./L. However, over the course of the periods with a low water level, zooplankton density declined by up to 54%, which highlighted the negative effects of elevated rates of competition caused by high densities of organisms.

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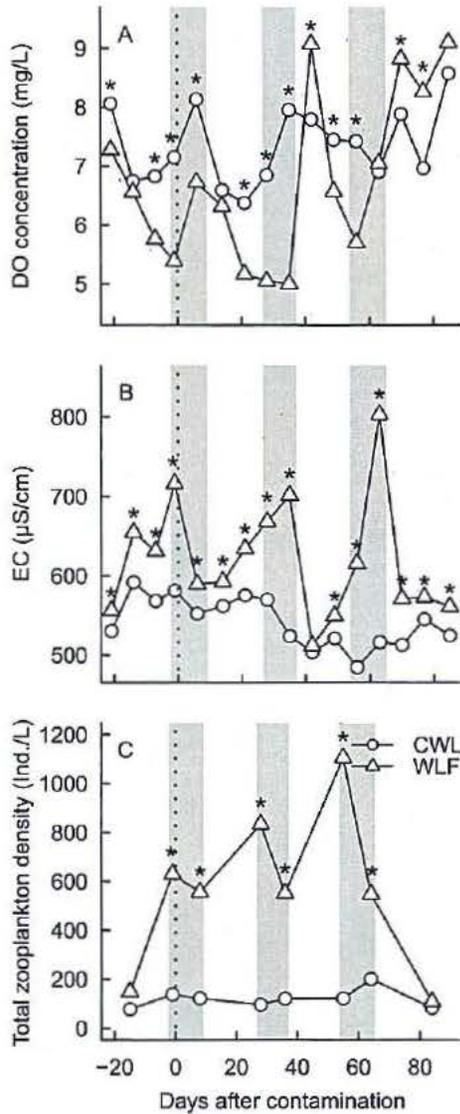


Figure 4.2: Water quality and total density following the constant water level (CWL) and water level fluctuation (WLF) treatments. A – Dissolved oxygen (DO) concentration (mg/L). B – Electrical conductivity (EC) ($\mu\text{S}/\text{cm}$). C – Total density of zooplankton (Ind./L) in the control series. Values in A and B are averaged over the different pesticide levels. Asterisks indicate significant differences between the treatments ($n = 21$ in WLF and $n = 24$ in CWL; $P < 0.05$, pairwise t -test or nonparametric multiple comparison test). The vertical dashed line indicates the time of contamination and the grey-shaded columns represent the periods of minimal water levels. The legend is valid for all subfigures.

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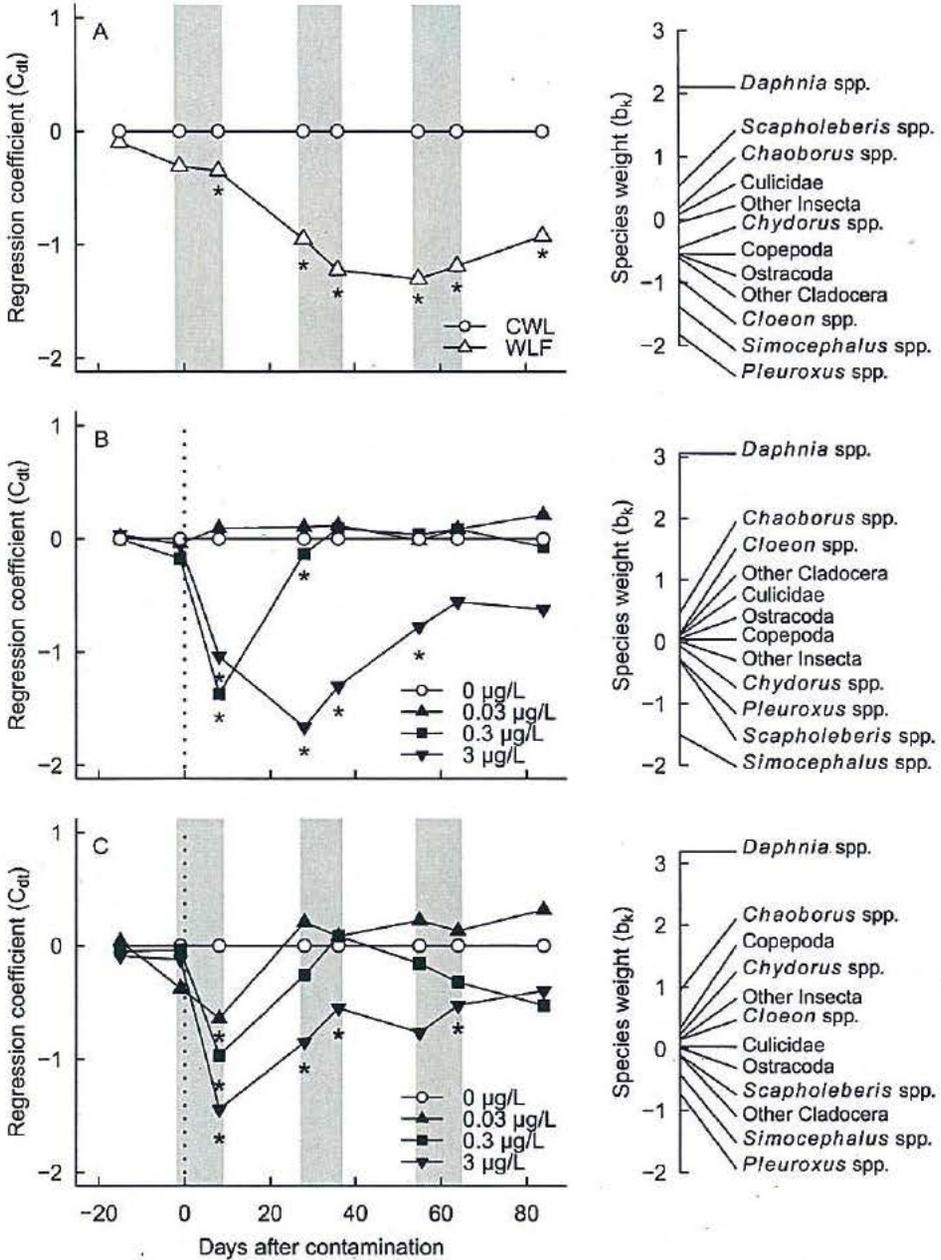


Figure 4.3: Principal response curves (PRC) that indicate the individual effects on the zooplankton community structure of water level fluctuations (A) and the individual effects of esfenvalerate following the CWL (B) and WLF treatments (C). Asterisks indicate significant differences in community structure from the control ($P < 0.05$, Monte Carlo permutation test following RDA).

4.4.3 Effects on the community structure

To understand the effects of the fluctuations in water level on community structure alone, we conducted a PRC analysis of the data obtained from the uncontaminated microcosm series of the CWL and WLF treatments (Fig. 4.3 A). Monte Carlo permutation tests indicated that community composition differed significantly between the two treatments ($P = 0.002$). Of the total variance, 35% could be attributed to the sampling date (horizontal axis) and 14% to the hydrological treatment. Of the variance attributed to the latter, 74% was displayed on the first PRC axis (vertical axis). In the microcosms that were subjected to the WLF treatment, *Daphnia* spp. and *Scapholeberis* spp. were present at relatively low levels. In contrast, *Pleuroxus* spp., *Simocephalus* spp., and *Cloeon* spp. were more abundant in these microcosms. The significant effects of the fluctuations in water level on the community structure were maintained until the end of the experiment, 24 days after the last period with a low level of water. To assess the effects of esfenvalerate on zooplankton communities under the different hydrological regimes, we performed PRC analysis for the CWL (Fig. 4.3 B) and WLF (Fig. 4.3 C) data sets. Both analyses showed significant toxicant-related effects compared with the controls. The statistical significance of the first PRC curves was confirmed by Monte Carlo permutation tests (both $P = 0.002$). Of the total variance in the CWL data set, 37% could be attributed to time and 26% to the pesticide treatment. It was also found that 29% of the variance explained by the pesticide treatment was displayed on the vertical axis. For the WLF data set, 41% of the variance could be assigned to time and 14% to the pesticide treatment, of which 19% was represented on the vertical axis. The effects of esfenvalerate on community structure varied between the two treatments. Following the CWL treatment, significant effects ($P < 0.05$) occurred at medium and high concentrations and were observed until 28 and 55 days after contamination, respectively (Fig. 4.3 B). Following the WLF treatment, significant effects ($P < 0.05$) were also observed at the lowest concentration (Fig. 4.3 C). Although observed at only one sampling time point, these differences occurred eight days after contamination and thus indicate significant short-term effects. Effects at medium and high concentrations were observed until 8 and 64 days after contamination, respectively. The LOEC were therefore 0.03 and 0.3 $\mu\text{g/L}$ for the communities under fluctuating and constant water levels, respectively. The taxon that was affected most in both treatments was *Daphnia* spp., the abundance of which decreased significantly as a result of exposure to the pesticide. In contrast to *Daphnia* spp., the abundance of *Simocephalus* spp. increased substantially following both the CWL and the WLF treatments, although the effects were less pronounced after the latter treatment. In addition to *Simocephalus* spp., the abundance of *Pleuroxus* spp. increased markedly following the WLF treatment. To understand the type of interaction between the two stressors, we performed a series of RDA for each sampling date with the entire data set, including all the concentrations of esfenvalerate and hydrological regimes. Monte Carlo permutation tests revealed no significant interaction between the pesticide treatment and the hydrological treatment (Table 4.2), which indicated that the effects of the two stressors on the community were additive, except on day 28 when the two stressors interacted antagonistically. Visual analysis of the toxicant's effects in CWL and WLF shows that there may have been synergism at the lowest tested concentration eight days after contamination, as indicated by the differences in LOEC and the characteristics of the effects (i.e. lack of effects at CWL and strong

and significant effects at WLF, Figs. 4.3 B and C). However, an additional Monte Carlo permutation test performed with the lowest concentration and control only did not reveal any significant interaction. To compare the sensitivities of the zooplankton species to both stressors individually, the species weights (b_k) of the PRC in Fig. 4.3 A and B were plotted against each other (Fig. 4.4). Figure 4.4 shows that both stressors affected the majority of species to approximately the same extent. The most extreme effects were seen for *Daphnia* spp. and *Simocephalus* spp. Effects on the other taxa were rather weak. The sensitivities of the species to the different stressors were correlated positively, as indicated by the regression line.

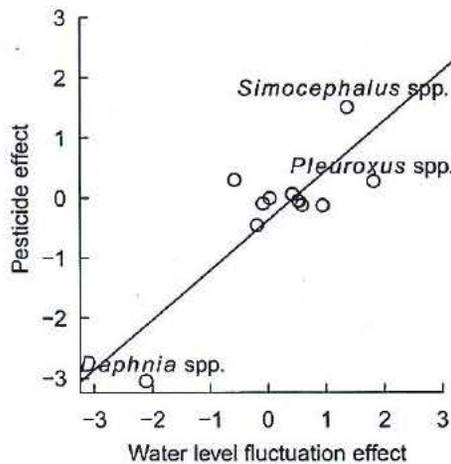


Figure 4.4: Correlation between the individual effects of the pesticide and water level fluctuations on the zooplankton species. The effects are represented by the species weights (b_k) derived from the PRCs of the zooplankton community exposed solely to water level fluctuations (x-axis) and pesticide (y-axis). The diagonal line represents the linear regression line. Each point depicts one species. The species that were most affected by the two stressors according to the PRC (*Daphnia* spp., *Simocephalus* spp., and *Pleuroxus* spp.) are marked with species names.

4.4.4 Effects on single-species dynamics

The most affected taxa of zooplankton showed contrasting dynamics: Both stressors had negative effects on *Daphnia* spp., but positive effects on *Simocephalus* spp. and *Pleuroxus* spp. (Fig. 4.5). The strength of the adverse effects of the insecticide on *Daphnia* spp. varied between the two hydrological treatments. For the WLF treatment, esfenvalerate had significant effects at all concentrations (Fig. 4.5 B), whereas for the CWL treatment, effects were only observed at medium and high concentrations of the toxicant (Fig. 4.5 A). Upon exposure to fluctuations in the water level, *Daphnia* spp. declined constantly and did not recover until 84 days after the contamination in the WLF setup (Fig. 4.5 C). In contrast to *Daphnia* spp., the abundance of *Simocephalus* spp. increased significantly as a result of fluctuations in the water level (Fig. 4.5 E). A similar increase in abundance

was observed in both the CWL and the WLF treatments 28 days after contamination with the highest concentration of esfenvalerate, which suggested an indirect effect on *Simocephalus* spp. due to release from competition with *Daphnia* spp. (Fig. 4.5 D, E). The effects on *Simocephalus* spp. were stronger in the CWL treatment than in the WLF treatment, which was probably because *Simocephalus* spp. reached carrying capacity in the latter as a result of the significant increases in abundance owing to fluctuations in the water level (Fig. 4.5 F). Similar to *Simocephalus* spp., levels of *Pleuroxus* spp. increased significantly as a result of the fluctuations in the water level (Fig. 4.5 I). Owing to the fact that *Pleuroxus* spp. was not present in the community before contamination, the effects of esfenvalerate were not pronounced. Nevertheless, we observed an increase in abundance of *Pleuroxus* spp. in the microcosms exposed to the highest insecticide concentration in the second part of the experiment following both the CWL (Fig. 4.5 G) and the WLF treatments (Fig. 4.5 H). This suggests an indirect effect of release from competition. Compared to the effects described above, the effects of the insecticide on the remaining taxa were less pronounced and hence are not considered for further discussion of population-level responses (but see Table S 4.6 in Supplementary Material for LOEC). As in the case for the community structure, we assessed the joint effects of the pesticide and fluctuations in the water level on the abundance of *Daphnia* spp. and *Simocephalus* spp., the two most affected taxa. The joint effects on *Pleuroxus* spp. were not assessed owing to the high frequency of zero values and thus the violation of the assumption of normality. To test for joint effects, we performed ANCOVA with the entire data set. The effects of the two factors on both *Daphnia* spp. and *Simocephalus* spp. were primarily additive (on four out of six sampling days) (Table 4.2). For *Daphnia* spp., antagonistic effects were found on two days, while for *Simocephalus* spp., antagonistic and synergistic effects were each found on one day (for details, see Fig. S 4.3 in Supplementary Material).

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Table 4.2: Statistical significance of the interactive effects of esfenvalerate and hydrological regime on community structure ($P < 0.05$, Monte Carlo permutation test) and abundances of *Daphnia* sp. and *Simocephalus* sp. ($P < 0.05$, ANCOVA).

| Endpoint | Days after contamination | Interaction (P values) | Type of interaction |
|-----------------------------------|--------------------------|------------------------|---------------------|
| Community structure (RDA) | | | |
| | 8 | 0.17 | Additive |
| | 28 | 0.04 | Antagonistic |
| | 36 | 0.15 | Additive |
| | 55 | 0.82 | Additive |
| | 64 | 0.28 | Additive |
| | 84 | 0.98 | Additive |
| <i>Daphnia</i> spp. (ANCOVA) | | | |
| | 8 | 0.36 | Additive |
| | 28 | 0.007 | Antagonistic |
| | 36 | > 0.001 | Antagonistic |
| | 55 | 0.41 | Additive |
| | 64 | 0.87 | Additive |
| | 84 | 0.53 | Additive |
| <i>Simocephalus</i> spp. (ANCOVA) | | | |
| | 8 | 0.46 | Additive |
| | 28 | 0.75 | Additive |
| | 36 | 0.03 | Antagonistic |
| | 55 | 0.89 | Additive |
| | 64 | 0.03 | Synergistic |
| | 84 | 0.18 | Additive |

n = 55, that is, 5 (10) and 6 (12) for contaminated (control) series in the CWL and WLF experiments, respectively.

4. Two stressors and a community – Effects of hydrological disturbance and a toxicant on freshwater zooplankton

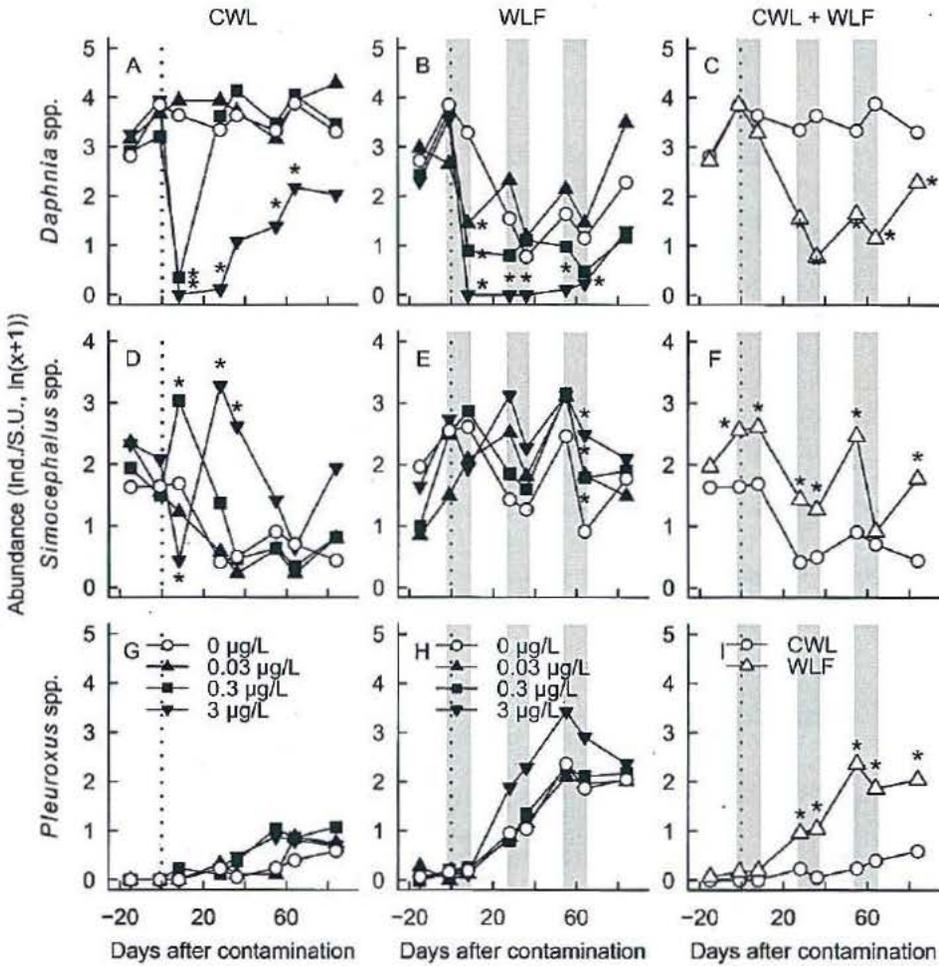


Figure 4.5: Effects of esfenvalerate in the CWL and WLF treatments as well as effects of the hydrological regime (control series of the WLF and CWL treatments) on the abundances of *Daphnia* spp. (Ind./S.U.; A–C), *Simocephalus* spp. (Ind./S.U.; D–F), and *Pleuroxus* spp. (Ind./S.U.; G–I). Abundances were $\ln(x+1)$ -transformed. Asterisks indicate significant differences from the control [$n = 5$ (10) and 6 (12) for contaminated (control) series of the WLF and CWL treatments, respectively; $P < 0.05$, pairwise t-test or nonparametric multiple comparison test]. The legends are valid for all subfigures in the same column.

4.5 Discussion

4.5.1 General

To our knowledge, the present study represents the first experimental analysis focused on the combined effects of a toxicant and fluctuations in water level on a biological community that develops as an open and self-regulated system and is shaped by interactions between species over several generations. The usefulness of comparing the present results with the previous studies on the combined effects of such stressors is limited because earlier investigations focused on amphibians and detected various interactive effects (from antagonistic to synergistic) depending on the endpoints (e.g., Boone and Semlitsch, 2002; Rohr et al., 2004). Owing to their long generation times and biphasic life cycles, it is technically difficult to consider the community development of amphibians over several generations. The present results showed that (i) community sensitivity to the toxicant was enhanced by the hydrological stress at low toxicant concentrations, as revealed by the difference in LOEC between the treatments, (ii) interactive effects of the two stressors were additive, and (iii) the two stressors had different effects on two abundant species, with both stressors affecting *Daphnia* spp. negatively and *Simocephalus* spp. positively. We suggest that we were not able to detect synergism due to the strong individual effects of the water level fluctuations (especially after the second low water period), which resulted in a strong reduction of the sensitive taxa and as a result could not be further exacerbated by the toxicant. These findings have important implications for both basic ecology and ecological risk assessment of toxicants and are discussed below (sections 4.5.5 and 4.5.6).

4.5.2 Esfenvalerate exposure

The exposure measurements (Table 4.1) indicate that the combined effects of the two stressors might be more synergistic than suggested by the present analyses. This is due to the fact that, at the medium and high concentration levels, the actual concentrations were markedly lower following the WLF treatment than following the CWL treatment. This can be attributed to higher adsorption of the highly lipophilic compound on the sediment, which resulted from the higher ratio of sediment surface to water volume in the former treatment. Therefore, at comparable measured concentrations between the CWL and the WLF treatments, stronger individual effects of the pesticide may be expected in the latter treatment. This in turn would result in an increased combined effect and thus (more) synergistic interactions at community and population levels. As described in section , exposure concentrations were assessed in similar experiments that were conducted in parallel and one year later. Even though the environmental conditions may have varied between the experiments, they expressed similar exposure trends; that is, in the WLF treatment, acute esfenvalerate concentrations were markedly lower than in the CWL treatment. Hence, the same trend is likely to apply for the actual experiment. In the present study, concentrations of esfenvalerate in the water were assessed for 48 hours. However, for pyrethroids to reach equilibrium in the system, more than two days are needed (Bondarenko et al., 2006). However, on the basis of the study of Schulz and Liess (2000), which showed that short-term exposure of the pyrethroid fenvalerate leads

to stronger effects than long-term exposure with an equivalent dose, we expect that the initial peak concentration was more relevant for toxicity to the zooplankton species than the lower concentrations in the later phase of the experiment.

4.5.3 Relevance and sensitivity of the test system

Comparison of the present community with the communities in previously reported studies using outdoor microcosms showed that the number of zooplankton taxa in the present study was not exceptional. For example, Daam et al. (2009) assessed the effects of a herbicide on a tropical zooplankton community consisting of 12 taxa and McKee et al. (2002) studied the effects of climate warming on 15 freshwater macrozooplankton taxa. To our knowledge, only a few studies have assessed the effects of esfenvalerate on invertebrate communities (Fairchild et al., 1992, 1994; Knillmann et al., 2012a; Lozano et al., 1992; Stampfli et al., 2011). Fairchild et al. (1994) observed an LOEC of 0.25 $\mu\text{g/L}$ for crustacean zooplankton and benthic macroinvertebrates. Slightly higher values were reported by Lozano et al. (1992), who observed an LOEC of 1 $\mu\text{g/L}$ or higher for Cladocera, Copepoda, and Ostracoda. The abundance of some genera, however, declined significantly at esfenvalerate concentrations below 1 $\mu\text{g/L}$ (e.g. *Aiona* spp.: 0.01 $\mu\text{g/L}$, nauplii: 0.08 $\mu\text{g/L}$, *Diaptomus* spp.: 0.2 $\mu\text{g/L}$). Similar to the present study, an LOEC of 0.03 $\mu\text{g/L}$ was previously found for zooplankton communities exposed to esfenvalerate in the treatment with the least favourable environmental conditions (i.e., a treatment with 'Shadow-No Harvesting' conditions, Stampfli et al., 2011) as well as for populations of *Daphnia* spp. under high interspecific competition (Knillmann et al., 2012a). Compared to the studies by the other authors mentioned above (Fairchild et al., 1992, 1994; Lozano et al., 1992), the community LOEC observed in the WLF treatment of the present study is lower. This may be explained by the additional stress (i.e. water level fluctuations) to which the microcosms were exposed in the present study. However, differences in time points at which the effects are measured, concentration ranges tested, and the statistical analysis from which the LOEC are derived (univariate vs. multivariate) may also influence the LOEC. According to ECOTOX, the database that provides single chemical toxicity information for aquatic and terrestrial life (<http://www.epa.gov/ecotox>), the freshwater organisms that are most sensitive to esfenvalerate (based on the LC_{50}) are insects. In fact, Beketov (2004) reported LC_{50} (96 h) of 0.0096 $\mu\text{g/L}$ and 0.0147 $\mu\text{g/L}$ for *Cloeon dipterum* and *Caenis miliaria*, respectively. Apart from insects, *Daphnia magna* and *Ceriodaphnia dubia* also showed high sensitivity to esfenvalerate, with respective LC_{50} (96 h) values of 0.029 $\mu\text{g/L}$ (Beketov, 2004) and 0.039 $\mu\text{g/L}$ (Yang et al., 2006). In the present study, insects were rather scarce. In contrast, *Daphnia* and *Ceriodaphnia* were well represented in the present community. Thus, in more insect-dominated systems, the effects of esfenvalerate may be more pronounced.

4.5.4 Mechanisms underlying the combined effects

There is strong evidence that the observed combined effects of esfenvalerate toxicity and fluctuations in water level were based on stress caused by the interplay of altered water quality and enhanced competition resulting from high densities of organisms, which occurred mainly during the periods when the water level was at its lowest. In terms of water

quality, in the WLF treatment, the physicochemical properties of the water temporarily approached the physiological limits that the organisms could endure. Our RDA analysis showed that DO concentration and EC were the most important physicochemical variables in terms of explaining the variance in the species data. The DO concentration decreased considerably to a minimum of approximately 5 mg/L following the WLF treatment. Although this value was not below the critical oxygen concentration of 2.7 mg/L for *Daphnia* spp. reported by Homer and Waller (1983), a markedly lower concentration, and thus critical oxygen stress, might have occurred during the night. The effects of low oxygen concentrations might differ among species. For example, LaBerge and Hann (1990) indicated that *Daphnia pulex* is less tolerant to low oxygen saturation than *Simocephalus vetulus*. This is in line with the present study, in which negative effects of low DO concentration were observed on *Daphnia* sp., but not on *Simocephalus* sp. (see RDA species scores in table S 4.5 in Supplementary Material). In contrast to the DO concentration, EC increased significantly up to 800 $\mu\text{S}/\text{cm}$ following the WLF treatment. While EC values around 800 $\mu\text{S}/\text{cm}$ are common in the study region (EC ranged from 560 to 900 $\mu\text{S}/\text{cm}$ in the ponds where the organisms were originally collected) and in Central European waters, and thus are not expected to have negative effects on aquatic organisms (Schletterer et al., 2010), there is evidence that DO concentrations (Hanazato and Dodson, 1995) might exacerbate the toxicity of pesticides to freshwater invertebrates at levels that are comparable to those observed following the WLF treatment. Therefore, temporarily suboptimal water quality may play a role in the effects of the fluctuations in water level on biota. Particular mention should be made of the EC, which may rise beyond the observed levels in natural systems, in which water evaporates naturally (in contrast to the manual removal of water in the present study), and thus becomes a stress factor. Concerning competition, evidence for the intensification of biological interactions is provided by the considerably higher densities of zooplankton that were observed following the WLF treatment than following the CWL treatment. Density and competition are well known to be important biotic factors that can influence both the sensitivity of organisms to toxicants (e.g. Boone and Semlitsch, 2002; Knillmann et al., 2012b) and their recovery post-contamination (e.g. Frost et al., 2006; Knillmann et al., 2012a; Liess and Foit, 2010). For example, Knillmann et al. (2012b) have shown that intraspecific competition can increase the sensitivity of *Daphnia* spp. to esfenvalerate by a factor of up to 100. In the present study, both exposure to toxicant and hydrological stress had strong negative effects on *Daphnia* spp. and positive effects on *Simocephalus* spp. It is likely that the effects on *Simocephalus* spp. were the indirect result of the release of competition. Such indirect effects based on between-species interactions have been reported frequently for toxicants (e.g. see review by Fleege et al., 2003; Wendt-Rasch et al., 2003), and are an important part of the mechanisms of multistressor effects at the community level (Rohr et al., 2006). An alternative mechanism underlying the observed combined effect of the two stressors could be a possibly higher exposure of the zooplankton via food (i.e. algae) in the WLF series (as indicated by the low DO concentration during early morning). However, Coats et al. (1989) found that the uptake of synthetic pyrethroids via food is six times less toxic than the cuticular uptake via water. Against this background, we do not expect a significant increase in sensitivity of the organisms in the WLF treatment due to exposure via food. Finally, although the reduction in the level of water following the WLF treatment was conducted as carefully as possible (see section 4.3.2), disturbance

of the zooplankton taxa resulting from the removal of water during the WLF treatment cannot be ruled out. The taxon most negatively affected by the fluctuations in the water level was *Daphnia* spp. (Fig. 4.3 A). However, its physiologically very similar relative, *Simocephalus* spp., was influenced positively by the hydrological disturbance. In fact, the majority of taxa were affected positively by the fluctuations in the water level. Therefore, we assume that the disturbance resulting from the removal of water was negligible.

4.5.5 Implications for basic ecology

As for empirical studies, the conceptual background for understanding the effects of multiple stressors at the community level is limited. A model that is frequently used to interpret such effects is the species co-tolerance concept developed by Vinebrooke et al. (2004). This model states that a positive correlation between species sensitivities (i.e. positive species co-tolerance, Fig. 4.4) increases the resistance to one stressor as a result of the exposure to another stressor. According to this concept, positive co-tolerance should reduce the effects of other stressors and thus result in antagonistic effects. Although species sensitivities were positively correlated in the present study, the joint effects of the toxicant and fluctuations in the water level on the community were predominantly additive. These findings indicate that the conceptual model, which is based on extinction patterns of species, might not be applicable to changes in community structure that are derived from species abundance profiles. These findings highlight the need for concepts that are rooted in community ecology and are based on an in-depth understanding of the biotic interactions (i.e. competition, predation) that occur within communities in order to deal with multiple stressors at the community level. Alterations in community structure in terms of species abundance profiles must also be considered (see also Rohr et al., 2006).

4.5.6 Implications for ecological risk assessment

The increase in the community sensitivity to the toxicant by a factor of up to 10 (LOEC of 0.03 vs. 0.3 $\mu\text{g}/\text{L}$ for the WLF and CWL treatments, respectively) caused by the fluctuation in water level indicates that this stressor can considerably enhance the susceptibility of freshwater biota to toxicants. Taking into account that hydrological disturbance is an ubiquitous stressor that is expected to increase worldwide due to increases in both the frequency and the intensity of extreme weather events, such as heavy precipitation and severe droughts (Pachauri and Reisinger, 2007), the combined effects observed in the present study are highly relevant for the ecological risk assessment of toxicants. To more precisely identify the factor by which sensitivity differs between conditions with and without hydrological stress, it might be necessary to test a concentration range with smaller distances between the concentrations. However, such precise values would have limited practical relevance, as in the risk assessment mesocosm studies dense concentration ranges in mesocosm studies are not used due to high costs of investigation. In the present study, community-level response was mainly driven by changes in the levels of *Daphnia* spp., *Simocephalus* spp., and *Pleurozrus* spp. These results, however, should not be interpreted as evidence that the community-level effect assessment can generally be relied on single dominant species as proxies for the entire community. Empirical knowledge about the accordance between population- and community-level responses remains limited, and simple

extrapolations from population- to community-level endpoints are unrealistic. Therefore, reliable assessment and prediction of community-level effects requires studies focused on entire communities. Although the current regulatory framework is mainly focused on population-level effects, the community-level response is undoubtedly important for common protection goals such as biodiversity, ecosystem functions, goods, and services (van Straalen, 2003). Previously, various studies indicated that additional stressors may also exacerbate the effects of toxicants on populations when investigated in realistic conditions and embedded in multi-species communities (Knillmann et al., 2012b; Liess and Beketov, 2011). However, information about modulation of the sensitivity of entire communities remains scarce. Together with a previous investigation (Stampfli et al., 2011), the present results indicate that (i) sensitivity may vary strongly depending on the environmental context not only at the organism and population levels, but also at the community level, and (ii) this variability can be assessed experimentally, and at least approximate ranges of the sensitivity modulations can be found empirically. These outcomes have basic importance for defining the “safety”, “uncertainty” or “assessment” factors applied in the risk assessment of toxicants to prevent negative effects resulting from unknown environmental and ecological factors and processes (Chapman et al., 1998). As for most substances, the data pool from which to predict ecosystem effects is strongly limited, these factors are in general defined arbitrarily based on expert judgements. A number of uncertainties must be addressed to establish safety factors, such as intra- and inter-laboratory variation of toxicity data, intra- and interspecies variations, short-term to long-term toxicity extrapolation, and laboratory data to field impact extrapolation (European Commission, 2003). However, it should be noted that defining the safety factors depending on the accordance between the different tests or replicates cannot ensure the coverage of the unknown factors present in the real-world systems, and this process essentially remains arbitrary. The present study together with the investigation by Stampfli et al. (2011), show that modulations of the community sensitivity by relevant and ubiquitous additional stressors can be estimated experimentally in outdoor micro- or mesocosms. Thus the safety factors should be based on empirical knowledge rather than arbitrary information (see also Liess and Beketov, 2011). Furthermore, identification of the safety factors should take into consideration results of field observational studies (Beketov and Liess, 2012; Schäfer et al., 2012; van Straalen and van Gestel, 2008).

4.6 Acknowledgements

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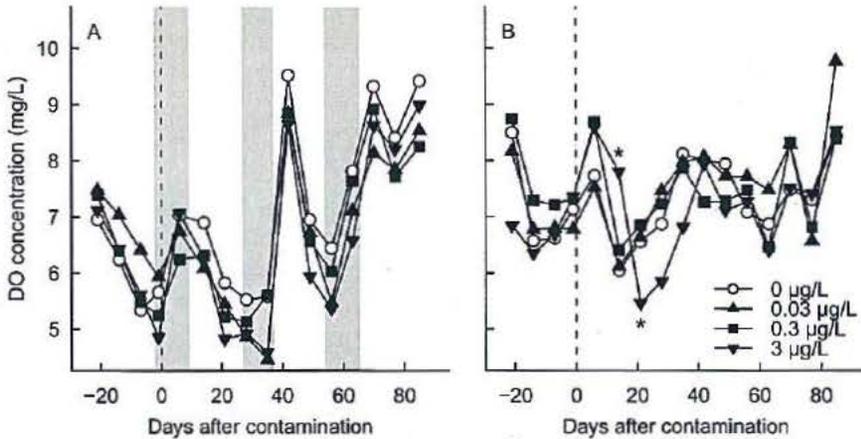
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Supplementary Material



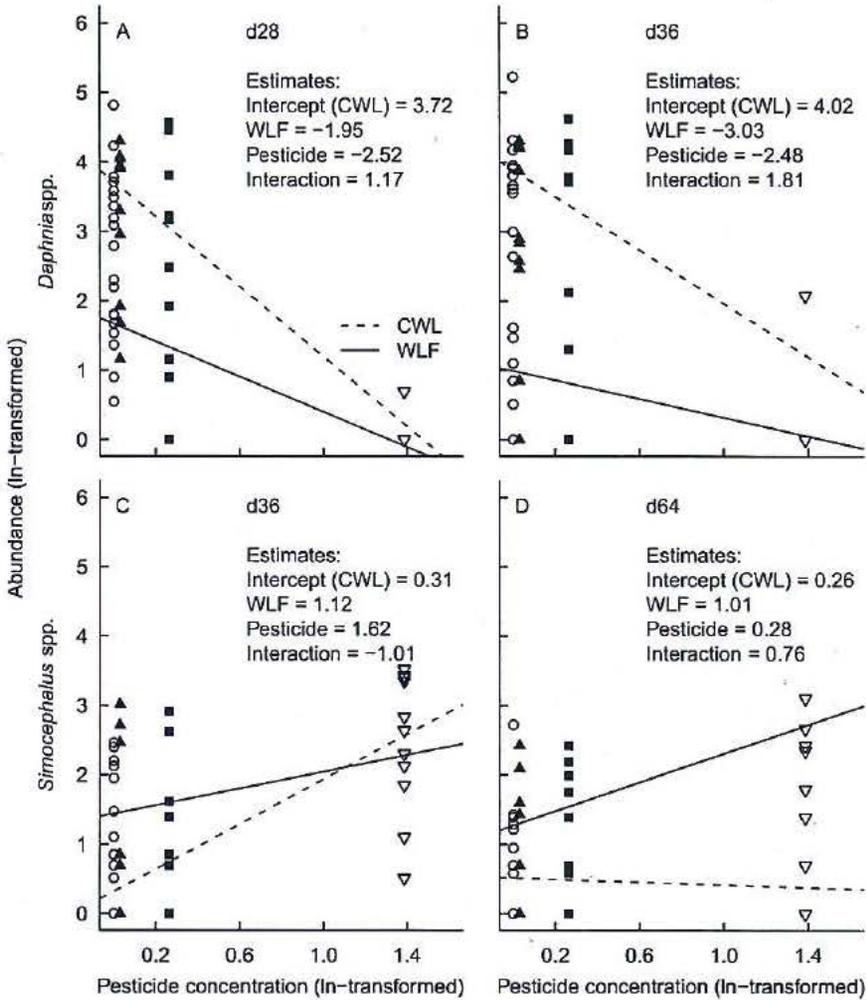
S 4.1: Dissolved oxygen (DO) concentration (mg/L) in the water level fluctuation (A) and the constant water level (B) treatments. Asterisks indicate significant differences between the treatments ($n = 3$, $P < 0.05$, pairwise t-test or nonparametric multiple comparison test). The vertical dashed line indicates the time of contamination and the grey-shaded columns represent the periods of minimal water levels. The legend is valid for both figures.

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S 4.2: Water level and water volume removed using the tube sampler in litres and as a percentage of the current water level in the WLF treatment for each sampling date. In the CWL treatment, the water level was maintained at 60 L at all sampling dates.

| Days after contamination | Water volume in L | Water volume removed in L (and % of the current water level) |
|--------------------------|-------------------|--|
| -22 | 60 | 1.1 (1.83) |
| -1 | 8 | 0.2 (2.5) |
| 8 | 8 | 0.2 (2.5) |
| 28 | 8 | 0.2 (2.5) |
| 36 | 7 | 0.19 (2.71) |
| 55 | 10 | 0.23 (2.3) |
| 64 | 10 | 0.23 (2.3) |
| 84 | 60 | 1.1 (1.83) |

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S 4.3: Results of analysis of covariance (ANCOVA) to test for interactive effects of the hydrological regime (CWL or WLF) and the pesticide treatment on total zooplankton density, and abundances of *Daphnia* spp. and *Simocephalus* spp. [$n = 55$, i.e. 5 (10) and 6 (12) for contaminated (control) series in the CWL and WLF experiments, respectively]. Only significant interactions ($P < 0.05$) are presented.

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S 4.4: Names of macroinvertebrate taxa identified in the present study and used in the PRC analysis, as well as their mean abundance (\pm standard deviation) in the control microcosms of the CWL and the WLF treatments averaged over all sampling dates (-22 to 84 days after contamination). As described in section 2.4, rare species were combined into groups. Abundances were $\ln(x+1)$ -transformed. n.c. = not considered; organisms that live on or just beneath the surface of the sediment (chironomids, oligochaets, etc.) or organisms that live on or underneath the surface of the water (water striders, water boatman, etc.) were not counted because the sampling technique was considered unsuitable for sampling these organisms.

| Taxon identified ^a | Species in PRC | Mean density | |
|-------------------------------|---------------------------|--------------|-------------|
| | | CWL | WLF |
| Branchiopoda | | | |
| <i>Daphnia</i> spp. | <i>Daphnia</i> spp. | 3.44 (1.11) | 2.22 (1.41) |
| <i>Simocephalus</i> spp. | <i>Simocephalus</i> spp. | 1.10 (1.08) | 1.88 (1.06) |
| <i>Scapholeberis</i> spp. | <i>Scapholeberis</i> spp. | 1.24 (1.16) | 1.13 (0.99) |
| <i>Chydorus</i> spp. | <i>Chydorus</i> spp. | 2.52 (1.32) | 2.97 (1.30) |
| <i>Pleuroxus</i> spp. | <i>Pleuroxus</i> spp. | 0.24 (0.59) | 1.18 (1.32) |
| <i>Ceriodaphnia</i> spp. | Other Cladocera | 0.10 (0.37) | 0.42 (1.04) |
| <i>Alona</i> spp. | Other Cladocera | | |
| Other Cladocera | Other Cladocera | | |
| Maxillopoda | | | |
| Cyclopoida | Copepoda | 2.11 (1.07) | 2.70 (0.89) |
| Calanoida | Copepoda | | |
| Harpacticoida | Copepoda | | |
| Ostracoda | Ostracoda | 3.01 (1.02) | 3.46 (0.85) |
| Insecta | | | |
| <i>Culex</i> spp. | <i>Culex</i> spp. | 0.10 (0.44) | 0.09 (0.29) |
| <i>Cloeon</i> spp. | <i>Cloeon</i> spp. | 0.17 (0.44) | 0.52 (0.71) |
| <i>Chaoborus</i> spp. | <i>Chaoborus</i> spp. | 0.69 (0.69) | 0.70 (0.72) |
| Zygoptera | Other Insecta | 0.01 (0.07) | 0.14 (0.37) |
| Anisoptera | Other Insecta | | |
| Coleoptera | Other Insecta | | |
| Chironomidae | n.c. | - | - |
| Gerridae | n.c. | - | - |
| Corixidae | n.c. | - | - |
| Notonectidae | n.c. | - | - |
| Hydrometridae | n.c. | - | - |
| Oligochaeta | n.c. | - | - |
| Mollusca | | | |
| Lymnaeidae | n.c. | - | - |
| Planorbidae | n.c. | - | - |
| Others | | | |
| <i>Hydracarina</i> spp. | n.c. | - | - |
| <i>Asellus</i> spp. | n.c. | - | - |
| <i>Hydra</i> spp. | n.c. | - | - |

^aIdentifications of a few random samples at levels lower than the genus revealed the presence of different species; therefore, taxon richness may be higher than indicated (e.g. for Ostracoda, the genus *Cypridopsis* and *Cypretta* were identified; for *Daphnia* spp., *Daphnia longispina* and *Daphnia pulex* were identified).

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S 4.5: Summary of the redundancy analysis (RDA) performed with the combined data set (i.e. CWL and WLF) using a reduced set of environmental variables derived by forward and backward model selection.

| | RDA1 | RDA2 | RDA3 | RDA4 |
|--------------------------------|-------|-------|-------|-------|
| Biplot scores/centroids | | | | |
| CWL | -0.43 | 0.1 | 0.05 | 0.02 |
| WLF | 0.54 | -0.13 | -0.07 | -0.02 |
| Insecticide concentration | 0.27 | 0.93 | -0.24 | -0.07 |
| EC | 0.74 | -0.07 | 0.35 | -0.39 |
| DO concentration | -0.28 | 0.1 | 0.53 | 0.58 |
| Species scores | | | | |
| <i>Daphnia</i> spp. | -1.02 | -0.59 | 0.051 | 0.09 |
| <i>Simocephalus</i> spp. | 1.05 | 0.01 | -0.25 | -0.11 |
| <i>Scapholeberis</i> spp. | -0.57 | 0.26 | -0.34 | 0.16 |
| Other Cladocera | 0.7 | -0.13 | 0.16 | 0.26 |
| <i>Chydorus</i> spp. | 0.47 | -0.54 | 0.17 | -0.07 |
| <i>Pleuroxus</i> spp. | 0.91 | 0.15 | 0.16 | 0.12 |
| Ostracoda | 0.63 | -0.2 | -0.35 | -0.03 |
| Copepoda | 0.41 | -0.23 | -0.25 | 0.35 |
| Culicidae | -0.05 | -0.22 | -0.4 | -0.19 |
| <i>Clocon</i> spp. | 0.53 | -0.05 | 0.25 | -0.16 |
| <i>Chaoborus</i> spp. | -0.23 | -0.03 | 0.18 | 0.02 |
| Other Insecta | 0.12 | -0.16 | -0.09 | -0.09 |

Inertia: 12 (Total), 1.59 (Conditioned), 1.74 (Constrained), 8.67 (Unconstrained)

Permutation test for RDA under reduced model: d.f. = 4, Variance = 1.74, F = 8.92, n permutation = 199, P = 0.005

4. Two stressors and a community – Effects of hydrological disturbance and a toxicant on freshwater zooplankton

S 4.6: Lowest-observed-effect concentration (LOEC) for all sampling days and taxa used in the PRC, derived by ANOVA followed by pairwise t-tests with pooled and unpooled variances for data with equal and unequal variances, respectively. For non-normally distributed data, the Kruskal-Wallis test, followed by a nonparametric multiple comparison test was conducted. Holm's correction was used for multiple comparison.

| Taxon | LOEC ($\mu\text{g/L}$) for the CWL and WLF treatments (CWL WLF) | | | | | | | |
|---------------------------|---|----|----------------|--------------------------|--------------|-------|-----------------|-------|
| | -22 | -1 | 8 | Days after contamination | | | | |
| | | | | 28 | 36 | 55 | 64 | |
| <i>Daphnia</i> spp. | NA | NA | 0.3 0.03 | 3 3 | >3 3 | 3 3 | 3 3 | >3 >3 |
| <i>Simocephalus</i> spp. | NA | NA | 0.3 >3 | 0.3 >3 | 3 >3 | >3 >3 | >3 0.03 | >3 >3 |
| <i>Scapholeberis</i> spp. | NA | NA | 3 >3 | 3 >3 | >3 >3 | 3 >3 | >3 >3 | >3 >3 |
| <i>Chydorus</i> spp. | NA | NA | >3 >3 | >3 >3 | >3 >3 | >3 >3 | >3 >3 | >3 >3 |
| <i>Pleuroxus</i> spp. | NA | NA | >3 >3 | >3 >3 | >3 >3 | >3 >3 | >3 >3 | >3 >3 |
| Other Cladocera | NA | NA | >3 >3 | >3 >3 | >3 >3 | >3 >3 | >3 >3 | >3 >3 |
| Copepoda | NA | NA | 3 3 | >3 >3 | >3 >3 | >3 >3 | >3 >3 | >3 >3 |
| Ostracoda | NA | NA | >3 3 | >3 >3 | >3 >3 | >3 >3 | >3 >3 | >3 >3 |
| <i>Culex</i> spp. | NA | NA | >3 >3 | >3 >3 | >3 >3 | >3 >3 | >3 >3 | >3 >3 |
| <i>Cloeon</i> spp. | NA | NA | >3 >3 | >3 >3 | >3 >3 | >3 >3 | >3 >3 | >3 >3 |
| <i>Chaoborus</i> spp. | NA | NA | 0.03 0.3 | >3 >3 | >3 >3 | >3 >3 | >3 >3 | >3 >3 |
| Other Insecta | NA | NA | >3 >3 | >3 >3 | >3 >3 | >3 >3 | >3 >3 | >3 >3 |

NA - not applicable.

Normal and bold fonts - significant negative and positive deviation from the control, respectively.

Chapter 5

Environmental stressors can enhance the development of community tolerance to a toxicant

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5.1 Abstract

Ecosystems are subject to a combination of recurring anthropogenic and natural disturbances, such as climate change and pesticide contamination. Biological communities are known to develop tolerance to recurring disturbances due to successive changes at both the community and organismal levels. However, information on how additional stressors may affect the development of such community tolerance is scarce to date. We studied the influence of hydrological disturbance on the reaction of zooplankton communities to repeated insecticide pulses in outdoor pond microcosms. The communities were exposed to three pulses of the insecticide esfenvalerate (0.03, 0.3, and 3 $\mu\text{g/L}$) and to the gradual removal of water and its subsequent replacement over three cycles. The communities developed tolerance to the toxicant, as indicated by their decreasing reaction to subsequent contamination, and this development was enhanced by hydrological disturbance. Elimination of the key taxa *Daphnia* spp. through the combined action of the two stressors was identified as the main mechanism responsible for the increase in community tolerance under a fluctuating water level. Under a constant water level, the abundance of *Daphnia* spp. did not decrease significantly, indicating that other mechanisms were responsible for the observed community tolerance. The present study shows, for the first time, that additional stressors can facilitate the development of community tolerance and that such facilitation is propagated through community-level mechanisms.

Keywords: Climate change; Community tolerance, Mesocosm, Multiple stressors, Pyrethroid pesticide

5.2 Introduction

Aquatic ecosystems face multiple threats, including chemical pollution, invasion by exotic species, and the reduction of natural habitats (Millennium Ecosystem Assessment, 2005). Global climate change is expected to further exacerbate the pressure on these ecosystems. Indeed, climate change models predict an increase in the frequency and intensity of extreme weather events, such as heavy precipitation and drought (Field et al., 2012). These changes are expected to severely alter the hydrology of aquatic ecosystems, causing extreme water level fluctuations that may exceed the physiological and behavioral adaptability of many organisms (Coops et al., 2003). In addition, a considerable increase in the impacts of agricultural pesticides may be expected due to the rise in pesticide use in response to the elevated prevalence of pests in many agricultural regions in Europe (Kattwinkel et al., 2011).

In freshwater systems, agricultural pesticide exposure often occurs in time-varying or repeated pulses (Liess et al., 1999). Such recurring disturbances result in successive changes in biological communities, thereby shaping their reaction to ensuing disturbances. According to the pollution-induced community tolerance (PICT) concept (Blanck et al., 1988), aquatic communities that have been exposed to a toxicant are expected to be more tolerant of subsequent exposures to that particular toxicant when compared to previously unexposed communities. This increase in community tolerance is attributed to the replacement of sensitive species with tolerant species.

A large number of studies have investigated the development of community tolerance to pesticides in aquatic ecosystems, with most examining microscopic and lower organisms with relatively short life cycles, such as bacteria, algae, and meiofauna (Bérard et al., 2002). The few studies that have assessed the effects of repeated contamination on communities of higher organisms, such as macroinvertebrates, have failed to demonstrate community tolerance (Boone et al., 2001; Daam et al., 2008; Hanazato and Yasuno, 1990; Relyea and Diecks, 2008).

Empirical data on how additional stressors, such as factors related to climate change (e.g., hydrological alterations, increased temperature), may affect the development of community tolerance to toxicants based on the replacement of sensitive species with tolerant ones are scarce to date. However, additional stressors could enhance the development of tolerance through the elimination of the components of a community that are sensitive to the toxicant. Conversely, additional stressors could increase community sensitivity by acting as an alternative selection pressure eliminating species that are tolerant to the toxicant. Such diversity of the potential effects and the lack of empirical studies hamper the ability to predict the development of community tolerance under multi-stress conditions.

The few existing studies of such additional stressors with regard to pesticides have shown that the examined stressors enhance community tolerance by increasing the uptake and toxic effects of a toxicant rather than by altering community-level mechanisms (Boivin et al., 2005). Furthermore, it has been demonstrated that community tolerance to a toxicant can occur as a side effect of the prior development of tolerance to other stressors due to similar defense mechanisms (Navarro et al., 2008).

We recently investigated the impacts of a single pulse of the insecticide esfenvalerate and hydrological disturbance on pond zooplankton communities (Stampfi et al., 2013). In that study, the species that was most sensitive to the toxicant was also most sensitive to the hydrological disturbance. Hence, we hypothesized that hydrological disturbance would enhance the development of community tolerance to recurring insecticide contamination by decreasing the abundance of pesticide-sensitive species and selecting more tolerant species.

Therefore, in the present study, we aimed to test whether an additional stressor might facilitate the development of community tolerance to a toxicant. Specifically, we sought to investigate whether hydrological disturbance could enhance the propagation of tolerance, as this type of disturbance acts similarly to the insecticide on *Daphnia* spp., which are most sensitive to the toxicant and dominate the community. The study was conducted as an outdoor microcosm experiment with freshwater zooplankton communities. We focused on the alterations of the structure of the entire community, complemented by an analysis of the dynamics of individual taxa.

5.3 Materials and methods

5.3.1 Experimental design

To investigate the effects of hydrological disturbance and repeated insecticide contamination on zooplankton communities, an outdoor microcosm experiment was conducted in 55 outdoor polyethylene ponds (height, 38 cm; radius, 25 cm; capacity, 80 L) at the

Helmholtz Centre for Environmental Research in Leipzig, Germany (51°21'13 N, 12°25'55 E). In mid-May 2009, the microcosms were filled with 60 L of tap water and inoculated with invertebrates, mainly zooplankton, and approximately 1.5 L of water from three small natural permanent ponds located within a 15-km radius of the experimental site. In each microcosm, an approximately 1-cm-thick layer of substrate consisting of a 1:1 mixture of sediment (from the above-mentioned surrounding natural permanent ponds) and sand and approximately 10 g of shredded leaves (mainly *Populus* sp.) were added. The experiment was conducted from May through October 2009.

We manipulated two factors experimentally in a fully crossed design: (1) the hydrological regime (constant water level (CWL) or water level fluctuations (WLF)) and (2) exposure to esfenvalerate (control, 0.03, 0.3, and 3 $\mu\text{g/L}$).

Six and five replicate mesocosms were established for each concentration of esfenvalerate in the CWL and the WLF treatments, respectively. As controls, twelve and ten replicate microcosms were established for the CWL and WLF treatments, respectively.

In parallel to this experiment, we conducted a similar experiment aimed at assessing the effects of hydrological disturbance and a single esfenvalerate contamination event (Stampfli et al., 2013).

5.3.2 Water level fluctuations

After an initial acclimation period of 20 days, the water level in the microcosms assigned to the WLF treatment was reduced gradually over 14 days from 60 L to 10 L, maintained at this level for 10 days, and subsequently returned to 60 L within three days. This procedure was repeated three times (Fig. 5.1).

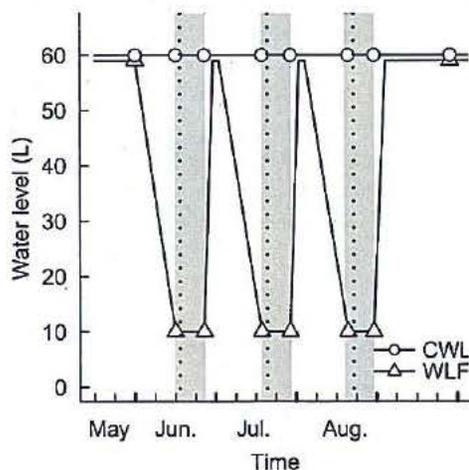


Figure 5.1: Sampling scheme and experimental design of the water level fluctuation treatment. Circles and triangles indicate the sampling time points, and vertical dashed lines indicate contamination with esfenvalerate. Grey-shaded columns highlight the periods of minimal water levels in the water level fluctuation treatment (WLF).

During the 14-day water removal period, the water level was reduced through the gentle

daily removal of the surface water to a predefined level using a glass beaker and involved the following steps: 5 L was removed on days 1-6, 3 L on days 7-11, 2 L on days 12-13, and 1 L on day 14. At low water levels, the removal of water was performed in several small steps to avoid strong perturbation of the remaining water or the capture of organisms. The water was then filtered through a sieve (55- μm mesh size), and the organisms that were unintentionally retained were returned to the microcosms. The microcosms were temporarily covered during heavy precipitation events. At the end of the low water level periods, the microcosms were refilled with tap water that had been adjusted to ambient temperature.

5.3.3 Esfenvalerate application and monitoring

The designated microcosms were exposed to Sumicidin Alpha (BASF, Limburgerhof, Germany), an emulsified concentrate containing 62 mg/L of the active substance esfenvalerate [(α S)- α -cyano-3-phenoxybenzyl (2S)-2-(4-chlorophenyl)-3-methylbutyrate]. The microcosms were contaminated with three different concentrations of the pesticide: 0.03, 0.3, and 3 $\mu\text{g/L}$ esfenvalerate. These concentrations were based on the 48-h lethal concentration 50 (LC_{50}) value (0.37 $\mu\text{g/L}$) for *Daphnia magna* determined in a preliminary laboratory study and reflected the concentrations observed in natural waterbodies, which range from trace concentrations to 0.166 $\mu\text{g/L}$ (Bacey et al., 2005) or even 0.76 $\mu\text{g/L}$ (Cooper et al., 2003).

The microcosms were contaminated with three pulses of esfenvalerate applied during the beginning of each minimum water level period (Fig. 5.1). The contamination events were conducted on the 18th of June, the 17th of July, and the 13th of August in 2009 after sunset to prevent immediate photodegradation. The water level was not manipulated for 10 days following each contamination event (i.e., no water was removed nor added), and the microcosms were covered temporarily during heavy rain showers. The microcosms were then refilled to the initial 60-L level over a period of three days (in steps of 5 L, 15 L, and 30 L); esfenvalerate was assumed to have disappeared completely from the water column during this refilling period (Stampfli et al., 2011). The procedure was performed three times. The esfenvalerate concentration was measured after each contamination event. However, due to technical failures, reliable analytical results were only obtained for the third contamination event. The measurements performed during the third contamination event were conducted by Eurofins Umwelt Ost GmbH (Jena, Germany) through solid-phase extraction, followed by gas chromatography-mass spectrometry (GC-MS; detection limit of 0.01 $\mu\text{g/L}$; Agilent Technologies Inc., Palo Alto, CA, USA).

5.3.4 Zooplankton and environmental parameters

Invertebrates were sampled every second week using a PVC tube (length, 31.7 cm; radius, 3.55 cm) with a lid. The tube was lowered quickly through the previously gently mixed water column and closed with a lid positioned in the center of the bottom of the microcosm. The content of the tube was then filtered through a sieve (180- μm mesh size), and the collected organisms were preserved in 70% ethanol. The abundance of cladocerans, copepods, ostracods, and insects in the samples were counted. The organisms

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were identified to the taxonomic level of class (Ostracoda, Arachnida), order (Odonata, Copepoda), or genus (Cladocera, Chaoboridae, Culicidae, Baetidae). Rare species (< 1% frequency overall) were grouped before the analyses to prevent spurious correlations (Field et al., 2012). As the sampling method used in the present study is most suitable for the collection of zooplankton (DeVries and Stein, 1991), all of the collected invertebrates are hereafter referred to as zooplankton. In the CWL treatment, one sampling unit (S.U.) corresponded to approximately 1.1 L water, or 1.8% of the total water volume in the microcosm. In the WLF treatment, the water volume and, hence, the volume sampled varied between the sampling time points. Thus, to allow comparison with the CWL treatment, all abundance data for the WLF treatment were multiplied by a factor ranging from 0.67 to 1 to represent 1.8% of the water volume at the sampling time. This unit was chosen to circumvent the problem of extrapolating the number of organisms to one liter for organisms with zero abundance and to compare equivalent proportions of the community (i.e., sampling 1 L in a system with a volume of 10 L (WLF treatment) and 60 L (CWL treatment) equates to 10% and 1.8%, respectively, of the community present). Unless otherwise stated, all zooplankton abundances are given as the number of individuals per 1.8% of the current total water volume (Ind./S.U.).

Environmental parameters, such as the electrical conductivity (EC), pH, dissolved oxygen (DO) concentration, water temperature, and turbidity, were also measured. EC and the DO concentration were the quantitative environmental parameters that explained most of the variance in the species data (Stampfli et al., 2013); therefore, we limited the information on environmental parameters presented to these two parameters. EC ($\mu\text{S}/\text{cm}$; HI-98312, Hanna Instruments, Woonsocket, RI, USA) and the DO concentration (mg/L; WTW Multi 340i Meter, WTW Instruments, Weilheim, Germany) were measured weekly. The measurements were performed between 7 and 9 am in a subsample of the microcosms (six replicates per treatment level in both the WLF and CWL treatments). The decreases in water levels resulted in a significantly lower DO concentration and a significantly higher EC.

5.3.5 Data analyses

The species data were $\ln(4x+1)$ -transformed prior to analysis (van den Brink et al., 2000), and the empty microcosms were removed from the analysis ($n = 2$, CWL). To analyze the effects of repeated insecticide exposure on zooplankton communities, we performed a principal response curve (PRC) analysis, followed by a set of redundancy analyses (RDAs) (van den Brink and Ter Braak, 1999) for the CWL and WLF experiments. The statistical significance of each of the two PRC models for the entire time series was tested through Monte Carlo permutation tests performed using an F-type test statistic based on the eigenvalue of the components (Lepš and Šmilauer, 2003; van den Brink and Ter Braak, 1999). The statistical significance of the effects of the toxicant at different concentrations and time points was tested by RDA using the nominal toxicant concentrations as an explanatory variable, followed by Monte Carlo permutations for each sampling date and toxicant concentration. This approach was employed to infer the lowest-observed effect concentration (LOEC).

To further compare the effects of multiple esfenvalerate applications on the zooplankton community in the CWL and WLF treatments, we computed the Bray-Curtis similarity

index between the control and each concentration level for each sampling date separately for the fluctuating water level and constant water level treatments. For statistical inference, the similarities were averaged over all sampling dates, and 95% confidence intervals (CI) were calculated. Non-overlapping CIs between the CWL and WLF treatments were interpreted as significantly different groups (Ramsey and Schafer, 2002). To rule out the possibility that the observed differences in community similarity between CWL and WLF resulted from a single dominant species (*Daphnia* spp. in the present study), we also computed the Bray–Curtis similarity indices for the community datasets without *Daphnia*.

The differences in taxon abundance between the control and each concentration level at each sampling time point were tested separately for each hydrological treatment using ANOVA, followed by pairwise *t*-tests with pooled and unpooled variances for data with equal and unequal variances, respectively. The Kruskal–Wallis test, followed by a non-parametric multiple comparison test (Giraudeau, 2011), was performed for non-normally distributed data. Holm's correction was used for multiple comparisons.

The PRC and RDA analyses were performed using CANOCO 4.5 for Windows (Wageningen, the Netherlands), and the other analyses were computed with the free software R, version 2.10.1 for Mac OS X (R Development Core Team, 2009).

5.4 Results

5.4.1 Effects on community structure

The development of community tolerance to esfenvalerate was observed in both water level treatments examined (Fig. 5.2), as indicated by the decreasing effects of esfenvalerate from the first to the last pulse observed in PRC. This decrease was more pronounced in the WLF treatment than the CWL treatment. In particular, in the WLF treatment, the second and third contamination events resulted in significant effects ($P < 0.05$) only at the highest concentration level (Fig. 5.2 B), whereas significant effects ($P < 0.05$) were also detected at intermediate concentrations after the second contamination event in the CLW series. This development of community tolerance was further reflected by the dynamics of the community LOECs (Fig. 5.3). These dynamics showed a gradual increase in LOECs in both treatments, indicating the development of community tolerance; equal or higher LOECs were observed in the WLF treatment than in the CWL treatment (except after the first contamination event), indicating lower community sensitivity in the former treatment (Fig. 5.3).

Evidence of enhanced community tolerance in the WLF treatment was also provided by the similarity of the communities between the control and contaminated microcosms. The similarity indices were significantly ($P < 0.05$) higher in the WLF compared to the CWL treatment, indicating that the communities exposed to the insecticide differed less from the non-exposed communities in the former treatment and were, therefore, less sensitive to repeated contamination events (Fig. 5.4). A similar picture emerged when the similarity analysis was conducted without *Daphnia* spp. (Fig 5.4), indicating that the significantly ($P < 0.05$) greater community similarity between the control and contaminated microcosms detected in the WLF treatment was not solely due to *Daphnia* spp.

5. Environmental stressors can enhance the development of community tolerance to a toxicant

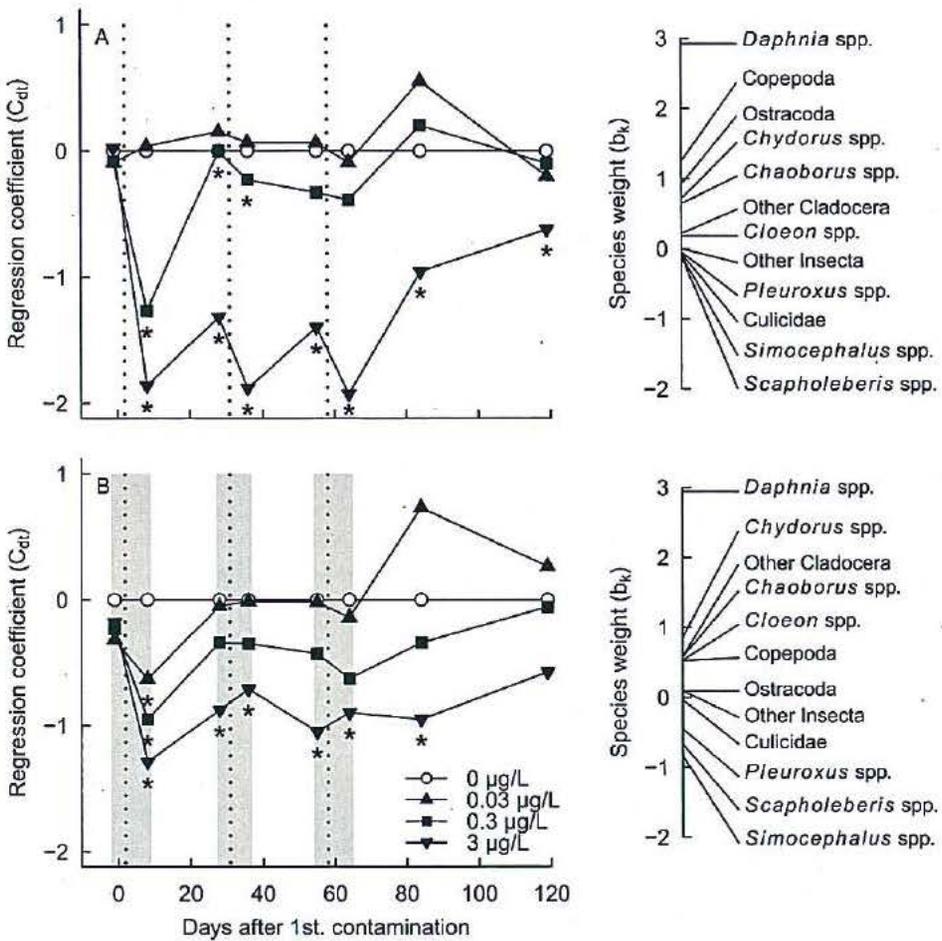


Figure 5.2: Principal response curves (PRC) indicating the effects of multiple insecticide exposures on the zooplankton community in the CWL (A) and WLF (B) treatments. Asterisks indicate significant effects of the toxicant at particular concentrations ($P < 0.05$, Monte Carlo permutation test following a redundancy analysis (RDA)). Vertical dashed lines indicate the time of contamination. Grey-shaded columns represent the minimum water level (10 L) period.

The recovery of the zooplankton communities following the pesticide applications was slightly faster in the WLF than the CWL treatment (Fig. 5.2). However, this phenomenon may be because the zooplankton community in the control microcosms subjected to the WLF treatment had not yet recovered from the water level fluctuations.

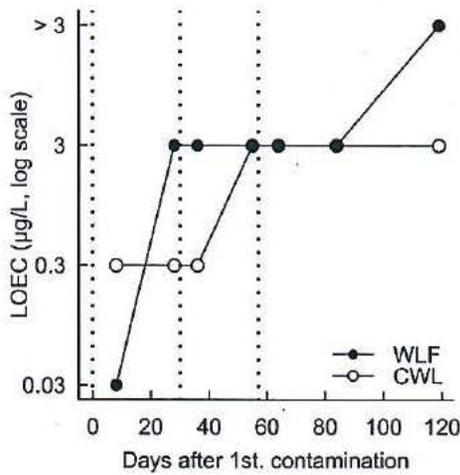


Figure 5.3: Lowest observed effect concentrations (LOECs) for different sampling time points in the CWL (open circles) and WLF (filled circles) treatments under multiple pesticide exposures, as derived through Monte Carlo permutation tests following RDA.

5.4.2 Population-level effects

To study the contribution of individual taxa to the observed community tolerance, we analyzed the dynamics of the taxa with high species weights (b_k), i.e., taxa with a high affinity for the overall community response, as displayed in the PRC diagram (Fig. 5.2). Based on the abundance dynamics, *Daphnia* spp. and *Simocephalus* spp. were found to be mainly responsible for the observed community tolerance in both the CWL and the WLF treatments (Fig. 5.5).

As indicated by PRCs (Fig. 5.2), *Daphnia* spp. were the most insecticide-sensitive taxa in both the CWL and WLF treatment communities. In both treatments, the populations of *Daphnia* spp. decreased to zero as a result of the first contamination event with the highest concentration of esfenvalerate and remained close to zero until the next contamination event (Fig. 5.5 A, B). Hence, the potential for further effects from subsequent contamination events was reduced in both hydrological treatments. At the intermediate concentration, the abundance of *Daphnia* spp. decreased significantly after the first contamination event in both hydrological treatments. Although these taxa recovered in terms of number of individuals in the CWL treatment, there was no recovery observed in the WLF treatment, thus reducing the capacity to react to the ensuing contamination events.

In contrast to what was observed for *Daphnia* spp., the dynamics of *Simocephalus* spp. were mainly characterized by increases in abundance, most likely due to the relaxation of competition with the dominant taxa *Daphnia* spp. (Fig. 5.5 C, D). Significant effects were mainly observed at the intermediate concentration in the CWL treatment; the most notable increases were observed on days 28 and 36 (Fig. 5.5 C), likely explaining the significance of the effect observed at the community level at this concentration at these time points, despite the small effect size (Fig. 5.2 A).

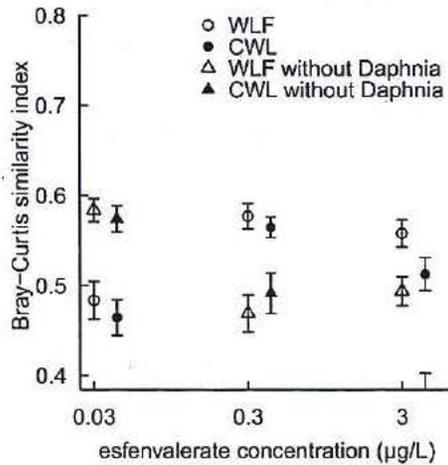


Figure 5.4: Average similarity (with 95 % confidence intervals) between the zooplankton communities with (circles) and without *Daphnia* spp. (triangles) in uncontaminated and contaminated microcosms in the constant water level treatment (CWL, filled symbols) and the fluctuating water level treatment (WLF, open symbols), as based on the Bray-Curtis similarity index (using $\ln(4x+1)$ -transformed species data). Non-overlapping confidence intervals of the mean indicate statistically significant differences between the groups (see section 5.3.5, Data analysis).

In contrast, the abundance of *Simocephalus* spp. decreased immediately after each contamination event in the WLF treatment and increased shortly thereafter. The decrease observed after the second contamination event with the intermediate concentration may partly explain the absence of significant effects at the community level at this concentration and time point (Fig. 5.2 B).

5.5 Discussion

5.5.1 General

The present study provides the first empirical evidence that an additional non-chemical stressor can enhance community tolerance to a toxicant. In particular, the additional stressor decreased the abundance of *Daphnia*, which were also the taxa that were most affected by the toxicant, reducing the potential effects of further contamination events. Prior to this study, it had only been shown that increased community tolerance may appear as the result of the enhanced physiological effects of a toxicant due to an additional stressor because of increased toxicant uptake (Boivin et al., 2005). In addition, the present study is one of the first to report community tolerance for higher aquatic organisms, such as zooplankton, which had previously been shown in only two studies (Clements, 1999; Millward and Grant, 2000). In contrast, several studies on higher organisms did not demonstrate community tolerance, i.e., a similar or higher community sensitivity

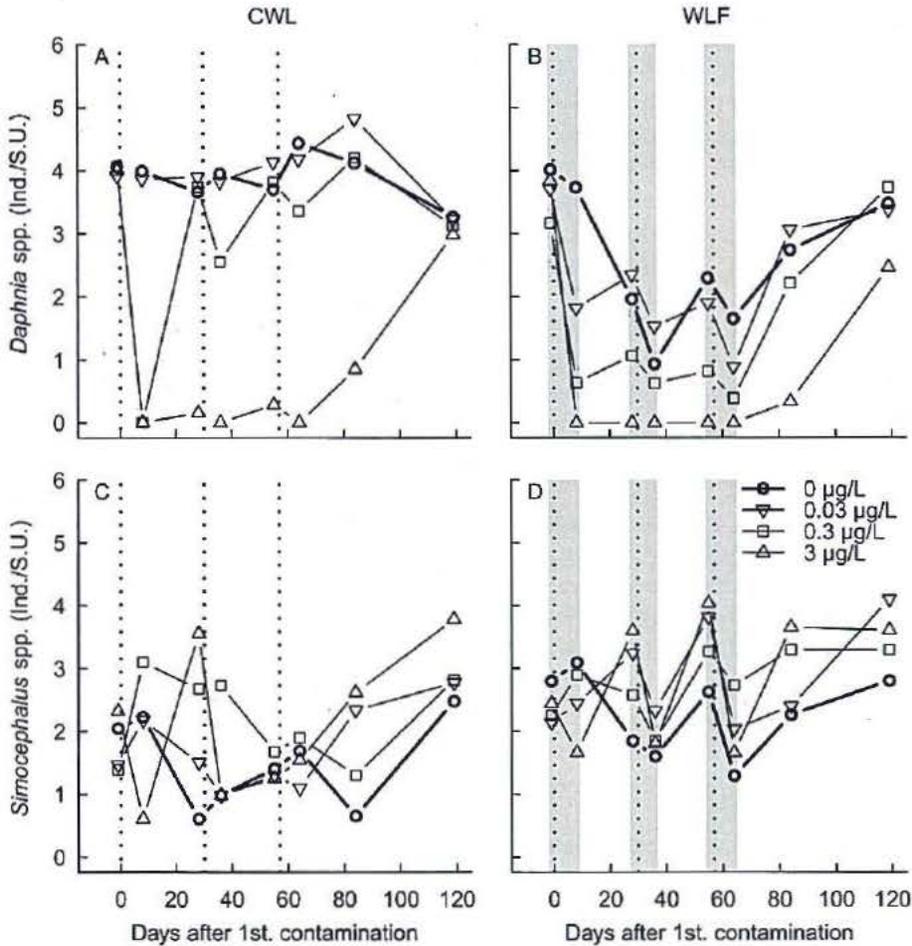


Figure 5.5: Abundance of *Daphnia* spp. and *Simocephalus* spp. in the CWL (first column; A, C) and WLF (second column; B, D) treatments exposed to multiple applications of esfenvalerate. The abundance was $\ln(x+1)$ -transformed. Grey-shaded columns represent the periods of minimal water levels (10 L), and vertical dashed lines represent the times of contamination events.

following repeated contamination events was observed (Boone et al., 2001; Daam et al., 2008; Hanazato and Yasuno, 1990; Relyea and Diecks, 2008).

5.5.2 Mechanisms underlying the enhancement of community tolerance

We suggest that the enhancement of community tolerance observed in the present study was based on the decline of *Daphnia* spp., as the water level fluctuations significantly decreased their abundance. This decrease was presumably due to an increase in compe-

tion, as indicated by the increasing overall density of organisms (Stampfi et al., 2013). The decrease in abundance originating from the water level fluctuations and the first contamination event explains the relative absence of further effects of subsequent insecticide applications on this key taxon. Therefore, the enhanced community tolerance resulted from the inability of *Daphnia* spp. to recover from the disturbance caused by the water level fluctuations and the first pesticide contamination event.

Conversely, in the absence of the additional stressor, there was no decrease in the total number of *Daphnia* spp.; thus, the observed community tolerance could not be attributed to *Daphnia* (cf. Fig. 5.5 A). We suggest that this taxon was in a growth phase after the first contamination event and was therefore less sensitive to the ensuing contamination event (Hanazato and Yasuno, 1990; Pieters and Liess, 2006). Another explanation could be that *Daphnia* developed physiological resistance, as demonstrated in a case study on the effects of the pesticide carbaryl on these taxa (Jansen et al., 2011). Substitution by other species of the same genus may be excluded as an explanation for the development of tolerance in *Daphnia* because the identification of *Daphnia* spp. to the species level did not reveal any change in species (data not shown). All of these mechanisms were likely obscured by the presence of the additional stressor.

In both hydrological treatments, the decrease of *Daphnia* spp. was associated with an increase in the abundance of the more tolerant *Simocephalus* spp. (Fig. 5.5 D). As a result of this increase, which was most likely an indirect effect caused by the release from competition, the proportion of (more) tolerant taxa in the community and thus community tolerance increased. We suggest that the increase in the abundance of *Simocephalus* spp. further suppressed the recovery of *Daphnia* spp. in the WLF treatment and therefore enhanced the community tolerance in this treatment. In fact, a study on *Daphnia* spp. performed within a community context showed that interspecific competition was prolonged under additional stresses, such as elevated temperature, and delayed the recovery of *Daphnia* spp. following esfenvalerate treatment (Knillmann et al., 2013). The observed replacement of *Daphnia* spp. by the more tolerant *Simocephalus* spp. is in agreement with the PICT concept, which attributes community tolerance to the replacement of sensitive species with tolerant ones (Blanck et al., 1988).

The fact that the enhanced community tolerance did not result from only one taxon, i.e., *Daphnia*, was confirmed by the Bray–Curtis similarity analysis of the community dataset without *Daphnia* spp., showing similar indices with and without *Daphnia* spp. Thus, the mechanisms underlying the enhanced community tolerance observed in the present study are based on rather complex community-level processes. In contrast, the above-mentioned previous study on the effects of temperature on the tolerance of bacterial communities to a toxicant (copper) (Boivin et al., 2005) showed that the enhancement of community tolerance may appear as a result of the intensification of physiological effects rather than changes in the community structure. In fact, the authors of that study suggested that the permeability of bacterial membranes and, hence, copper uptake in bacterial cells and the associated toxic effects were increased at higher temperatures, thereby enhancing the development of copper tolerance.

Similar community-level mechanisms may explain the results of a study by Navarro et al. (2008), which showed that the long-term exposure to UVR induced structural changes in a periphyton community that contributed to an increase in tolerance to UVR and, collaterally, to Cd. Thus, community-level mechanisms similar to those observed here may

have occurred, though this remains open to speculation because the effects of previous Cd exposure on the development of tolerance to Cd was not tested. Indeed, the observed co-tolerance was instead attributed to common defense mechanisms, such as the induction of antioxidant enzymes.

5.5.3 Community tolerance in higher organisms

The present study is one of the first to report community tolerance in such higher aquatic organisms as zooplankton. The observed tolerance is in accordance with the findings of several PICT studies (Bérard et al., 2002), including the only two studies on this topic in higher organisms (Clements, 1999; Millward and Grant, 2000). However, the present results contrast with those of several studies on repeated exposure in zooplankton (Daam et al., 2008; Hanazato and Yasuno, 1990) and amphibians (Boone et al., 2001; Relyea and Diecks, 2008).

For example, a study on frogs comparing single and multiple exposures showed that small weekly applications of the insecticide malathion caused greater impacts on many of the examined response variables than single-pulse applications at a 25-fold higher concentration (Relyea and Diecks, 2008). This finding was explained by the fact that the multiple pulses held the community in a state of continued disturbance, which further reinforced the trophic cascade initiated by the direct toxic effect of the insecticide on the zooplankton assemblage in the community.

Further studies comparing the effects of single pulses versus multiple pulses showed tendencies toward stronger effects. These results may be because the applied concentrations were relatively high with respect to the LC_{50} of each taxon (Boone et al., 2001; Hanazato and Yasuno, 1990), or because the time interval between the applications was relatively short relative to the life cycles of the organisms present (Daam et al., 2008). In the current study, the time interval between the pesticide applications was 30 days, which is approximately equal to the life span of *Daphnia* spp. under laboratory conditions (Anderson and Jenkins, 1942). Regarding the administered pesticide concentrations, the intermediate pesticide concentration was approximately equivalent to the 48-h LC_{50} of *Daphnia magna*, with the low and the high concentrations being a factor of 10 lower and higher, respectively. Thus, the determination of community tolerance through repeated pesticide contaminations may depend on various factors, such as the concentration level, exposure duration relative to the life cycle duration, interval between exposures, and frequency of exposure.

5.6 Conclusions

The present investigation indicates that non-chemical stressors can play an important role in the development of community tolerance to toxicants by acting through community-level mechanisms, thereby facilitating the development of community tolerance. This outcome implies that it is of major importance to consider the effects of non-chemical stressors on the development of community tolerance to toxicants when attempts are made to link community sensitivity in mesocosm experiments to the effects in the field or when PICT is applied in biomonitoring under natural conditions as they may alter the

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development of community tolerance. However, as this study provides the first empirical example of such effects, further investigations are needed to support their consideration in applied ecology.

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Chapter 6

Discussion

In this thesis, the influence of the environmental context, in particular solar radiation, community density, and water level, on the sensitivity of zooplankton communities to the model insecticide esfenvalerate was studied in two outdoor microcosm experiments. The validity and consistency of the results obtained by manipulating solar radiation and community density were assessed by comparing the outcomes of the experiment with those of a parallel experiment conducted in another biogeographical region. Furthermore, the effects of repeated exposure to a toxicant on zooplankton communities under fluctuating hydrological conditions were studied. In the following sections, the major results of these studies are presented and discussed in the light of their applications to ecological risk assessments of pesticides in the context of global climate change.

6.1 Summary of the results

6.1.1 Environmental context determines community sensitivity of freshwater zooplankton to a pesticide (Chapter 2)

The aim of this study was to examine the effects of food availability and competition on the community level impact of the insecticide esfenvalerate.

We performed an outdoor microcosm experiment in which we experimentally manipulated solar radiation by shading the microcosms and community density by harvesting organisms, which resulted in three treatments that represented a gradient of competition for food. The treatment in which the microcosms were not shaded but harvested represented the treatment with the lowest level of competition, while the treatment in which the microcosms were shaded but not harvested represented the highest degree of competition. The treatment in which the microcosms were neither shaded nor harvested was considered as intermediate. Microcosms in each treatment were exposed to 0, 0.03, 0.3 and 3 $\mu\text{g/L}$ of the insecticide esfenvalerate. The impact of the insecticide on the entire community under different levels of competition was analysed using multivariate statistical methods such as the redundancy analysis and the principal response curves, which are based on redundancy analysis, adjusted for overall changes in community response over time as observed in control microcosms (van den Brink and Ter Braak, 1999).

The results show that community sensitivity varied considerably between treatments.

6. Discussion

Table 6.1: Lowest observed effect concentrations (LOECs; $\mu\text{g/L}$) for the different environmental treatments and different sampling dates of the experiment conducted in Leipzig, Germany. The LOECs are derived from Monte Carlo permutation tests following redundancy analysis.

| Days after contamination | High food availability – low competition ^a | Medium food availability – medium competition ^b | Low food availability – high competition ^c |
|--------------------------|---|--|---|
| -9 | NA | NA | NA |
| 4 | 3 | 0.03 | 0.03 |
| 11 | 3 | 0.03 | 0.03 |
| 16 | 3 | 3 | 0.3 |
| 44 | 3 | 3 | 3 |
| 59 | 3 | 3 | 3 |
| 71 | 3 | 3 | 3 |

^a No shading – harvesting

^b No shading – no harvesting

^c Shading – no harvesting

In the treatment with high competition, significant effects of the insecticide on the community were observed at the lowest concentration of $0.03 \mu\text{g/L}$. In contrast, in the treatment with relatively weak competition, significant effects were only observed at the highest concentration level of $3 \mu\text{g/L}$ (Table 6.1). Thus, community sensitivity to the toxicant differed by up to a factor of 100 among the treatments. Despite the differences in acute sensitivity to the toxicant, no differences in community recovery rates among the different treatments were observed. This lack of difference in acute sensitivity was attributed to the fact that the majority of the species in the microcosms were not affected by the pesticide in the long-term.

To conclude, the study shows that communities exposed to high competition may be more sensitive to pesticides than communities exposed to relatively less competition, and the sensitivity may differ by up to a factor of 100.

6.1.2 Community sensitivity to a xenobiotic can be predictable – A cross-Eurasia experiment revealed strong and consistent modulations (Chapter 3)

In this study, we compared the sensitivity to esfenvalerate of zooplankton communities exposed to different levels of competition in two biogeographical regions.

We compared the results of the experiment presented in Chapter 2 with a similar experiment conducted in parallel in Karasuk, southwestern Siberia (Russia), within the framework of the German-Russian project ECOLINK. In particular, we compared the LOECs and NOECs of the communities at the different locations. Furthermore, we compared the structure of the zooplankton community between the two biogeographical regions by computing the Bray-Curtis similarity indices for each treatment.

As in Germany, community sensitivity in Russia between the experimental treatments

Table 6.2: Lowest observed effect concentrations (LOECs; $\mu\text{g/L}$) for the different environmental treatments and different sampling dates of the experiment conducted in Karasuk, Russia. The LOECs are derived by Monte Carlo permutation tests following redundancy analysis.

| Days after contamination | High food availability – low competition ^a | Medium food availability – medium competition ^b | Low food availability – high competition ^c |
|--------------------------|---|--|---|
| -8 | NA | NA | NA |
| 5 | 3 | 0.3 | 0.03 |
| 10 | 3 | 0.3 | 0.03 |
| 14 | 3 | 3 | 0.03 |
| 36 | 3 | 3 | 0.03 |
| 61 | 3 | 3 | 3 |

^a No shading – harvesting

^b No shading – no harvesting

^c Shading – no harvesting

represented a gradient of competition for food that differed up to a factor of 100 based on the LOEC (Table 6.2). In both regions, the high competition treatment, due to shading and absence of harvesting, was the most sensitive. Remarkably, this treatment exhibited the greatest dissimilarity in community structure between the regions. This indicates that the stress caused by the combination of the two abiotic factors may overrule any differences in the response of communities to insecticide exposure related to differences in species composition.

In summary, the present study shows that the direction and magnitude of alterations in community sensitivity was reproducible and consistent across different biogeographic regions despite the differences in community structure.

6.1.3 Two stressors and a community – Effects of hydrological disturbance and a toxicant on freshwater zooplankton. (Chapter 4)

The aim of this study was to investigate the influence of hydrological stress on the toxicity of the insecticide esfenvalerate in a zooplankton community.

We conducted an outdoor microcosm study in which zooplankton communities were exposed to esfenvalerate contamination as a single pulse with fluctuations in the water level. Water level fluctuations consisted of the gradual removal of water followed by its subsequent replacement over three cycles. Esfenvalerate concentrations were 0, 0.03, 0.3 and 3 $\mu\text{g/L}$. The impact of the insecticide on the entire community under different hydrological regimes was analysed using multivariate statistical methods, such as redundancy analysis and principal response curves, a special case of the first method. Furthermore, the statistical significance of the interactions between the toxicant and the hydrological treatment for the entire community and the most sensitive taxa was assessed using redundancy analysis followed by Monte Carlo permutation tests and analysis of covariance,

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Table 6.3: Lowest observed effect concentration (LOECs; $\mu\text{g/L}$) and combined effect of esfenvalerate and hydrological regime on community structure and the abundance of *Daphnia* spp. The LOECs are derived by Monte Carlo permutation tests following redundancy analysis and ANOVA followed by pairwise t-tests for community structure and the abundance of *Daphnia* spp., respectively. Significance of interaction was derived by RDA and ANCOVA for community structure and abundance of *Daphnia* spp., respectively.

| Endpoint | Days after contamination | LOEC Treatment with constant water level | LOEC Treatment with water level fluctuations | Type of interaction |
|---------------------|--------------------------|--|--|---------------------|
| Community structure | 8 | 0.3 | 0.03 | Additive |
| | 28 | 0.3 | 3 | Antagonistic |
| | 36 | 3 | 3 | Additive |
| | 55 | 3 | 3 | Additive |
| | 64 | 3 | 3 | Additive |
| | 84 | > 3 | > 3 | Additive |
| <i>Daphnia</i> spp. | 8 | 0.3 | 0.03 | Additive |
| | 28 | 3 | 3 | Antagonistic |
| | 36 | > 3 | 3 | Antagonistic |
| | 55 | 3 | 3 | Additive |
| | 64 | 3 | 3 | Additive |
| | 84 | > 3 | > 3 | Additive |

respectively.

The results show that the effects of the toxicant on the community and the key taxon *Daphnia* spp., were greater in the presence of hydrological disturbance. Specifically, the LOECs for both the community and *Daphnia* spp. were 0.03 and 0.3 $\mu\text{g/L}$ for the microcosms with fluctuating and constant water levels, respectively. Despite these differences in sensitivity, interactions between the two stressors were not significant, i.e., the effects were additive for both community structure and the abundance of *Daphnia* spp. (Table 6.3). Recovery time of the community following exposure to the highest concentration of pesticide was longer under fluctuating water levels. Based on principal component analysis, competition and water quality were considered to be the major factors underlying the observed effects of fluctuations in the water level.

To conclude, the present study indicates that changes in hydrological regime may increase the sensitivity of communities to toxicants, especially at low concentrations of toxicants. Although the interactions between the two stressors in the present study were not statistically significant, the results indicate that the combined effects might be more than additive, i.e., synergistic.

6.1.4 Environmental stressors can enhance the development of community tolerance to a toxicant (Chapter 5)

In this study, we aimed to investigate whether zooplankton communities exposed to repeated esfenvalerate contamination events developed tolerance to the insecticide and whether this tolerance development was enhanced by fluctuating water levels. This hypothesis was based on the results of the experiment presented in Chapter 4, which showed that although esfenvalerate and water level fluctuations were expected to affect zooplankton organisms through different mechanisms, both stressors caused a strong reduction in the abundance of the same dominant taxon, *Daphnia* spp. Hence, we hypothesised that hydrological disturbance would enhance the development of community tolerance to recurring insecticide contamination by decreasing the number of pesticide-sensitive species and selecting more tolerant species. To test this hypothesis, an outdoor microcosms experiment was conducted in which zooplankton communities were exposed to three sequential pulses of esfenvalerate under conditions of constant or decreased periodical water level. We quantified the alterations to community structure using multivariate statistics such as PRC and similarity analysis. Analysis of the dynamics of individual taxa was used to complement the analysis of the community's response.

The results show that the zooplankton community developed tolerance to the insecticide esfenvalerate, and this development was enhanced by hydrological disturbance. The latter result was indicated by the higher community LOEC in the WLF treatment compared to the CWL treatment (Table 6.4). Further evidence was provided by the significantly greater similarity in community structure between the uncontaminated and contaminated microcosms in the WLF compared to the CWL treatment. The higher similarity in the WLF treatment indicates that the communities exposed to esfenvalerate were less different than the non-exposed communities and hence were less disturbed by the repeated exposures than the CWL treatment. Elimination of the key taxa *Daphnia* spp. through the combined action of the two stressors was confirmed to be the main mechanism responsible for the increase in community tolerance under a fluctuating water level. In contrast to the WLF treatment, the abundance of *Daphnia* spp. in the CWL treatment did not continuously decrease over the multiple applications, i.e., *Daphnia* spp. recovered to the control level shortly after each contamination event, except under the highest concentration. This indicates that other mechanisms may have been responsible for the observed community tolerance in the CWL treatment.

To summarise, the present investigation indicates that non-chemical stressors may facilitate the development of community tolerance to toxicants by acting through community-level mechanisms.

Table 6.4: Lowest observed effect concentrations (LOECs; $\mu\text{g/L}$) for the two hydrological treatments and the different sampling dates under multiple esfenvalerate contamination events. The LOECs are derived by Monte Carlo permutation tests following redundancy analysis. The days refer to time since the first contamination. The second and third contamination events were conducted after the sampling on days 28 and 64, respectively.

| Days after first contamination | LOEC treatment with constant water level | LOEC treatment with water level fluctuations |
|--------------------------------|--|--|
| 8 | 0.3 | 0.03 |
| 28 | 0.3 | 3 |
| 36 | 0.3 | 3 |
| 55 | 3 | 3 |
| 64 | 3 | 3 |
| 84 | 3 | 3 |
| 119 | 3 | > 3 |

6.2 Discussion of the major results and implications for pesticide risk assessment

6.2.1 Influence of additional stressors on the sensitivity of zooplankton communities to pesticides

The studies presented in Chapter 2-5 reveal that additional environmental stressors can increase the sensitivity of entire communities to pesticides. In particular, the maximum calculated values of NOEC and LOEC for the different treatments differed by more than factors of 10 and 100, respectively. The main difference in community sensitivity occurred immediately after pesticide contamination (i.e., 4 and 8 days). To date, only two studies have quantified and compared the NOEC or LOEC of communities exposed to additional environmental stressors and those experiencing no additional stress (Roessink et al., 2005; van Wijngaarden et al., 2005a). In these studies, similar differences in NOEC of > 2.5 (100 ng/L and > 250 ng/L, Roessink et al., 2005) and ≥ 10 (0.1 $\mu\text{g/L}$ and ≥ 1 $\mu\text{g/L}$, van Wijngaarden et al., 2005a) were found. In contrast to our studies, the main difference in community sensitivity in these two studies was observed a considerable time after contamination (i.e., 21 days) and at pesticide concentrations higher than the LOEC. Based on these outcomes, the authors suggested that the environmental context does not play an important role in determining community sensitivity. This position was further supported by studies that compared sensitivity of communities from different geographical regions that are characterised by different environmental conditions and did not find any significant differences in community sensitivity (Daam et al., 2009; López-Mancisidor et al., 2008a, 2008b). The lack of difference in community sensitivity found in these studies may be explained by the adaptations of the communities to the different environments. In contrast to these studies, the communities in our experiments were exposed to the toxicant shortly after the manipulation of the environmental factors. Thus, the communities may

not have been adapted to the new environmental conditions when exposed to the toxicant. This hypothesis is supported by previous studies that have shown that trends in the dynamics of populations and developmental stages may alter the sensitivity as well as the recovery dynamics of populations affected by toxicants (e.g., reviews by Fleeger et al., 2003; Hanazato, 1998).

In our studies, the differences in community sensitivity were generally observed shortly after contamination, but recovery did not seem to differ among the treatments. This may be due to the dominance of non-affected species in the test systems. In fact, Knillmann et al. (2012a) analysed the effects of esfenvalerate on *Daphnia* spp. in the community context in the same experiment and detected significant differences in the abundance of *Daphnia* spp. up to 6 weeks after contamination. Thus, even though recovery from the insecticide at the community level may not appear to be affected by the additional stressors, there may still be significant effects of the additional stressors on recovery from the insecticide at the population level.

6.2.2 Abiotic factors

In the present work, solar radiation (Chapter 2, 3) and water level (Chapter 4, 5) were experimentally manipulated. The manipulation of these abiotic factors in turn resulted in changes of other abiotic factors that contributed to altered community sensitivity to the insecticide esfenvalerate.

Shielding the microcosms from solar radiation (Chapter 2, 3) resulted in a reduction in the amount of sunlight, which in turn caused a significant reduction in water temperatures and resulted in lower phytoplankton productivity. On the contrary, in the water level fluctuation experiment (Chapter 4, 5), phytoplankton productivity in the microcosms exposed to fluctuating water levels increased as a result of the greater penetration of solar radiation into the water and the resulting elevated water temperature.

In all of the experiments, the changes in abiotic factors were generally considered to be in the range of tolerance of the aquatic organisms. For example, in the experiment in which solar radiation and community density were manipulated (Chapter 2), comparisons of the community structure in the control series (i.e., the series that was not contaminated with esfenvalerate) of the different treatments did not reveal significant differences, indicating that manipulating solar radiation and community density did not significantly affect the communities. In the experiment in which the hydrological regime was manipulated (Chapter 4, 5) environmental parameters such as DO concentration, EC, pH and temperature were generally within the natural range of such habitats. However, when the water reached minimum levels (10 L), DO concentrations decreased to levels as low as 5 mg/L, which is in the critical range of oxygen concentration for *Daphnia* spp. (2.7 mg/L Homer and Waller, 1983), and EC levels significantly increased to maximum values of up to 800 $\mu\text{S}/\text{cm}$. While there is evidence that DO concentration might exacerbate the toxicity of pesticides to freshwater invertebrates at levels comparable to those observed in the water level fluctuation treatment (Hanazato and Dodson, 1995), EC values such as those observed in the present study are common in the study region and in Central European waters.

Abiotic factors may also indirectly influence the effects of pesticides on aquatic communities by altering biotic factors such as species interactions (e.g. competition, predation).

6.2.3 Species interactions

In both experiments, the manipulation of abiotic factors resulted in a change in phytoplankton production, which in turn may have altered competition between the grazing species. In fact, changes in competition were identified as a major mechanism underlying the observed differences in community sensitivity.

In the first experiment (Chapter 2), three different environmental treatments representing different levels of food availability, and thus competition for food, were established. The treatment, which was designed to produce the highest stress level due to having the smallest amount of food and the highest level of competition, produced the greatest community sensitivity. By contrast, the treatment expected to have the lowest stress due to having the most food and the lowest levels of competition indeed showed the lowest community sensitivity. Consequently, community sensitivity to the insecticide esfenvalerate increased with increasing competition and was highest under competition. Further evidence for the importance of competition was provided by Knillmann et al. (2012a, 2012b) who, based on the same microcosm experiment, analysed the effects of the pesticide on *Daphnia* spp. The authors found a significantly positive correlation between the density of *Daphnia* spp. before contamination, i.e., under intraspecific competition, and the effect of the pesticide on this taxon (Knillmann et al., 2012b). Furthermore, a significant negative correlation between the density of the related taxon *Simocephalus* spp. (i.e., interspecific competition) and the recovery of *Daphnia* spp. was also found (Knillmann et al., 2012a). In the water level fluctuation experiment (Chapter 4, 5), competition was also identified as playing a major role in determining community sensitivity. In this experiment, the reduction of water level resulted in a strong increase in overall organism densities, which on average reached between 630 to 1104 individuals/L compared to 201 individuals/L under constant (60 L) water levels (see Fig 4.2, Chapter 4). In the WLF treatment, zooplankton density declined by up to 54%, highlighting the negative effects of elevated rates of competition induced by high densities of organisms. Similar to the experiment with shading and harvesting, in the water level fluctuation treatment, which was characterised by temporarily high organism densities and thus high competition levels, the communities were more sensitive to the single application of pesticides. These results are in line with previous studies, primarily at the population level on food availability and competition that showed that species exposed to competition are more sensitive to toxicants (e.g., Beketov and Liess, 2005; Liess, 2002; Rose et al., 2002).

Remarkably, in the presence of multiple pesticide exposures, communities exposed to water level fluctuations were less sensitive to the insecticide than the communities held under constant water levels (Chapter 5). This result may be explained by the strong decrease in the abundance of the dominant taxon *Daphnia* spp. as a result of the first pesticide contamination and the strong competition that prevailed under low water level conditions. As a result, the potential effect of further pesticide contamination events was strongly reduced, and the community appeared to be less sensitive. While this is in accordance with the findings of several PICT studies (see review by Bérard et al., 2002), to date no such outcome has been reported for communities exposed to additional stressors. In fact, prior to the present study, it had only been shown that increased community tolerance may appear as the result of increased toxicant uptake (Boivin et al., 2005).

In addition to competition, predation has been recognised as an important factor struc-

turing aquatic communities and influencing the effects of pesticides on aquatic ecosystems. Several studies have shown that predation may exacerbate the sensitivity of organisms to toxicants (Beketov and Liess, 2006; Relyea and Hoverman, 2008). In the present studies, predation was not found to be affected by the abiotic factors or to influence the response of aquatic organisms to the insecticide. However, this is most likely because the community consisted mainly of crustaceans. Common predators of these organisms are phantom midge larvae, notonectids, odonates and planktivorous fish. Compared to the abundance of crustaceans, these predators were either comparably scarce or not present. However, the lack of predators may have resulted from the sampling technique used for the initial collection of organisms that was aimed at colonising the microcosms and is mostly suited for collecting zooplankton (DeVries and Stein, 1991).

6.3 Importance of the present results for the future ecological risk assessment of pesticides

6.3.1 Representativity of the present outcomes for the field

To study the effects of pesticides and environmental factors, we conducted artificial outdoor microcosm experiments. Microcosms or mesocosms are increasingly used in risk assessment of pesticide effects on communities (van den Brink et al., 2005). However, the identification of community-level effects of pesticides in micro- and mesocosms is generally hampered by high between-replicate variation and a scattered low-abundance distribution of the majority of the taxa (Beketov et al., 2008; Knauer et al., 2005; Liess and Beketov, 2011; Sanderson et al., 2009; Wang and Riffel, 2011). Additionally, because of limited resources, micro- and mesocosm studies are usually characterised by a small number of replicates (Knillmann et al., 2012a) and restricted sampling effort (Campbell et al., 1999; de Jong et al., 2008; Giddings et al., 2002). As a result, the actual effects of pesticides in micro- and mesocosms may be underestimated, especially at low concentrations, which can subsequently lead to under-protective ecological standards (Sanderson et al., 2009).

An analysis of outdoor microcosms reviewed by Fleeger et al. (2003) showed that the average number of replications in outdoor microcosm studies is three (Knillmann et al., 2012a). In the present study, approximately twice the average number of replicates, i.e., five in the experiment with fluctuating water levels and six in the experiment with solar radiation and harvesting, was used for each environmental treatment and pesticide concentration level. Controls were replicated 10-12 times. Replication reduces variability in experimental results, which increases their significance and the level of confidence with which conclusions can be drawn. Consequently, the reliability of the results from the microcosm experiments is high.

Esfenvalerate was used as the model pesticide. The concentrations of esfenvalerate used in the experiments (0.03, 0.3, 3 $\mu\text{g/L}$) were within the range of concentrations previously detected in natural ecosystems (0.166 $\mu\text{g/L}$, Bacey et al., 2005), (0.76 $\mu\text{g/L}$, Cooper et al., 2003). The medium concentration used in our studies approximately represents the 48-h LC_{50} value of *Daphnia magna* (0.37 $\mu\text{g/L}$) as determined by a preliminary laboratory study.

In all of the microcosm experiments, significant effects on the community were ob-

served at the lowest concentration applied, 0.03. Thus, significant effects occurred at concentrations approximately 10 times smaller than the laboratory derived LC₅₀ value for *Daphnia magna*. Based on the concept of ecological redundancy, which assumes that more than one species performs a given role within an ecosystem (Walker, 1992), lower effects may be expected in natural ecosystems. In the present study, however, the majority of organisms were short-lived. Effects in natural ecosystems, in which longer living organisms such as stoneflies or dragonflies are more abundant, may be even stronger. This is in line with recently published studies on the risk assessment of pesticides in streams which have detected higher pesticide effects in the field compared to those estimated from stream mesocosms (Beketov et al., 2013; Schäfer et al., 2012). The authors attributed the higher pesticide effects to factors such as exposure to multiple stressors and mixtures of toxicants as well as the statistical approaches used to define environmentally safe concentrations (Schäfer et al., 2012).

6.3.2 Significance of the present results in terms of on-going climate change

Temperature and moisture regimes are major variables that determine the distribution, growth and productivity of flora and fauna (Bates et al., 2008). Both of these variables are projected to change in the coming decades. For instance, global air temperature is estimated to rise 0.2°C per decade (Pachauri and Reisinger, 2007). Similarly, precipitation is projected to be concentrated into more intense raining events with longer periods of little precipitation in between (Bates et al., 2008). As a result of these changes, pesticide input into aquatic ecosystems is projected to increase (Kattwinkel et al., 2011).

Considering the results of the studies presented in Chapter 2-5, changes in the environmental context of aquatic ecosystems are expected to significantly affect the sensitivity of aquatic communities to pesticides. In particular, alterations in community sensitivity to pesticides by a factor of up to 100 (based on the LOEC) may occur. In the present studies, these alterations were mainly driven by pronounced changes in the abundance of *Daphnia* spp. and the resulting effects on other species. Thus, *Daphnia* spp. played a major role in determining community sensitivity. In fact, *Daphnia* spp. is often considered to be a keystone species in freshwater ecosystems. Therefore, in communities where keystone species are significantly affected by stressors, pronounced changes in community sensitivity are expected to occur. Conversely, in communities where stressors mainly affect subordinate species, community sensitivity may be less affected.

Even though the changes in abiotic and biotic factors were not based on particular climate change scenarios, they are still likely to occur. For instance, alterations in the penetration of solar ultraviolet radiation into aquatic ecosystems, in particular due to shading, may result from changes in the surrounding vegetation. Similarly, the density of a community may be influenced by changes in the quantity or quality of available food or the presence of competitors or predators. Water level fluctuations are a natural characteristic of aquatic ecosystems. They are projected to increase and, as a result, will likely exceed the levels with which many organisms can cope through physiological and behavioural adaptations (Coops et al., 2003). Based on the outcomes of the present studies, such extreme water level fluctuations may increase the sensitivity of communities to pesticides in the short-term (Chapter 4). In the long-term, however, such fluctuations

may result in enhanced development of community tolerance to pesticides (Chapter 5). In conclusion, climate change may render aquatic communities more sensitive to pesticides (Chapter 2-4) or may enhance community tolerance to pesticides due to the replacement of sensitive species with more tolerant ones (Chapter 5), particularly if keystone species are affected. Either case might be accompanied by fundamental changes in community structure, which in turn may affect the function of aquatic ecosystems.

6.3.3 Implications for pesticide risk assessment using outdoor micro- or mesocosms

Outdoor micro- or mesocosms are increasingly used in pesticide risk assessment. A framework of criteria and relevant endpoints for acceptable aquatic outdoor cosm studies for testing chemicals is provided by the OECD guidance document (OECD, 2006). The aim of these higher tier risk assessments is to produce knowledge about the ecological relevance of the results from laboratory studies or to measure effects of the chemical under more environmentally realistic conditions for exposure (OECD, 2006). As a result, the uncertainty factors applied to higher tier studies may be reduced when compared to those applied to lower tier studies, such as single-species laboratory tests. In fact, the use of an uncertainty factor of 1 for aquatic outdoor microcosms or mesocosms has been proposed by various experts (Sabine Dusquene, UBA, pers. communication).

The findings reported in this work have two major implications for micro- or mesocosm studies used in higher tier risk assessment studies:

(1) The abiotic environmental context of aquatic ecosystems needs to be taken into account. The present studies show that changes in abiotic environmental factors such as water level or solar radiation might influence the sensitivity of communities to pesticides both directly and indirectly through alterations of species interactions, which in turn affect community sensitivity. Therefore, abiotic environmental factors either need to be considered in micro- or mesocosms studies, such as by using a full factorial design with at least 2 levels for each abiotic factor, or through the incorporation of appropriate uncertainty factors.

(2) A literature research on community-level studies (table 1.1) revealed that several of studies did not examine the effect of the multiple stressors at the community level, i.e., they did not use a community endpoint. Community endpoints should be included in the analysis of the outcomes as they provide valuable information about the community by reflecting the responses of individuals. In the present studies, changes in community structure were derived from redundancy analysis and principal response curve analysis. This endpoint was found to be a sensitive indicator of the combined effects of a toxicant and an additional stressor.

In conclusion, the present results as well as the outcomes of previous investigations clearly indicate the need to include more ecological knowledge and methods in the practice of risk assessment of pesticides. This is particularly relevant and timely as the current risk assessment practice falls short of protecting aquatic systems and their biodiversity (Beketov et al., accepted; Schäfer et al., 2012).

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Curriculum Vitae

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Beiträge an die Manuskripte

Geschätzter Beitrag der einzelnen Autoren an den Publikationen der kumulativen Dissertation „Der Einfluss von Umweltbedingungen auf die Sensitivität von Zooplanktongemeinschaften gegenüber Pestiziden“:

1) Stampfli, N.C. (SN), Knillmann, S. (KS), Liess, M. (LM), Beketov, M.A. (BM) (2011). „Environmental context determines community sensitivity of freshwater zooplankton to a pesticide“. In: *Aquatic Toxicology* 104 (1-2), pp. 116-120.

| Feldarbeit | Proben-aufbereitung (v.a. Arten-identifikation) | Datenaufbereitung und -analyse | Interpretation der Daten | Schreiben des Manuskripts | Korrektur-lesen und Kommentieren |
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2) Beketov, M.A. (BM), Stampfli, N.C. (SN), Yurchenko, Yu.A. (YA), Belevich, O.E. (BO), Knillmann, S. (KS), Noskov, Yu.A. (NY), Liess, M. (LM). „Community sensitivity to a xenobiotic can be predictable – A cross-Eurasia experiment revealed strong and consistent modulations“. In preparation.

| Feldarbeit | Proben-aufbereitung (v.a. Arten-identifikation) | Datenaufbereitung und -analyse | Interpretation der Daten | Schreiben des Manuskripts | Korrektur-lesen und Kommentieren |
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3) Stampfli, N.C. (SN), Knillmann, S. (KS), Liess, M. (LM), Noskov, Y.A. (NY), Schäfer, R.B. (SR), Beketov, M.A. (BM) (2013). „Two stressors and a community – effects of hydrological disturbance and a toxicant on freshwater zooplankton“. In: *Aquatic Toxicology*, 127, pp. 9-20.

| Feldarbeit | Proben-aufbereitung (v.a. Arten-identifikation) | Datenaufbereitung und -analyse | Interpretation der Daten | Schreiben des Manuskripts | Korrektur-lesen und Kommentieren |
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| Feldarbeit | Proben-aufbereitung (v.a. Arten-identifikation) | Datenaufbereitung und -analyse | Interpretation der Daten | Schreiben des Manuskripts | Korrektur-lesen und Kommentieren |
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Eigenständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig und ohne unerlaubte Hilfsmittel verfasst und keine anderen als die angegebenen Quellen verwendet habe.

Nathalie C. Stampfli
Basel, den 19.07. 2013

