EFFECTS OF PESTICIDES TO MACROPHYTES AND MACROINVERTEBRATES AND AQUATIC-TERRESTRIAL FOOD WEB COUPLING IN STREAM MESOCOSMS

by

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Overview of Publications

This cumulative dissertation is based on the following four scientific publications:

1. Wieczorek, MV, Bakanov, N, Stang, C, Bilancia, D, Lagadic, L, Bruns, E and Schulz, R. 2016. Reference scenarios for exposure to plant protection products and invertebrate communities in stream mesocosms. *Sci. Total Environ.* 545-546: 308-319. Doi: 10.1016/j.scitotenv.2015.12.048 [**Appendix I**]

2. Wieczorek, MV, Bakanov, N, Lagadic, L, Bruns, E and Schulz, R. in press. Response and recovery of the macrophytes *Elodea canadensis* and *Myriophyllum spicatum* following a pulse exposure to the herbicide iofensulfuron-sodium in outdoor stream mesocosms. *Environ. Toxicol. Chem.* Doi: 10.1002/etc.3636 [**Appendix II**]

3. Wieczorek, MV, Bakanov, N, Bilancia, D, Szöcs, E, Stehle, S, Bundschuh, M and Schulz, R. Structural and functional effects of a short-term pyrethroid pulse exposure on invertebrates in outdoor stream mesocosms (Submitted to *Environ. Pollut.*) [**Appendix III**]

4. Wieczorek, MV, Kötter, D, Gergs, R and Schulz, R. 2015. Using stable isotope analysis in stream mesocosms to study potential effects of environmental chemicals on aquatic-terrestrial subsidies. *Environ. Sci. Pollut. Res.* 22: 12892–12901. Doi: 10.1007/s11356-015-4071-0 [**Appendix IV**]

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Abstract

Agricultural land-use may lead to brief pulse exposures of pesticides in edge-of-field streams, potentially resulting in adverse effects on aquatic macrophytes, invertebrates and ecosystem functions. The higher tier risk assessment is mainly based on pond mesocosms which are not designed to mimic stream-typical conditions. Relatively little is known on exposure and effect assessment using stream mesocosms. Thus the present thesis evaluates the appliacability of the stream mesocosms to mimic stream-typical pulse exposures, to assess resulting effects on flora and fauna and to evaluate aquatic-terrestrial food web coupling. The first objective was to mimic stream-typical pulse exposure scenarios with different durations ($\leq$ 1 to $\geq$ 24 hours). These exposure scenarios established using a fluorescence tracer were the methodological basis for the effect assessment of an herbicide and an insecticide. In order to evaluate the applicability of stream mesocosms for regulatory purposes, the second objective was to assess effects on two aquatic macrophytes following a 24-h pulse exposure with the herbicide iofensulfuron-sodium (1, 3, 10 and 30 µg/L; n = 3). Growth inhibition of up to 66 and 45% was observed for the total shoot length of *Myriophyllum spicatum* and *Elodea canadensis*, respectively. Recovery of this endpoint could be demonstrated within 42 days for both macrophytes. The third objective was to assess effects on structural and functional endpoints following a 6-h pulse exposure of the pyrethroid ether etofenprox (0.05, 0.5 and 5 µg/L; n = 4). The most sensitive structural (abundance of *Cloeon simile*) and functional (feeding rates of *Asellus aquaticus*) endpoint revealed significant effects at 0.05 µg/L etofenprox. This concentration was below field-measured etofenprox concentrations and thus suggests that pulse exposures adversely affect invertebrate populations and ecosystem functions in streams. Such pollutions of streams may also result in decreased emergence of aquatic insects and potentially lead to an insect-mediated transfer of pollutants to adjacent food webs. Test systems capable to assess aquatic-terrestrial effects are not yet integrated in mesocosm approaches but might be of interest for substances with bioaccumulation potential. Here, the fourth part provides an aquatic-terrestrial model ecosystem capable to assess cross-ecosystem effects. Information on the riparian food web such as the contribution of aquatic (up to 71%) and terrestrial (up to 29%) insect prey to the diet of the riparian spider *Tetragnatha extensa* was assessed via stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$). Thus, the present thesis provides the methodological basis to assess aquatic-terrestrial pollutant transfer and effects on the riparian food web.

Overall the results of this thesis indicate, that stream mesocosms can be used to mimic stream-typical pulse exposures of pesticides, to assess resulting effects on macrophytes and invertebrates within prospective environmental risk assessment (ERA) and to evaluate changes in riparian food webs.

Zusammenfassung

Landwirtschaftliche Flächennutzung kann zu zeitlich begrenzten Pestizideinträgen in angrenzende Fließgewässer und damit zu negativen Effekten auf Invertebraten, Makrophyten und ökosystemare Funktionen führen. Zumeist werden für die Effekterfassung Stillgewässermesokosmen verwendet, die allerdings nicht dazu geeignet sind, typische Prozesse in Fließgewässern zu simulieren. Relativ wenig ist bisher über die Expositions- oder Effekterfassung mittels Fließmesokosmen bekannt. Daher prüft die vorliegende Arbeit die Möglichkeit, Fließmesokosmen zu nutzen, um kurze Pestizidexpositionen nachzustellen und daraus resultierende Effekte auf Flora und Fauna zu erfassen und aquatisch-terrestrische Interaktionen zu untersuchen. Das erste Ziel war es, realistische Pestizidexpositionen mit unterschiedlicher Verweildauer ($\leq$ 1 bis $\geq$ 24 Stunden) in Fließmesokosmen zu etablieren. Diese Expositionsszenarien stellten die methodische Basis für die Effekterfassung eines Herbizids und eines Insektizids im Rahmen dieser Arbeit dar. Um die Anwendbarkeit der Fließmesokosmen für regulatorische Zulassungsprozesse zu untersuchen, war das zweite Ziel dieser Arbeit, Effekte einer 24-stündigen Herbizidexposition (iofensulfuron-sodium; 1, 3, 10 und 30 µg/L; n = 3) auf zwei aquatische Makrophyten zu untersuchen. Vorübergehende Wachstumshemmungen der Gesamtsprosslänge von bis zu 66% bei *Myriophyllum spicatum* bzw. 45% bei *Elodea canadensis* wurden erfasst. Eine Erholung dieses Sprosswachstums beider Makrophyten konnte während einer 42-tägigen Erholungsphase nachgewiesen werden. Das dritte Ziel war die Erfassung struktureller und funktioneller Effekte einer 6-stündigen Insektizidexposition mit dem Pyrethroid Etofenprox (0.05, 0.5 und 5 µg/L; n = 4). Der sensitivste strukturelle (Abundanz von *C. simile*) und funktionelle (Fressrate von *A. aquaticus*) Endpunkt zeigte signifikante Effekte bei 0,05 µg/L Etofenprox. Da diese Konzentration unterhalb der in Feldstudien erfassten Effekte lag, könnten auch kurzzeitige Expositionen Invertebratenpopulationen und ökosystemare Funktionen in Fließgewässern schädigen. Solche Belastungen von Fließgewässern können zu einer Reduktion schlüpfender merolimnischer Insekten und potentiell zu einem Transfer von Schadstoffen in angrenzende Nahrungsnetze führen. Testdesigns, um solche ökosystem-übergreifenden Effekte zu erfassen, sind bisher noch nicht für Mesokosmen entwickelt worden. Der vierte Teil dieser Arbeit präsentiert ein aquatisch-terrestrisches Modellökosystem, das geeignet ist, ökosystemübergreifende Effekte zu erfassen. Der Beitrag von aquatischen (bis zu 71%) und terrestrischen (bis zu 29%) Beuteinsekten zur Ernährung der uferbewohnenden Spinnen *Tetragnatha extensa* konnte mittels stabiler Isotopenverhältnisse ($\delta^{13}C$ and $\delta^{15}N$) nachgewiesen werden. Daher stellt die vorliegende Arbeit die methodische Basis dar, um den

aquatisch-terrestrischen Schadstofftransfer und Effekte auf das Nahrungsnetz von Uferökosystemen zu untersuchen.

Insgesamt konnte gezeigt werden, dass Fließmesocosmen dazu geeignet sind, fließgewässertypische Pestizideinträge zu simulieren sowie die resultierenden Effekte auf Makrophyten und Invertebraten im Rahemen der Umweltrisikoeinschätzung zu erfassen und Änderungen in Ufernahrungsnetzen zu untersuchen.

1 Introduction

As a consequence of the worldwide intensification of agricultural practice and the associated increase of pesticide use (Tilman et al. 2001), pesticide residues are frequently found in non-target ecosystems such as edge-of-field surface waters (Stehle and Schulz 2015). Edge-of-field surface waters are defined as water bodies adjacent to pesticide treated agricultural areas (EFSA 2013). There are fundamental differences between standing and flowing water bodies concerning exposure dynamics and the fate of pesticides within the water phase, mainly due to different hydrologic conditions (Mohr et al. 2007). Although there is a considerable proportion of small edge-of-field streams in agricultural areas, most of the prospective higher tier test approaches are based on pond mesocosms which are not designed to mimic flowing water conditons. Even though stream mesocosm approaches regained interest for scientific and regulatory purpose in the last years, knowledge on effects on invertebrates and macrophytes following stream-typical pesticide pulses is still scarce and the implementation in regulatory processes was not put into practice up to now. The present thesis thus focuses on stream mesocosm approaches to contribute to the further development of mimicking stream-typical pulse exposures and to adapt effect assessment procedures of insecticides and herbicides to stream mesocosm.

Typical pulse exposures of pesticides vary in their duration between one hour (Schulz and Liess 1999; Leu et al. 2004; Rabiet et al. 2010; Sangchan et al. 2012), multiple hours (Richards and Baker 1993; Leu et al. 2004) and day(s) (Sangchan et al. 2012). For instance, pulses of sorptive pesticides (e.g. insecticides) typically occur following runoff events with durations of few hours (Leu et al. 2004; Rabiet et al. 2010; Rasmussen et al. 2013; Stehle et al. 2013). Due to their predominantly high solubility, herbicides may enter edge-of-field streams through runoff and additionally via drainage, resulting in compound pulses with durations from several hours to days (Leu et al. 2004; Rabiet et al. 2010). In order to set the basis for an effect assessment of herbicides and insecticides on aquatic macrophytes and invertebrates, the first part of the present thesis focuses on mimicking several realistic and stream-typical pulse exposures using stream mesocosms. Although pulse durations of insecticides and herbicides in edge-of-field streams are short, pulse exposures may adversely affect aquatic flora (Cedergreen et al. 2005) and fauna (Schulz and Liess 1999; Schulz 2004; Rasmussen et al. 2013).

Herbicide-induced adverse effects on aquatic macrophytes in streams (Graymore et al. 2001; Cedergreen et al. 2005) are critical due to the fact that aquatic macrophytes are essential for many aquatic invertebrates. For instance, aquatic macrophytes provide shelter from

predators as well as stream current and serve as habitats for reproduction (Walker et al. 2013). Furthermore, aquatic macrophytes are of importance for stream ecosystem functioning by influencing the nutrient cycle and physico-chemical parameters such as pH and oxygen (Kaenel et al. 2000; Clarke 2002). Moreover, aquatic macrophytes are, to a certain degree, determinants that may modify hydrological conditions of streams by e.g. retaining sediment particles and reducing velocity (Gregg and Rose 1982; Wharton et al. 2006; Franklin et al. 2008). From an ecotoxicological point of view, aquatic macrophytes and the surface-associated biofilm are of importance for mitigating exposure-related adverse effects via sorption, transient storage, degaradation and phytoremediation of pesticides (Schulz 2004; Dosnon-Olette et al. 2009; Thomas and Hand 2011; Brogan and Relyea 2013).

The recent effect assessment of herbicides is based mostly on laboratory experiments (e.g. Cedergreen et al. 2005) and pond mesocosms (Cedergreen et al. 2004; Vervliet-Scheebaum et al. 2010). There are some stream mesocosm approaches assessing herbicide effects on macrophytes (Mohr et al. 2007; King et al. 2015). However, knowledge on effects of short pulse exposures on macrophytes studied using a replicated test design is scarce or not present. The present thesis provides a specifically designed stream mesocosm approach to address herbicide effects on macrophytes and thus represents a new methodological approach for regulatory effect assessment.

Besides adverse effects on aquatic macrophytes caused by herbicides, insecticide pulse exposures may adversely affect aquatic life in edge-of-field streams. For instance, insecticide pulses may cause catastrophic drift of aquatic invertebrates (Heckmann and Friberg 2005; Lauridsen and Friberg 2005; Beketov and Liess 2008), induce mortality (Jergentz et al. 2004) and, in the last instance, may cause structural changes of an invertebrate community (Schäfer et al. 2007; Beketov et al. 2013; Stehle and Schulz 2015). Furthermore, pesticide-induced adverse effects on detritivores stream invertebrates may impact ecosystem functions such as leaf breakdown, which is an essential for heterotrophic food webs (Schäfer et al. 2007).

In the past, scientific knowledge on adverse effects of insecticide pulse exposures on invertebrates was mostly evaluated experimentally with laboratory beaker and microcosm experiments (Schulz and Liess 2001b; Rasmussen et al. 2008; Rasmussen et al. 2013). Although the process understanding of pesticide effects on individuals, populations, behavioral endpoints and subsequent recovery can be gained with these approaches, stream mesocosm approaches enable a more realistic and comprehensive evaluation on various levels of complexity up to community and ecosystem effects. Especially the implementation of short-pulsed exposures with durations of less than 12 hours, differentiates the stream

mesocosm approaches of the present thesis from other stream systems with a comparable level of complexity (Liess and Beketov 2011; Mohr et al. 2012). The presented approach facilitates a prospective and realistic effect assessment of stream-typical pulse exposures of insecticides.

Insecticide risk assessment mainly focuses on adverse effects and ecological linkages either in the aquatic or terrestrial environment. However, especially the reciprocal exchange between aquatic and terrestrial ecosystems is of ecological importance (Nakano and Murakami 2001; Baxter et al. 2005; Walters et al. 2008). Hence, allochthonous inputs of terrestrial invertebrates contribute to stream food webs and emerging merolimnic invertebrates (referred to as subsidies) contribute to terrestrial food webs and are thus of importance for predators such as spiders, bats or birds (Baxter et al. 2005). Especially for terrestrial predators preferentially focusing on hatching merolimnic insects (Kato et al. 2003; Blanchette et al. 2014) and for predators of less productive habitats (Sanzone et al. 2003; Paetzold et al. 2005), the inter-ecosystem transfer of energy is essential (Ballinger and Lake 2006) and thus key element of aquatic-terrestrial food web coupling (Schulz et al. 2015).

Such riparian food webs might be at risk from contaminant-induced reductions of hatching merolimnic insects (Schulz and Liess 2001a; Schmidt et al. 2013) or an aquatic-terrestrial transfer of aquatic contaminants to adjacent riparian ecosystems (Walters et al. 2008; Walters et al. 2010; Daley et al. 2011). Hence, pollution of stream systems may cause adverse alterations of the insect-mediated transfer of energy and lead to effects within the adjacent food webs. A comprehensive aquatic-terrestrial effect assessment of pollutants is not yet integrated in mesocosm approaches. Here, the present thesis developes an aquatic-terrestrial model ecosystem including the riparian spider species *Tetragnatha extensa* (representative riparian predator). By analyzing the stable isotopes ratios δ15N and δ13C of aquatic and terrestrial insects and those of *T. extensa* (representative riparian predator), the dietary composition of the riparian predators within the aquatic-terrestrial model ecosystem can be evaluated. Such a test design provides the conceptual and methodological basis for ecotoxicological approaches aiming at aquatic-terrestrial pollutant transfer and cross-ecosystem effects.

2 Study site

All studies of the present thesis were conducted at the Landau Stream Mesocosm Facility (Figure 1) at the University of Koblenz-Landau, Campus Landau, Germany (e.g. Elsaesser et al. 2013; Wieczorek et al. 2015). The stream mesocosm facility comprised in total 16 independent high density concrete channels (Figure 1; length = 45 m; width = 0.4 m; average water depth = 0.26 m). Constant water flow of 1 to 3 L/s was maintained in a flow-through mode using water from a storage reservoir (Figure 2) or in a recirculation mode using pumps, respectively. Dependent on individual test approaches, stream mesocosms were equipped either with artificial sediment comparable to OECD-substrate or with sieved topsoil (silty loamy sand). For the studies of the present thesis, streams were equipped with the submerged macrophyte species *Elodea canadensis* Michx., *Elodea nuttallii* (Planch.) H. St. John, *Myriophyllum spicatum* L., and the helophyte *Berula erecta* (Huds.) Coville.

Figure 1: The stream mesocosm facility of the University Koblenz-Landau; Photo: Matthias Wieczorek

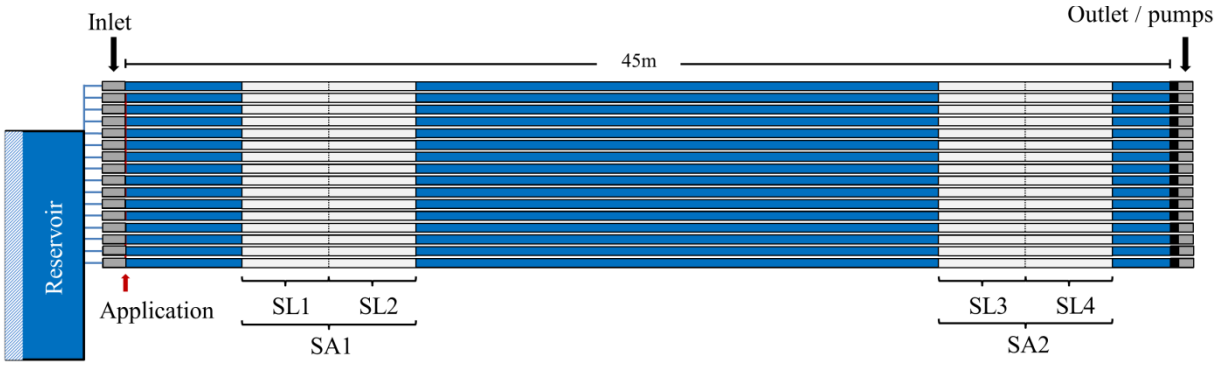

Figure 2: Schematic overview of the Landau stream mesocosm facility. The sampling areas (SA1 and SA2) with in total four sampling locations (SL1-4) for emergence and invertebrate sampling are presented as used during the insecticide effect assessment (**Appendix III**). Drifts nets (indicated by the black bars) were installed at the outlet of the channels.

3 Research objectives and thesis outline

The main objective of the present thesis was to evaluate the impact of short-term pesticide exposure events on representative flora and fauna of edge-of-field streams using stream mesocosms (Figure 3). For this purpose, both aquatic macrophytes and invertebrates representative for different trophic levels were integrated in the stream mesocosms as major compartments of typical edge-of-field streams (**Appendix I**). In order to provide controlled pesticide exposure events similar to that reported for streams in field studies, typical pulse exposure scenarios with durations in the range of ≤ 1 to ≥ 24 hours were established using a fluorescence tracer (research objective RO1; **Appendix I**). This methodological basis was applied in the studies on the effect assessment of the herbicide iofensulfuron-sodium (RO2; **Appendix II**) and the insecticide etofenprox (RO3; **Appendix III**). Both studies were conducted to gain knowledge on response and recovery of macrophytes and invertebrates following pulsed pesticide exposures. Furthermore, the applicability of steam mesocosms for regulatory registration purposes of pesticides was evaluated using the ecological threshold (ETO) and recovery option (ERO) according to EFSA (2013). To go beyond the regulatory perspective which considers aquatic and terrestrial ecosystems mainly separately, the present thesis introduced an aquatic-terrestrial model ecosystem (RO4; **Appendix IV**) capable to identify cross-ecosystem effects. The use of the stable isotope ratios $\delta^{13}C$ and $\delta^{15}N$ in this study uncovered how aquatic and terrestrial prey insects may contribute to the diet of the riparian spider species *T. extensa*. This new approach thus enables to qualitatively assess a transfer of pollutants and pollutant-induced effects from an aquatic environment to adjacent riparian food webs.

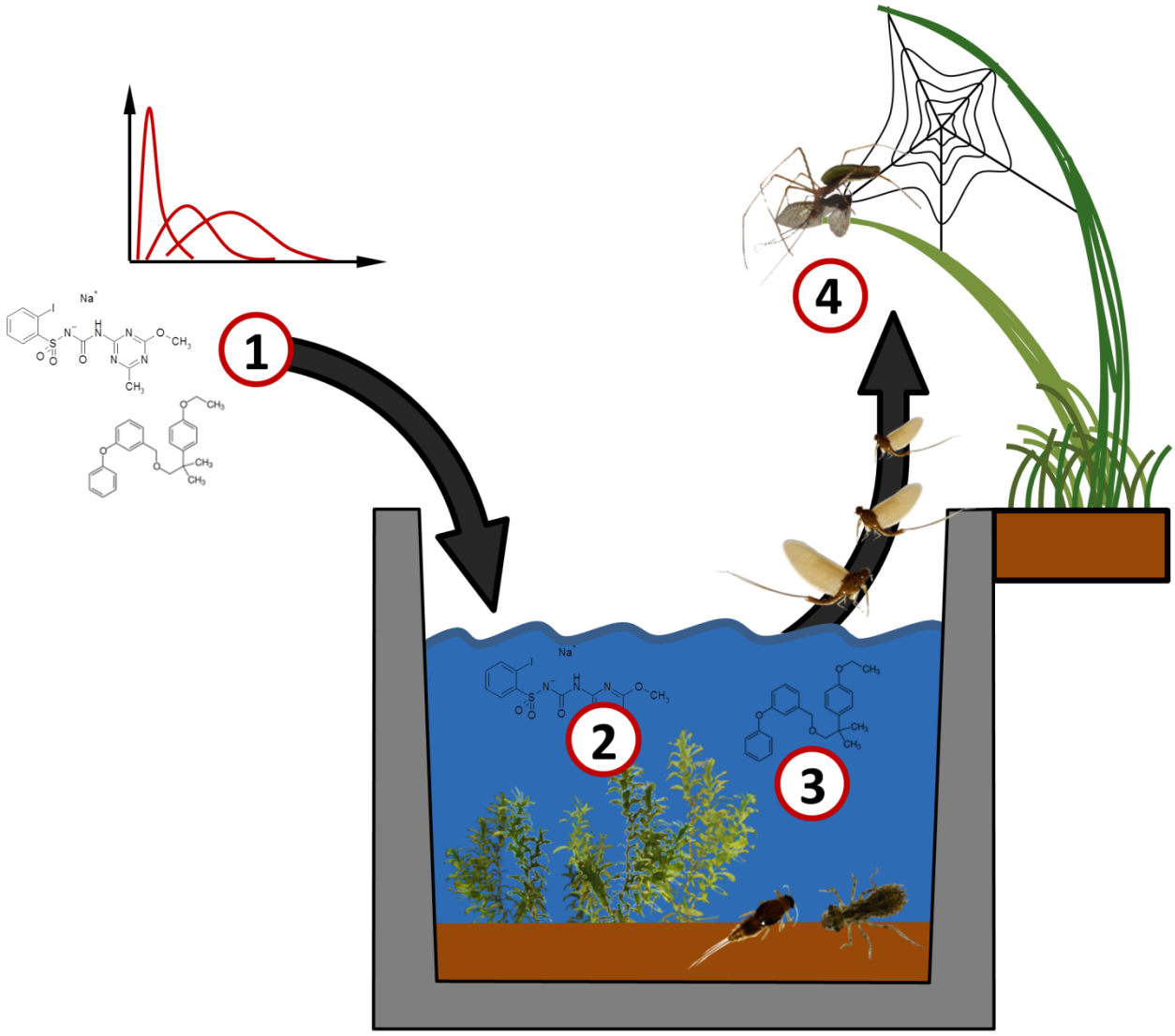

Figure 3: Schematic overview of the research objectives (RO), thesis outline and the provided publications (Appendix I – IV)

1 RO1: Reference scenarios for exposure to plant protection products and invertebrate communities in stream mesocosms [**Appendix I**]

2 RO2: Effect assessment of a pulsed 24-h sulfonylurea herbicide exposure using aquatic macrophytes [**Appendix II**]

3 RO3: Structural and functional effects of a pyrethroid pulse exposure on invertebrates in outdoor stream mesocosms [**Appendix III**]

4 RO4: Using stable isotope analysis to study potential effects of environmental chemicals on aquatic-terrestrial subsidies [**Appendix IV**]

4 Results and discussion

4.1 Reference scenarios for exposure to plant protection products

As a methodological basis for effect assessments using stream mesocosms, the first study of the present thesis aimed at establishing substance-specific exposure dynamics for edge-of-field streams. Using the non-sorptive tracer uranine, three stream-typical exposure scenarios (pulse durations ≤ 1 to ≥ 24 hours) were established using flow-through and/or recirculating flow conditions (Appendix I).

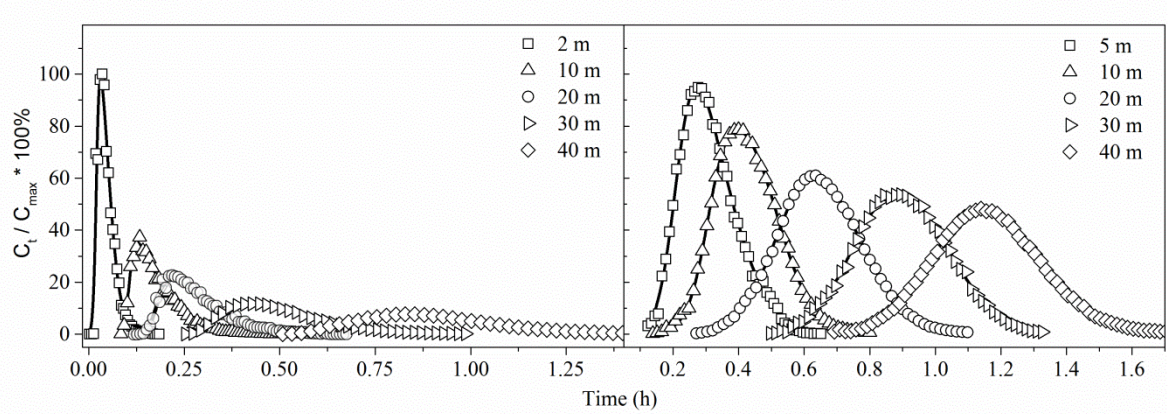

Figure 4: Dynamics of the tracer uranine during two pulse exposures (duration of tracer injection: 5 s (left side) and 10 min (right side)). The concentrations of each approach were displayed relatively by dividing the time-dependent concentrations C_t by the maximum concentration C_{max}. Figure taken from Appendix I (modified).

In the present thesis, exposure scenarios with pulse durations of ≤ 1 hour (Figure 4) were appropriate to adress exposure characteristics that occur in small edge-of-field streams after brief runoff or spray drift events as shown by Rabiet et al. (2010). The pulse exposures shown in Figure 4 enabled to mimic longitudinal stream gradients which are characterized by declining maximum concentrations and increasing pulse durations with increasing flow length of the streams (Appendix I). The underlying processes of the exposure dynamics shown in Figure 4 can mainly be ascribed to processes such as transient storage and longitudinal dispersion (Nepf et al. 2007; Nepf 2012a; Nepf 2012b; Sukhodolova and Sukhodolov 2012; Stang et al. 2014). Although a non-sorptive tracer was used for the exposure assessments, the underlying exposure dynamic can be transferred to a pesticide application. For instance, the exposure dynamic of three pesticides penflufen, pencycuron and triflumuron (K_{oc} = 290 - 30,000 and log K_{ow} = 3.3 - 4.9) was comparable to those observed for uranine (Figure 4; right side) but revealed additional reductions of the maximum concentrations due to sorptive processes (Stang et al. 2014). Thus, the use of the tracer technique enables to estimate the exposure dynamics prior to pesticide applications or enables a real-time estimation of solute transport if the pesticides and trace substances are injected simultaneously and measured *in situ*. The combinatory use of tracer and pesticides enables a comprehensive understanding of

environmental fate processes underlying pulse exposures due to the fact that hydraulic processes such as longitudinal dispersion and sorption can be distinguished (Stang et al. 2014).

The applicability of exposure events comparable to those in Figure 4 might be limited for ERA, as the Aquatic Guidance Document (EFSA 2013) suggests realistic to worst case conditions, which might not be fulfilled for these pulse durations of ≤ 1 hour. In order to fulfill these realistic or worst case conditions and thus to enhance the applicability within the ERA, an exposure scenario (pulse duration ≥ 1 hour) hereafter referred to as 'hour-scale' was established (Figure 5) for the stream mesocosm.

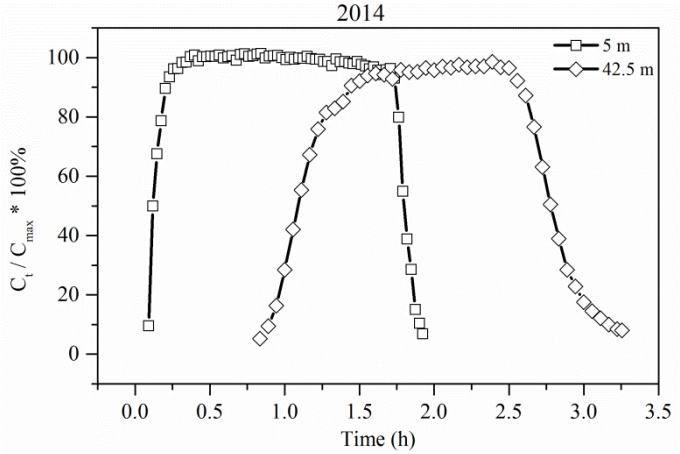

Figure 5: Dynamic of the 'hour-scale' exposures (duration of the tracer injection = 105min). The concentrations of each approach were displayed relatively by dividing the time-dependent concentrations C_t by the maximum concentration C_{max}. Figure taken from Appendix I.

Prolonged injection times of the 'hour-scale' scenario (Figure 5) resulted in comparable maximum concentrations of uranine at both sampling locations. Hence, longitudinal concentration gradients due to longitudinal dispersion and transient storage were less pronounced compared to those in Figure 4. The 'hour-scale' exposure scenario was applied in an effect study (Appendix III) using a 6-hour injection of the highly sorptive insecticide etofenprox (K_{oc} ≈ 18,000; $\log K_{ow}$ = 6.9). Hereby, an approximately 35% decrease of the maximum pesticide concentration at the outlet compared to the inlet (Appendix III) indicated a longitudinal concentration gradient predominately due to sorptive processes. As the 'hour-scale' scenario with constant concentration levels was approximated to stagnant laboratory approaches, comparability of results between laboratory and mesocosm effect assessments might be facilitated in the case that sub-processes are replicated on laboratory scale.

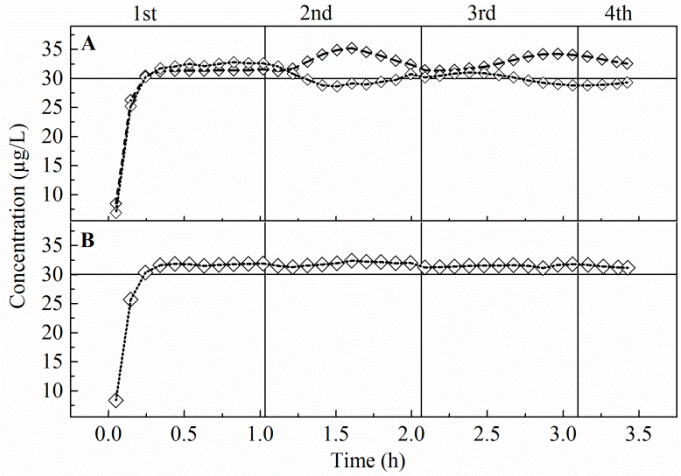

Figure 6: Concentration dynamics of uranine during and following the application phase of the day-scale exposure using recirculating flow conditions. (A) displays an incompletely adapted and (B) a successfully adapted application technique. The water was recirculated once (1st) during the application with full injection rates, during the second cycle (2nd) with a step-wise reduced injection rate and for two more recirculation cycles (3rd and 4th) without tracer injection. Figure taken from Appendix I.

In order to adress long-lasting runoff events, subsurface drainage and other continuous exposure events, an exposure scenario using a pulse duration of ≥ 24 hours, hereafter referred to as 'day-scale', was established for the mesocosm using recirculating flow conditions (Figure 6). Within the present thesis a step-wise reduced injection method was used to prevent oscillating concentration maxima in the hours following the application in recirculating mode (Figure 6 A; Appendix I). In order to mimic pulse exposures (pulse duration ≥ 24 hours) comparable to those reported for herbicides (Leu et al. 2004), the applicability of the day-scale scenario is recommendable for substances with a low tendency for sorption such as herbicides and soluble insecticides (e.g. neonicotinoids).

The day-scale scenario was applied in the course of a 24-h exposure event using the highly soluble herbicide iofensulfuron-sodium (Appendix II; Wieczorek et al. in press). The subsequent flushing with unpolluted water (flow-through mode) enabled to mimic a time-limited pulse exposure event in edge-of-field streams. Furthermore, the scenarios comparable to Figure 6 might be applied in order to simulate or validate exposure scenarios resulting from e.g. FOCUS stream models (FOCUS 2001).

Overall, this first part of the present thesis provided the methodological basis to mimic a wide range of pesticide pulse exposures representing pulse durations which can be considered as realistic or worst case in edge-of field streams.

4.2 Effect assessment of a herbicide pulse exposure

Up to now, the assessment of herbicide effects using a replicated study design was realized mostly on basis of laboratory and stagnant micro- and mesocosm studies (e.g. Maltby et al. 2010). Since these study designs may not reflect the conditions of stream ecosystems, the second study of the present thesis (Appendix II) provides an approach to address stream-typical herbicide pulse exposures and their effects on aquatic macrophytes. Hereby, standard procedures (e.g. Maltby et al. 2010; Vervliet-Scheebaum et al. 2010) typically used for pond mesocosm approaches were adapted to the stream mesocosm design. In accordance with pulse durations for herbicide events in the field (Leu et al. 2004), the 'day-scale' exposure scheme (Figure 6; Appendix I) was used to establish a constant herbicide pulse exposure of 24 hours. The single-pulsed 24-h exposure with the herbicide iofensulfuron-sodium was in line with the previously known duration of a single FOCUS stream scenario and thus considered to sufficiently represent realistic or worst case conditions (EFSA 2013).

The study design (Appendix II) comprised potted shoots of the macrophytes *E. canadensis* and *M. spicatum* in the stream mesocosm. The use of the morphological endpoints growth of main, side and total shoot length, dry weight, maximum root length and side shoot number represented a broad range of morphological macrophyte endpoints. The recovery of macrophyte exposed to the herbicide iofensulfuron-sodium for 24 hours was monitored on basis of morphological endpoints over a period of 42 days following herbicide exposure.

Overall, the 24-h herbicide exposure was appropriate to demonstrate short-term adverse effects on the two macrophyte species (Figure 7). The maximum herbicide-induced growth inhibition was 45% for *E. canadensis* on day 7 and 66% for *M. spicatum* on day 14, determined for the endpoint 'total shoot length' (Figure 7; Appendix II). Furthermore, a concentration-response relationship was demonstrated for the shoot endpoints main, side and total shoot length of *M. spicatum* on several sampling dates (Figure 7). Contrary this finding, no concentration-response relationship was observed for shoot endpoints of *E. canadensis* and for dry weight endpoints of both macrophytes.

M. spicatum seemed to be more susceptible to the iofensulfuron-sodium exposure compared to *E. canadensis*. This difference in susceptibility might be partly explained by the results of the macrophyte tissue analytics. The concentration of the iofensulfuron-sodium was up to 4.5-fold higher in *M. spicatum* than in *E. canadensis*. Differing herbicide residues may be explained with macrophyte-specific differences in absorption, translocation within the macrophytes, metabolic transformation by e.g. inactivation of acetolactate synthase inhibitors (Brown 1990) and/or excretion of the herbicide. According to Brown (1990), the tolerance of

macrophytes to sulfonylurea herbicides was correlated positively to the macrophyte-specific response time for metabolic herbicide transformation and breakdown. Although this might indicate that *E. canadensis* had the more efficient metabolic processes compared to *M. spicatum*, this could not be verified sufficiently with our study design.

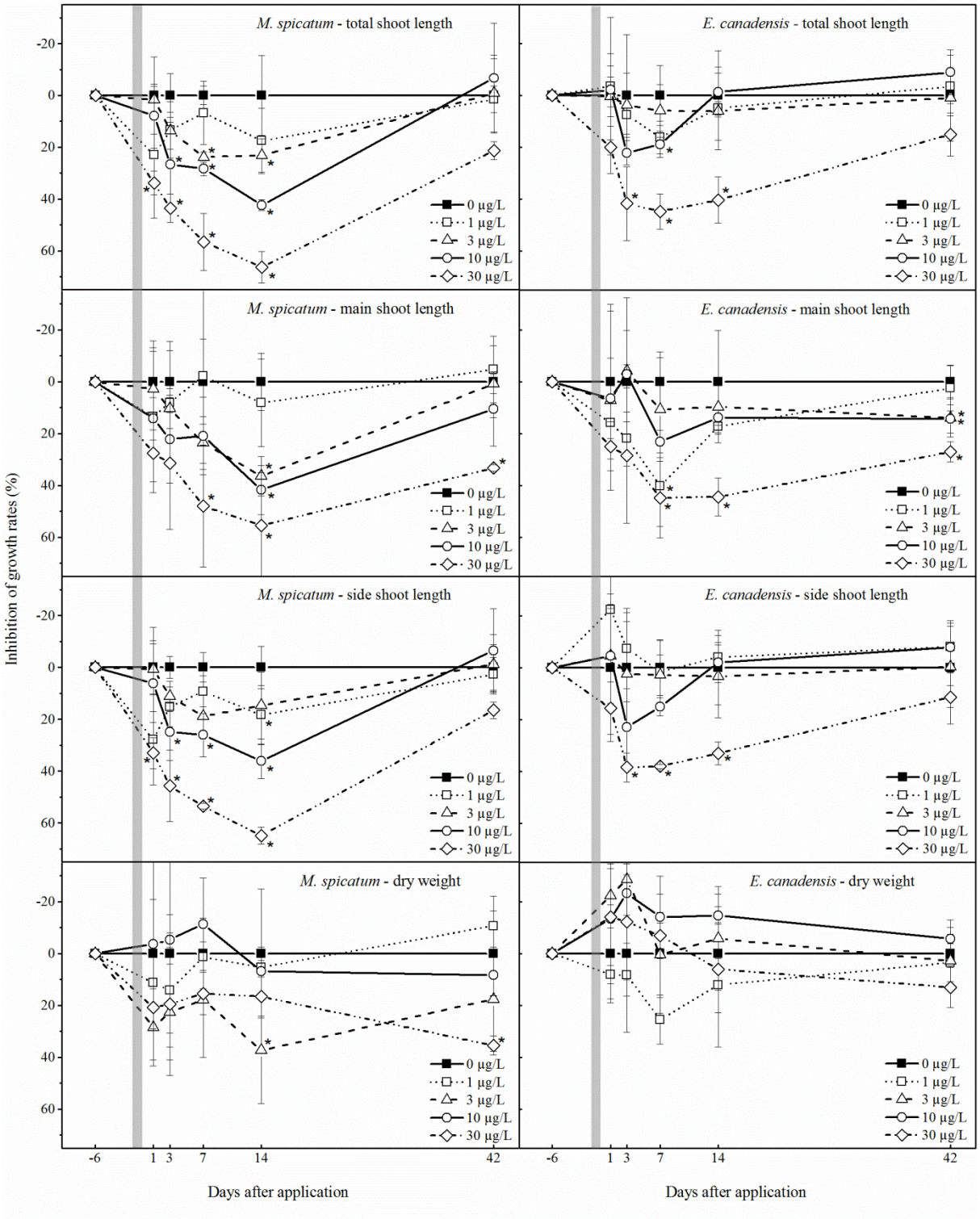

Figure 7: Average percent inhibition of shoot and dry weight growth rates (± SD) of *M. spicatum* and *E. canadensis* relative to the control. Statistically significant differences between treatments and the control are indicated by asterisks. The 24-h exposure phase to iofensulfuron-sodium is marked by the grey vertical bar. Figure taken from Appendix II.

To evaluate the applicability of stream mesocosms within the regulatory registration of pesticides according to EFSA (2013), the present study results were evaluated with regard to the applicability of the ETO and ERO. The ETO accepts negligible effects on aquatic macrophytes (recovery is not considered) whereas the ERO accepts transient effects on potentially vulnerable species (population level) during a 56-days recovery period. According to the aquatic guidance document (EFSA 2013) in total eight sensitive or vulnerable macrophyte species should be included in the higher tier macrophyte effect assessment to apply the ETO and ERO. In the present study, two macrophyte species were evaluated with regard to their suitability for including them in ERA procedures using stream mesocosms (Appendix II).

In order to apply the ETO according to EFSA (2013), no statistically and/or ecologically significant effects (effect class 1) or effects solely on single sampling dates (effect class 2) are acceptable. Within the evaluation of present results using the ETO, effect classes 1 and 2 (EFSA 2013) were determined at up to 1 and 3 µg/L for *M. spicatum* (total shoot length) and *E. canadensis* (side shoot number), respectively. Effect endpoints without a concentration-response relationship or ecological significance as demonstrated for *E. canadensis* main shoot length (Appendix II) were excluded.

In the present study, several growth endpoints revealed effect classes > 2 but recovered within the recovery period of 42 days and thus supported the applicability of the ERO for the two aquatic macrophytes. The application of the ERO resulted in no observed ecologically adverse effect concentrations (NOEAECs) of 10 and 30 µg/L for *M. spicatum* and *E. canadensis*, respectively. Thus the regulatory acceptable concentrations were on a higher level using the ERO compared to the ETO. However, the recovery period of the present study was shorter than the suggested 56 days by EFSA (2013). A prolonged recovery period of at least 56 day might have supported the recovery of *M. spicatum* endpoints. However, if pulse series comparable to those reported by Leu et al. (2004) are predicted to occur in the field, the experimental design should consist of multiple-pulse exposures. In the present study, recovery of macrophyte growth started on day 7 and 14 for *E. canadensis* and *M. spicatum*, respectively. Thus, a second exposure of iofensulfuron-sodium in the period between day 7 and 14, might have resulted in enhanced inhibitory effects on macrophyte growth and, thus, the time for recovery might have been prolonged.

Overall, the present study revealed an applicability of stream mesocosms for higher tier macrophyte risk assessment. However, more research is needed to identify in total eight

sensitive and vulnerable aquatic macrophyte species to fulfill the requirements of the aquatic guidance document (EFSA 2013).

4.3 Effect assessment of a pyrethroid pulse exposure

The third study of the present thesis used a 6-h pulse exposure (Figure 5; Appendix I) using 0.05, 0.5 and 5 µg/L (n = 4) of the pyrethroid ether etofenprox. As etofenprox was measured with durations of up to 7 hours (Tanabe and Kawata 2009) and aqueous concentrations between 0.04 and 0.2 µg/L (Tanabe et al. 2001; Añasco et al. 2010) in field studies, realistic or worst case conditions as suggested by the aquatic guidance document were fulfilled by the present study design (EFSA 2013).

Overall, the 6-h etofenprox pulse exposure caused significant adverse effects below the 48-h LC50 *D. magna* (0.44 µg a.s./L) for all investigated endpoints (Table 1). The structural endpoint abundance of *C. simile* (Figure 8) and the functional endpoint *in situ*-measured feeding rates of *Asellus aquaticus* (Table 2) were identified as the most sensitive endpoints.

Table 1: Overview of LOECs for the structural and functional endpoints of the pyrethroid etofenprox on aquatic invertebrates. Table taken from Appendix III.

Endpoint	LOEC (µg/L)
Structural	
Abundance	0.05
Drift	0.5
Emergence	na
Community	0.5
Functional	
Feeding rate	0.05

[na] the endpoint was not statistically evaluated

Structural endpoints - Out of the 11 evaluated invertebrate populations, *C. dipterum*, *C. simile*, *C. fusca/villosa* and *P. nymphula* were adversely affected in the highest etofenprox treatments for at least two consecutive samplings (Figure 8). This is in line with Rico and Van den Brink (2015) who classified these families as more sensitive to pyrethroids compared to the average pyrethroid sensitivity of invertebrate families based on the ECOTOX database of the US Environmental Protection Agency (USEPA). The statistically significant decreased *C. simile* abundances by approximately 60% in the 0.05 µg/L treatment relative to the control revealed effects at 10-fold lower concentrations compared to the laboratory 48-h EC_{50} of *D. magna*. This result is in line with findings of Rasmussen et al. (2013) presenting eight times lower LC_{50} concentrations for *C. dipterum* compared to the 48-h EC_{50} for *D. magna* using pyrethroids.

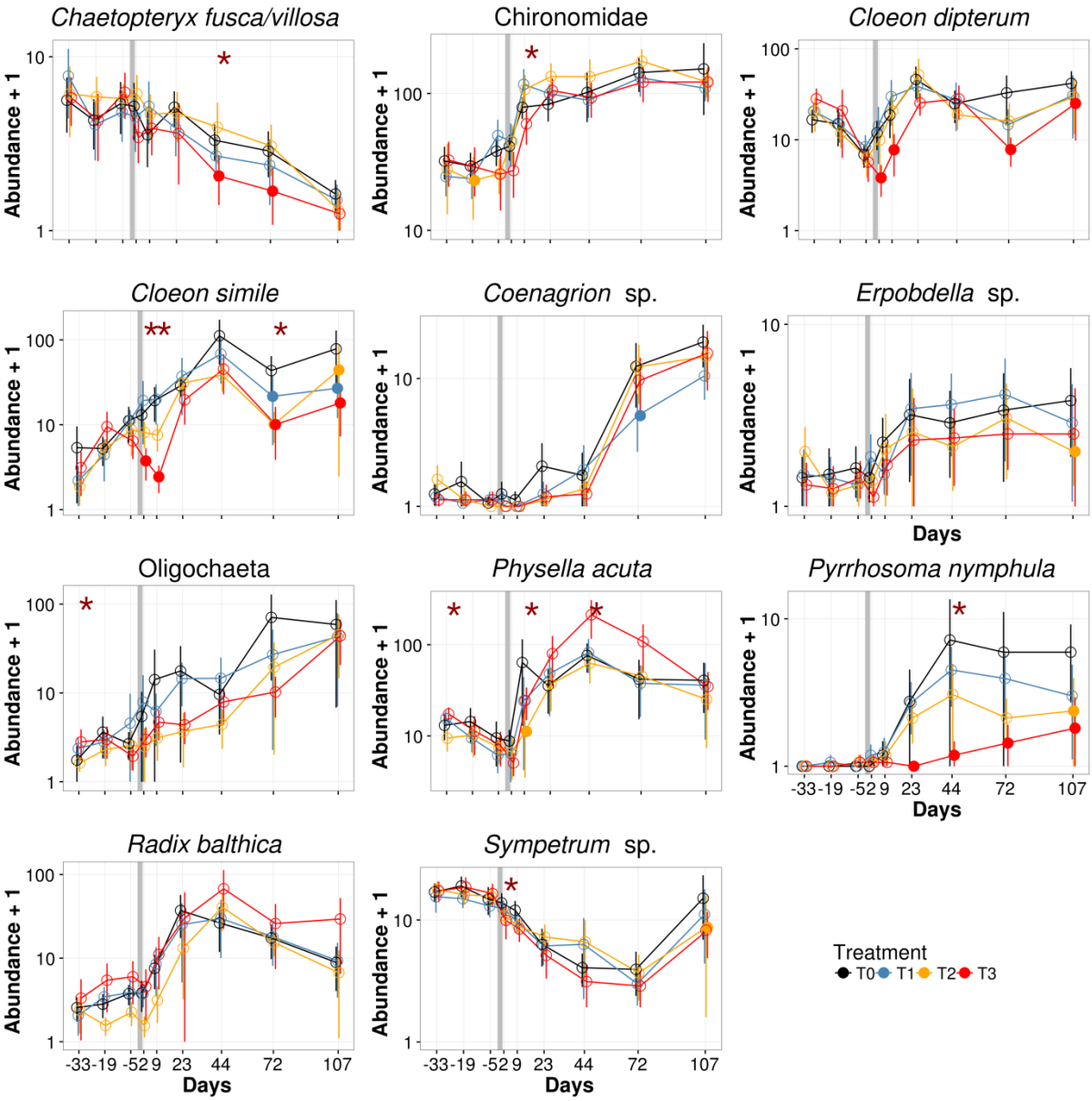

Figure 8: Population dynamics of eleven taxa considering only populations with mean abundances in control channels greater than 1 (based on all assessed control samples). Mean abundances are displayed per 0.05 m^2 using 95% confidence intervals (CI) on a logarithmic scale. Filled circles mark statistically differences of treatments ($p < 0.05$) compared to control (Dunnett-contrasts). Stars above sampling dates represent statistically significant overall treatment effects (Likelihood-Ration Test). The etofenprox treatment (T0 – T3) is indicated by the vertical grey bar. Figure taken from Appendix III.

At the community level, a slight effect of etofenprox exposure on community composition was found for the last sampling date, 107 day after pesticide amendment to the stream mesocosms (Figure 9, left). Only 6% of the variation was explainable by the etofenprox treatment, 32% by time and in total 62% remained unexplained. Similar to the findings at the population level, adverse effects were found for *C. dipterum*, *C. simile*, *C. fusca/villosa* and *P. nymphula* in the 5 µg/L treatment (Figure 9) indicated by positive species scores. No statistically significant differences of the community composition in the 0.05 and 0.5 µg/L treatment were determined within the 107 days compared to the control. However differences

in community composition between control and 0.05 and 0.5 µg/L treatments increased over time, which might be an indication for long-term effects.

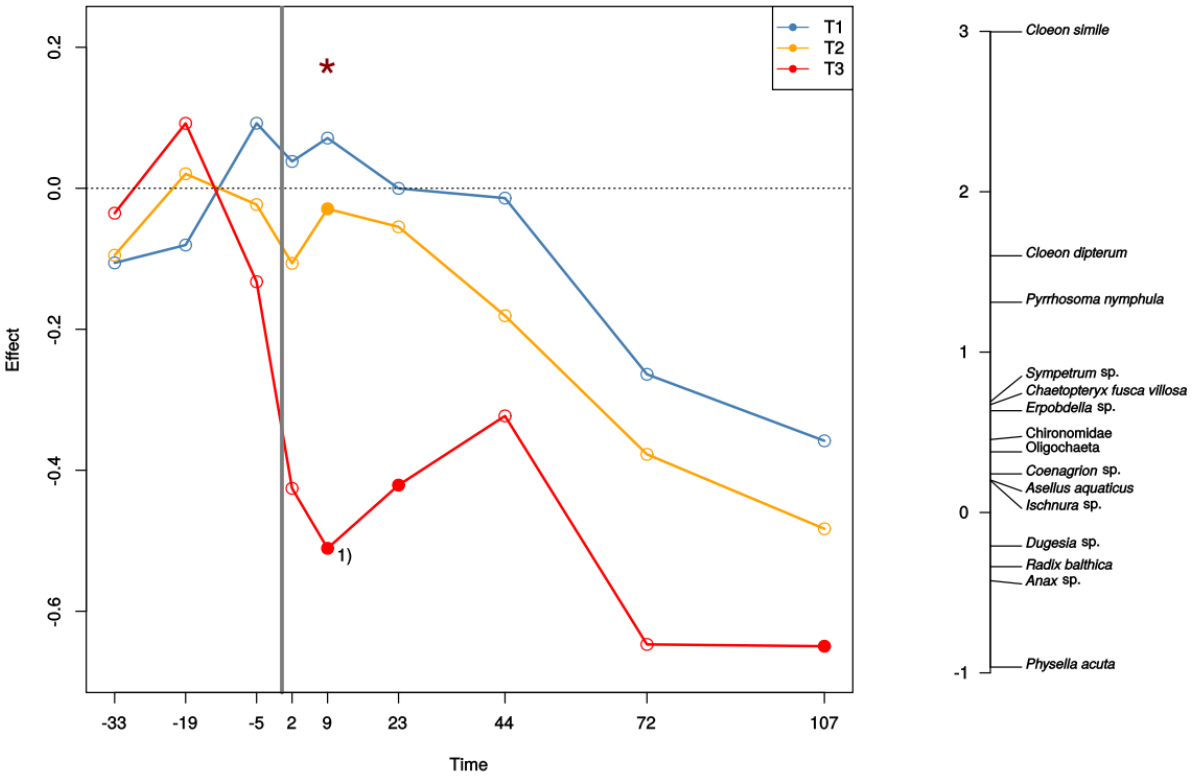

Figure 9: Effect of etofenprox exposure on the macroinvertebrate communities (left part) displayed by Principal Response Curves. The control is represented by the dotted horizontal line. The vertical line marks the etofenprox injection at day 0. In total 3.3% of variation is displayed by first axis. The star above the sampling date represents a statistically significant overall treatment effect. Filled circles mark statistically different PCA scores of treatments at each date compared to control. 1) Significant differences on the second PCA axis. Species weights (greater than 0.2) are displayed in the right part of Figure 9. Figure taken from Appendix III.

Recovery - No recovery was demonstrated for the 5 µg/L at the community level and for all concentrations for *C. simile* at the population level. Caquet et al. (2007) demonstrated recovery of e.g. Baetidae, Caenidae, Ecnomidae and overall biodiversity between 62 and 149 days for pyrethroid-treated pond mesocosms. External recolonization via merolimnic invertebrates e.g. Baetidae and damselflies was enabled by the present study design. Stream-internal recolonization was partially enabled as the mesocosms were flushed with water of the adjacent reservoir (Figure 2) during 3 hours prior to and during the 48-h-period following the etofenprox exposure (mesh size of the spillway = 1 mm). However, as no indications for recovery were observed at population and community level, the study duration of 107 days might not have been long enough to enable recovery.

Table 2: Overview of mortalities (%) and feeding rates of *A. aquaticus* (mg/mg/d ± SD) at the end of the experimental period of in total seven days. A nested design with 10 individual replicates in each of the streams ensuring an independent replication of 4. Statistically significant differences between treatments and controls are indicated by asterisks based on nested ANOVA analysis and chi square testing, respectively. Table taken from Appendix III.

		Treatment		
	control	0.05 µg/L	0.50 µg/L	5.00 µg/L
Mortality (%)	8 (±10)	15 (±10)	39 (±44)*	100*
Feeding rate (mg/mg/d)	0.25 (±0.04)	0.14 (±0.03)*	0.04 (±0.02)*	-

Functional endpoints – The functional endpoint feeding rate of *A. aquaticus* was determined as the most sensitive sublethal endpoint. In the 0.05 and 0.5 µg/L treatments, feeding rates were significantly decreased by 44 and 84%, respectively (Table 2). Hence, this study demonstrated adverse effects on the feeding rate of *A. aquaticus* at concentrations 10-fold below the 48-h EC_{50} value (mortality) of *D. magna*. This significant reduction of the feeding rates revealed the hazardous potential of stream-typical pulse exposures of pyrethroids on the invertebrate-associated leaf litter decomposition and thus functioning of stream ecosystems.

The adverse effects on the feeding rate (functional endpoint), as well as on abundance of *C. simile* (structural endpoints) were observed at pyrethroid level of 0.05 µg/L which is in the range of the predicted environmental concentrations of surface waters (0.024 µg/L) and below reported field concentrations (up to 0.2 µg/L; Tanabe et al. 2001). Our results indicate that predicted and measured field concentrations might adversely affect stream ecology. The ecological significance of *in situ* based feeding rates for ecosystem functions was indicated by Maltby et al. (2002), demonstrating a positive correlation between total leaf decomposition and *in situ* feeding rates for the detritivorous *Gammarus pulex*. As the feeding rates of *A. aquaticus*, considered as representative for detritivorous invertebrates, were roughly inhibited by 44% in the 0.05 µg/L treatment it can be hypothesized that realistic etofenprox exposures (Tanabe et al. 2001) might alter basic functional processes of heterotrophic food webs. Decreased feeding rates might also result in adverse effects at the population level and, furthermore, alter inter-species competition of *A. aquaticus* and other detritivorous shredders (Whitehurst 1991). However, the overall adverse effects in natural stream ecosystems are dependent on the functional redundancy due to other detritivorous invertebrates which might have differing sensitivities to an insecticide pulse exposure and thus strongly influence ecosystem resilience (Maltby et al. 2002; Johnson 2007).

Overall, the response of the majority of invertebrates was restricted to the highest treatment and thus rather non-sensitive. However, the present study revealed significant effects on structural and functional endpoints at the 0.05 µg/L etofenprox concentration. The determined LOECs (0.05 µg/L) of structural and functional endpoints (Table 1) and the assumption of an assessment factor of 10 for functional endpoints led to a hypothetical regulatory acceptable concentration (RAC) of 0.005 within the present study. This RAC is in line with the official tier I RAC based on laboratory single species data (EFSA 2008).

4.4 Aquatic-terrestrial food web coupling

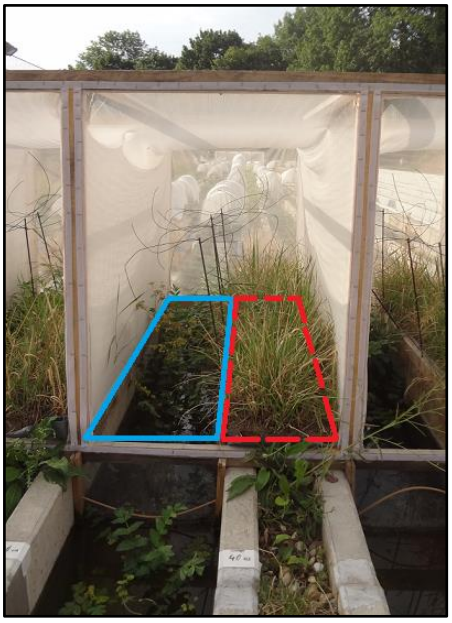

Figure 10: Photograph of one mesh-separated compartment with a schematic outline of the aquatic model ecosystem (solid line) and the terrestrial model ecosystem (dotted line). Figure taken from Appendix IV. Photo: Matthias Wieczorek

The aquatic-terrestrial model ecosystem consisted of a meadow section and an aquatic section of the stream mesocosms surrounded by a mesh cage (Figure 10). Throughout the sampling period, emergence of aquatic and terrestrial insects was observed and the riparian spiders *T.* extensa (Figure 11) were enabled to feed on both, since web-building structures (see vertical sticks with horizontal plastic wire in Figure 10) covered equally the aquatic and terrestrial area. The dry weights of emerging merolimnic insects observed in the present study (Appendix IV) were in the range of those reported by field studies (Kato et al. 2003; Paetzold et al. 2005). The dry weights of insects emerging in the terrestrial model ecosystem were at the lower end of those reported by Gergs and Rothhaupt (2014). Here, more research is needed to increase emergence rates of terrestrial insects. Overall, the aquatic-terrestrial model ecosystem mimicked riparian ecosystems which are predominately characterized by aquatic emergence. As the present approach focused on the inter-habitat transfer of prey organisms of aquatic origin to adjacent riparian habitats, allochthonous inputs from terrestrial ecosystems into the streams, e.g. via leaf litter (Tank et al. 2010) and terrestrial insects (Kawaguchi and Nakano 2001; Kawaguchi et al. 2003; Baxter et al. 2005) were excluded. The design of the aquatic-terrestrial model ecosystem was equipped with structural elements (wire constructions) in order to mimic typical construction sites for spider webs in riparian vegetation e.g., overhanging grass (Figure 10).

Figure 11: Photograph of the riparian predator *T. extensa* with the merolimnic prey *Cloeon* sp. Photo: Matthias Wieczorek

The food supply of 6.3 mg dry weight of prey species per spider per day was considered as sufficient for *T. extensa* as the minimum daily intake of 0.87 and 2.34 mg prey was reported for *T. elongata* (Gillespie and Caraco 1987). Due to the easy handling, the present study revealed *T. extensa* as appropriate model predator for the experimental assessment of cross-ecosystem effects. Overall, tetragnathid spiders can be considered representative for riparian predators (Wise 1995) due to their global distribution (Walters et al. 2010). Their usability as indicator species for aquatic pollution and the aquatic-terrestrial transfer of contaminants was shown by Walters et al. (2008), Raikow et al. (2011) and Otter et al. (2013).

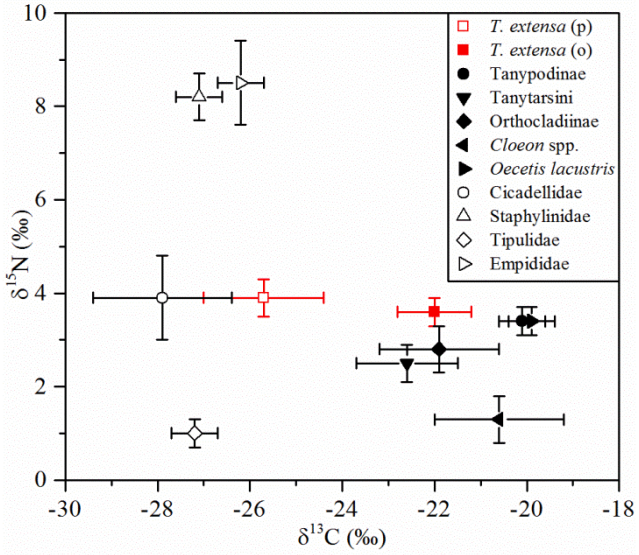

Figure 12: Average stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$ ± SD) of *T. extensa* (red symbols: prosoma (p; open symbol) and opisthosoma (o; filled symbol)) and merolimnic (filled black symbols) and terrestrial prey (open symbol) species. Figure taken from Appendix IV.

The use of stable isotopes ratios ($\delta^{13}C$ and $\delta^{15}N$) enabled insights into the food web structure of the model ecosystem (Figure 12). Aquatic prey organisms showed statistically significant higher δ13C ratios compared to those of terrestrial prey organisms, which is in line with findings of Akamatsu et al. (2004) and Akamatsu and Toda (2011). Such difference in δ13C ratios of merolimnic and terrestrial insects which is important for the statistical

evaluation of diatary composition of riparian spiders might be explained by different collection sites of prey organisms.

The evaluation with SIAR (stable isotope analysis in R; Appendix IV) revealed that aquatic prey species *Cloeon* spp. and terrestrial prey Tipulidae were major prey species of *T. extensa*. Based on isotope ratios of the spiders' opisthosoma (Figure 13), the diet of *T. extensa* was composed in total of 71% merolimnic and 29% terrestrial prey. Hereof, *Cloeon* spp. and Tipulidae contributed with 62 and 26%, respectively, to the diet of *T. extensa*. This high contribution of merolimnic insects to the diet of *Tetragnatha* sp. is in line with findings of several field studies (Henschel et al. 2001; Kato et al. 2003; Akamatsu et al. 2004). Thus, the present study reflects typical foraging behavior of orb-weaving spiders with a horizontal webs within river habitats (Sanzone et al. 2003; Akamatsu and Toda 2011).

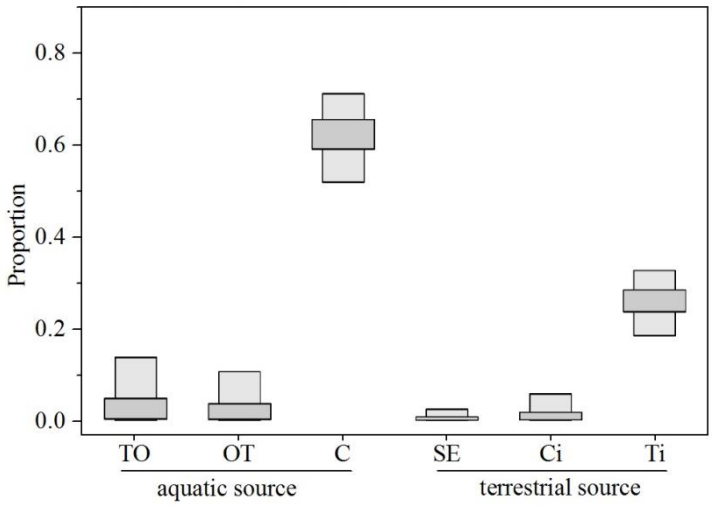

Figure 13: Figure 6: SIAR output with 95 (light grey) and 50% (dark grey) credibility intervals, showing the estimated prey contribution to the opisthosoma of *T. extensa*. Abbreviations of merolimnic and terrestrial prey organisms: TO = Tanytarsini and Orthocladiinae, OT = *Oecetis lacustris* and Tanypodinae, C = *Cloeon* spp., SE = Staphylinidae and Empididae, Ci = Cicadellidae and Ti = Tipulidae. Figure taken from Appendix IV.

The present aquatic-terrestrial model system and the stable isotope analysis might be integrated in an effect assessment of substances with bioaccumulation potential in order to analyze cross-ecosystem transfer of pollutants and potentially effect transmission to riparian predators. The combinatory use of the stable isotope analysis and a quantitative analysis of pollutants of the aquatic and terrestrial prey insects and the receptors *T. extensa* enables the possibility to qualitatively assess the pollutant transfer from the aquatic to the terrestrial compartment. Thus, pollutant residues measured in *T. extensa* could be referred to a certain prey source.

5 Conclusion

Edge-of-field streams are characterized by exposure conditions differing from those of lakes and ponds. However, stream-typical exposure conditions are underrepresented in the current higher tier effect assessment of pesticides. The present thesis provides concepts to address pesticide exposure events representative for a wide range of typical pulse durations observed in of small edge-of-field streams. The implementation of such pulse scenarios within the ERA enables higher tier effect assessment of pesticides with respect to realistic or worst case exposure conditions of edge-of-field streams.

As being one of the first replicated higher tier macrophyte effect assessments using stream mesocosms, the results of the present thesis highlighted the applicability of ETO and ERO for the two macrophytes *M. spicatum* and *E. canadensis*. Higher tier macrophyte effect assessment using stream mesocosms and pulse exposure conditions can be recommended for substances with a fast mode of action. To support the results of the present thesis, the usability of additional sensitive or vulnerable macrophytes species should be evaluated for stream mesocosm approaches.

The effect assessment of the etofenprox pulse exposure highlighted the potential of the stream mesocosms to demonstrate stream-typical effects on structural and functional endpoints. The hypothetical RAC determined for etofenprox of the present study was on the same level compared to the official tier-1 RAC (EFSA 2008). Contrary to the present findings, van Wijngaarden et al. (2015) demonstrated that > 90% of tier-1 and tier-2 RACs of insecticides were lower compared to ETO-RACs derived from micro- and mesocosm studies. Thus, the present thesis reveals that an effect assessment of insecticides using stream mesocosms not necessarily leads to less sensitive RACs compared to lower tier RACs. As the hypothetical RAC of the etofenprox assessment was based on the *in situ*-measured feeding rates of *Asellus aquaticus*, such functional endpoints at individual level are recommendable as supportive concepts for stream mesocosm approaches.

The experimental coupling of aquatic and terrestrial model ecosystems using riparian spiders enables the qualitative assessment of insect-mediated pollutant and effect transmission to adjacent ecosystem. Furthermore, riparian spiders such as *T. extensa* might contribute to the elaboration of new micro- or mesocosm approaches specifically addressing biomagnification and secondary poisoning (EFSA 2013). In order to facilitate the implementation within ERA the presented aquatic-terrestrial model ecosystem might be simplified to spider cages solely surrounded by the aquatic environment.

Overall, the results of the present thesis suggest that effect assessments using stream mesocosms within the scope of ERA can contribute to a realistic pesticide assessment of stream-typical pulse exposures on macrophytes and invertebrates and potentially to qualitatively evaluate effect transfer to the riparian food web.

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8 Declaration

I hereby declare that I autonomously conducted the work presented in this PhD thesis entitled "EFFECTS OF PESTICIDES TO MACROPHYTES AND MACROINVERTEBRATES AND AQUATIC-TERRESTRIAL FOOD WEB COUPLING IN STREAM MESOCOSMS". All assistances and contributors are clearly declared. This thesis has never been submitted elsewhere for an exam, as a thesis or for evaluation in a similar context to any department of this University or any scientific institution. I am aware that a violation of the aforementioned conditions can have legal consequences.

Landau, 20.01.2017

Place, date

Signature

Appendix I: Matthias Wieczorek, Christoph Stang, Eric Bruns and Ralf Schulz conceived and designed the experiments. The experiments were conducted by the first, second and third author. The first author statistically analyzed the data. The first draft was written by the first author and all other authors contributed to the final version of the manuscript.

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Appendix II: Matthias Wieczorek, Eric Bruns and Ralf Schulz conceived and designed the experiments. The experiments were conducted by the first and second author. The first author statistically analyzed the data. The first draft was written by the first author and all other authors contributed to the final version of the manuscript.

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Appendix III: Matthias Wieczorek, Mirco Bundschuh and Ralf Schulz conceived and designed the experiments. The experiments were conducted by the first, second and third author. The fourth author statistically analyzed the data. The first draft was written by the first author and all other authors contributed to the final version of the manuscript.

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Appendix IV: Matthias Wieczorek, Denise Kötter and Ralf Schulz conceived and designed the experiments. The experiments were conducted by the first and second author. The first author statistically analyzed the data. The first draft was written by the first author and all other authors contributed to the final version of the manuscript.

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9 Appendices

***Appendix I*: Scientific publication 1**

Reference scenarios for exposure to plant protection products and invertebrate communities in stream mesocosms

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Abstract

In the past, current tier-3 risk assessment for plant protection products (PPPs) for aquatic organisms in edge-of-filed surface waters was mainly based on pond-like mesocosm approaches. However, transient and dynamic PPP exposure scenarios as observed in lotic systems are hardly achievable in pond-like mesocosm approaches. The present compilation of studies performed at the Landau stream mesocosm facility provides knowledge on dynamic PPP exposure scenarios at different time scales (i.e. peak-, hour- and day-scale) under flow-through and recirculating conditions which are expected to be relevant in the field for deviating substance-related exposure scenarios. The use of the non-sorptive fluorescent tracer uranine, revealed the hydraulic processes universally underlying peak- and hour-scale exposures in the stream mesocosms and demonstrated an optimized application technique for the recirculating mode to enable day-scale exposure followed by a post-exposure flushing event with unpolluted water. Furthermore, the present study highlights the importance of aquatic macrophytes for realistic PPP exposure on peak- and hour-scale but at the same time for macrophyte-related structures favoring the establishment of aquatic invertebrates which is a keystone for ecotoxicological stream mesocosm testing. As the field relevance of the tier-3 risk assessment for PPPs for invertebrates might be qualitatively advanced by the presence of potentially sensitive and/or vulnerable species, those species were especially considered. Thus, the establishment of aquatic invertebrates in non-dosed streams was evaluated with respect to (*i*) the presence of different aquatic macrophytes and (*ii*) the duration of the pre-experimental period. The present study highlights the beneficial influence of complex-structured macrophytes and prolonged pre-experimental periods on the abundance of invertebrate taxa such as mayflies and damselflies. Furthermore, population dynamics were evaluated statistically by simulating PPP-related declines of 30, 50 and 70%. Thereby, minimum detectable difference (MDD) classes of mostly ≥ III were found for 12 out of 15 taxa for at least two consecutive sampling dates.

Introduction

The presence and fate of plant protection products (PPPs) in stream ecosystems is driven by complex application-, catchment-, or substance-related transport patterns towards and within respective flowing surface waters (Kirchner et al., 2001; Schulz, 2004). Therefore, exposure profiles of PPPs may vary in their characteristics such as the height of the concentration peak, the duration and frequency of the peak exposure, the intervals between

peaks, and the presence and level of a day-scale background concentration (Brock et al. 2010; Gordon et al. 2012; Edwards and Moore 2014).

According to the Aquatic Guidance Document recently published by the European Food Safety Authority (EFSA 2013), chemical exposure scenarios in mesocosm testing should provide pulse durations being equal or larger than predicted for field exposures and reflect realistic to worst case exposure scenarios in edge-of-field surface waters. For lotic edge-of-field waters, three different time-scales have been identified, namely: (i) peak-scale exposures at or below an hourly scale (Schulz and Liess 1999; Leu et al. 2004; Rabiet et al. 2010; Sangchan et al. 2012), (ii) hour-scale exposures on a multiple hour scale (Richards and Baker 1993; Leu et al. 2004), (iii) and continuous, day-scale exposures on a daily or weekly scale (Sangchan et al. 2012, Bayona et al. 2014, Bayona et al. 2015). Nevertheless, the current regulatory mesocosm practice is mainly based on pond-like mesocosms where hour-scale exposures, as expected to be relevant in lotic edge-of-field waters, are hardly achievable in case of stable compounds (e.g. insecticides) and, thus, frequently represent unrealistic worst case scenarios for lotic systems.

Few examples exist, where such exposure scenarios have been implemented in stream mesocosms using flow-through conditions (Ippolito et al. 2012; Stang et al. 2013; Elsaesser et al. 2013; Stang et al. 2014), a post-treatment flushing phase with uncontaminated water (Berghahn et al. 2012) and recirculating conditions (e.g. Mohr et al. 2007; Beketov and Liess 2008; Liess and Beketov 2011). Especially exposure durations below 12 hours as expected to be relevant in the field were scarce (e.g. Stang et al. 2013; Elsaesser et al. 2013; Stang et al. 2014). None of the studies presented in detail the compilation of wide-ranging realistic exposure scenarios on time-scales below 12 hours using the flow-though conditions including the underlying hydrological conditions and its applicability in ERA.

To fill this gap, the present study provides several short exposure scenarios (peak- and hour-scale) using the flow-through mode in vegetated and non-vegetated stream mesocosm. Furthermore, an exposure scenario characterized by substance exposures on day-scale using the recirculating mode combined with a subsequent flushing period with unpolluted water provides a promising opportunity for ERA (e.g. for macrophytes) in stream mesocosms to mimic temporal exposure scenarios such as resulting from FOCUS models (FOCUS 2001) and being expected to be relevant in the field. Thereby, an optimized application technique was applied to prevent oscillating concentration peaks following an application in recirculating mode.

Hence, the present stream mesocosm approach provides a methodological compilation for standardized realistic exposure scenarios (peak, hour-scale and day-scale) using the unique combination of controllable dynamic flow-through and recirculating conditions. Overall, flow-through stream mesocosms could provide more appropriate conditions within ERA for lotic systems to cover realistic exposure scenarios on various time scales.

The exposure in stream mesocosm should always be linked to ecology, as taxon abundance and the composition of invertebrate communities of stream mesocosms mimicking edge-of-field lotic surface waters are essentially for the successful ecotoxicological testing following short PPP pulses. Compared to pond-like systems, stream mesocosm approaches enable an enhanced realism of PPP exposure but to enhance the field of mesocosm testing a stream mesocosm should demonstrate at least a comparable community complexity to those of pond mesocosms and preferably demonstrate a special adaption to lotic taxon compositions. Unfortunately, many fundamental aspects of stream mesocosm establishment and their impact on taxon abundance and richness of invertebrate communities remain often unclear in existing studies (Brock et al. 2009), *e.g.* the importance of aquatic macrophyte species for invertebrate establishment and the duration of the pre-experimental period.

The overall species abundance and richness are highly dependent on physico-chemical parameters and habitat-inherent aspects (Briers, 2014) such as food supply (detritus), morphological structures which enable growth of periphyton, protection from predators, habitat structures for reproduction (Scheffer et al. 1984; Lalonde and Downing 1991; Tokeshi and Arakaki 2011) and structures for oviposition by adult insects (Guillermo-Ferreira and Del-Claro 2011). In particular, the spatial distribution, abundance and taxon richness of epiphytic invertebrates are, among others, driven by structurally complex elements such as aquatic macrophytes (Scheffer et al. 1984) and their intrinsic morphological complexity (Lillie and Budd 1992; Taniguchi et al. 2003; Hansen et al. 2010; Tokeshi and Arakaki 2011). These ecological interdependencies are likely to be valuable for stream mesocosm establishment. Hence, the present study investigates the establishment of aquatic invertebrates in uncontaminated control stream mesocosms with respect to (*i*) the presence of three aquatic macrophyte species with different morphologies (habitat structure) and (*ii*) different pre-experimental periods (successional time). The different stream mesocosm approaches were evaluated with regard to invertebrate community composition (richness and abundance). As the field relevance of the tier-3 risk assessment for PPPs for invertebrates might be qualitatively advanced by the presence of potentially sensitive and/or vulnerable species, the current studies evaluate the presence of potentially sensitive species as defined by Rico and

van den Brink (2015), which includes EPT taxa (Rubach et al. 2010), and of species classified as SPEcies At Risk (SPEAR) according to Liess and von der Ohe (2005). Furthermore, the invertebrate data were evaluated according to the minimum detectable difference (MDD) approach as presented within the Aquatic Guidance Document (EFSA 2013). By calculating the MDD values, it is possible to present the discriminatory power and the possibility to distinguish between effects and no effects based on a specific data set (Brock et al. 2014). The underlying taxon abundances (per 1/10 m^2) presented in the supporting information might enable additional information for the applicability in the ERA.

Overall, this study provides methodological insights into dynamic exposure scenarios with different time scales, the resulting location-dependent differences of concentrations and retention times with increasing stream length and the development and composition of invertebrate communities in control stream mesocosms. This combinatory evaluation highlights implications of peak- or hour-scale exposures for ecotoxicological testing in lotic systems and the integrative role of aquatic macrophytes in stream mesocosm.

Materials and methods

Stream mesocosm design

All studies were conducted at the Landau stream mesocosm facility (SW Germany) of the University of Koblenz-Landau consisting of 16 independent high density concrete channels (length = 45 m; width = 0.4 m; water depth = 0.23 - 0.26 m). Further information, concerning the stream mesocosms, are described in Elsaesser et al. (2013), Stang et al. (2014) and Wieczorek et al. (2015).

To evaluate different hypothetical PPP exposure scenarios, pre-studies with a non-sorptive and non-toxic tracer substance were conducted between 2010 and 2014 (Table 1). Furthermore, two independent test designs were used in 2011 and 2012 to evaluate the establishment of invertebrate communities in the streams. Therefore, four different test designs were established consisting of three unreplicated (S-2011-A, B, C) and one replicated approach (S-2012-D to D4; n = 4) (Table 2). Overall, this study design provided the option to compare long pre-experimental establishment periods (≈ 9 months; stream B and C in 2011) with short ones (≤ 2 months; stream A in 2011 and D in 2012).

In order to mimic small lotic surface waters, the stream mesocosms were equipped with substrate as described in Stang et al. (2014) (height approx. 0.1 m) and aquatic macrophytes in a way that reproducible conditions could be set for exposure scenarios and invertebrate

community implementation. Thereby, the setup of the streams varied in macrophyte species and horizontal and/or vertical macrophyte coverage in order to meet the experimental requirements of the present study (Tables 1 and 2).

Table 1: Exposure durations (peak-, hour- and day-scale), the underlying application techniques of the tracer applications between 2010 and 2014, displayed together with the underlying macrophyte characteristics and the resulting resident times for the exposure studies.

Duration of application (scale of the exposure)	Test year	Technical application procedure	Macrophytes			Residence times[c] (min)	
			Species	Horizontal coverage	Vertical coverage[a]	inlet	outlet
3 s (peak-scale)	2010	tilting mechanism	*E. nuttallii*	91%	half water column	6 (unveg) 15 (veg)	18 (unveg) 52 (veg)
10 min (peak-scale)	2011	peristaltic pumps (24 teflon tubes per channel)	*E. nuttallii*	77%	half water column	18(unveg) 25 (veg)	30 (unveg) 49 (veg)
105 min (hour-scale)	2014	peristaltic pumps (6 teflon tubes per channel)	*M. spicatum* *E. nuttallii*	57-100% 49-87%	*M. spicatum* patches with complete vertical coverage (60%) and *E. nuttallii* patches with up to half vertical coverage (40%)	111 (veg)	143 (veg)
60 min + 60 min[b] (day-scale)	2013	peristaltic pumps (6 teflon tubes per channel)	*M. spicatum* and *E. canadensis*	100%	complete water column	-	-

a = Macrophyte structure and its orientation within the water column according to Stang et al. (2014)
b = Stepwise reduced application
c = A concentration threshold of 5% of the maximum moving average was used as a lower limit; unveg = unvegetated and veg = vegetated streams

In total, three macrophyte species, namely the submerged *Elodea nuttallii* (PLANCH.) H. St. John, *Elodea canadensis* Michx., *Myriophyllum spicatum* L., and the helophyte *Berula erecta* (HUDS.) Coville, were established in the streams from 2010 to 2014 (Tables 1 and 2). As the structure of aquatic macrophytes within the water column was shown to influence solute dispersion processes (Nepf 2012a; Stang et al. 2014), both horizontal and vertical coverages of macrophyte were considered (Table 1). Thus, habitat-relevant morphological macrophyte structures, e.g. simple (broad-leafed) versus complex structures (Hansen et al. 2010), were implemented for the studies on invertebrate communities (Table 2). To account for time of invertebrate acclimation and macrophyte succession, the duration of the pre-experimental period is given in Table 2.

Table 2: Stream mesocosm setup and sampling periods of the invertebrate establishment studies performed in 2011 and 2012. The underlying stream setup characteristics in terms of the water renewal procedure the used macrophyte species, the related macrophyte structure and coverage and the pre-experimental period are displayed.

Parameter	2011[a]			2012[b]	
	S-2011-A	S-2011-B	S-2011-C	S-2012-D2	S-2012-D4
Sampling period	June 14 to September 2			May 30 to August 23	
Water renewal	Daily 1500 L per channel			Complete daily water renewal (from June 18 to August 19)	
Macrophyte species	*M. spicatum*	*E. nuttallii*	*E. nuttallii*	*B. erecta*	
Macrophyte structure[c]	complex	complex	complex	simple	
Macrophyte coverage	22%	50-60%	100%	40% (consisting of patches with ≤ 60% macrophyte coverage)	
Pre-experimental period (month)	≤ 2	9[d]	9	≤ 2	

a = In 2011 nutrient parameters were measured once
b = In 2012 nutrient parameters were measured eight times
c = According to Hansen et al. (2010)
d = Macrophyte coverage was reduced initially in April 2011 and twice during the experimental period

In order to provide organic material for shredders, leaf material was added to the streams. In 2011, an initial amendment (106 ± 21 leafs per m2) of *Fagus sylvatica* L. leaves to stream S-2011-A and S-2011-B collected from the pristine Hainbach stream (Bundschuh et al. 2011; 49°14′N; 08°03′E) and *Acer platanoides* L. to stream S-2011-C collected from the stream mesocosm facility area. In order to maintain the initial leaf amount being present at the experimental start consistently across all streams and over time, adapted amounts of *Acer platanoides* L. leaves were added weekly into all streams to substitute loss due to decomposition processes and shredders. In 2012, dried leaf material (> 95% *Alnus glutinosa* (L.) Gaertn.), which was pre-soaked in tap water for 12 h, was added to the streams on a monthly basis. Further details on leaf addition in 2012 and the general procedure of leaf litter addition are described in Wieczorek et al. (2015).

To prevent high solar radiation, 67% (2011) and 50% (2012) of the stream surface was covered with white cotton mesh (reduction of solar radiation = 40%, Wieczorek et al. 2015). The achieved level of solar radiation was within a range between open pasture streams (≥ 10% shading) and streams with patchy riparian shade (≤ 70% shading) (Wieczorek et al. 2015).

Each stream was run in a recirculation mode and water was renewed as indicated in Table 2. The physico-chemical parameters, namely conductivity, pH, oxygen saturation, and temperature, were measured twice a week at 9 a.m. and 4 p.m. (WTW Multi 340i) in each channel. Ammonium, nitrate, nitrite, phosphate and total hardness were measured once in

2011 and at 8 dates in 2012, in all channels using visocolor Test-Kits (Macherey-Nagel, Düren, Germany).

Exposure profile experiments

Reference exposure scenarios were evaluated using the non-sorptive, fluorescent tracer uranine (Sigma–Aldrich, Steinheim, Germany). Uranine was measured *in situ* with fiber-optic fluorometers (FOFs, Hermess Messtechnik, Stuttgart, Germany) at two sampling locations below the inlet (5 and 40 m in 2010/11; 5 and 42.5 m in 2014; placement of the probes in the middle of the water column). Due to high photosensitivity of the FOFs and rapid photodegradation of uranine (Hadi et al. 1997), tracer experiments were conducted during night time. The dimensionless fluorescence signals (measurement intervals = 5 s) were subsequently quantified using an external calibration (Stang et al. 2014).

Four approaches were specifically designed that encompassed several application durations (peak-, hour- and day-scale exposures) using different application procedures (Table 1). The tracer was applied using a tilting mechanism in 2010 or 24-channel peristaltic pumps (Ismatec IPC 24, IDEX Health & Science GmbH, Wertheim, Germany) in 2011, 2013 and 2014. The tracer was linearly applied over the whole stream width, in order to minimize lateral dispersion. Depending on the duration and dynamics of the application scenario, the substance amendment was conducted either in flow-through (2010, 2011 and 2014) or recirculation mode (2013). In all approaches, the water flow rate was set to 1 L s^{-1}.

Constant application rates in scenarios with recirculation mode during an initial period of time may lead to oscillating concentrations above and below the nominal concentration, due to substance addition to recirculated and, thus, already treated water. To achieve a constant plateau concentration in day-scale scenarios in the recirculation mode (2013), the application procedure had to be adapted. The first step was a constant amendment phase for one recirculation cycle. In the second step, a subsequent stepwise reduced amendment phase starting with the arrival of already treated water after a complete recirculation cycle and ending at the nominal plateau concentration.

Establishment of aquatic invertebrates

Different aquatic invertebrate communities were established in 2011 and 2012. The taxa originated from passive and targeted introduction into the mesocosms and natural colonization. Passive introduction of invertebrates occurred along with leaf material from the

Hainbach stream added to streams S-2011-A and S-2011-B and to all streams in 2012. Furthermore, passive introduction occurred with the addition of aquatic macrophytes and the sediment associated with their roots. Considering the replicated approach in 2012, the passive introduction of invertebrates was assumed to be equal as each channel received the same quantities of plant material in a random manner.

Introduction of invertebrates in 2011 and 2012 was restricted to the species *Cloeon* spp., *Gammarus fossarum* and *Asellus aquaticus*. In both years, *Cloeon* spp. and *G. fossarum* were collected from the Hainbach stream and *A. aquaticus* at the Klingbach River (49°07'N; 08°06'E). In April 2011, approximately 4500 individuals of *Cloeon simile* were randomly added to each of the three streams. Furthermore, 4500 individuals of *A. aquaticus* were added to stream S-2011-C. The addition of *G. fossarum* was restricted to the streams S-2011-A and S-2011-B. In 2012, the intended randomized addition of these three species was done as described in Wieczorek et al. (2015). The occurrence of other taxa resulted from natural colonization from both a storage pond (200 m^3) next to the channels and nearby surface waterbodies.

Initial organism drift was avoided by dissecting the streams into 10-m long sections separated by water permeable dividers (frames with polyester (PES) mesh; mesh size = 1 mm) for two weeks before the experiments started. Beyond that, channel outlets were equipped with water permeable dividers (PES mesh; mesh size = 1 mm) to avoid loss of taxa during the sampling period.

Invertebrate sampling

To evaluate the abundance and community composition of aquatic invertebrates, samplings of invertebrates with aquatic life stages and imagines of merolimnic invertebrates (emergence sampling) were conducted. In 2011, sampling was done once a week at four locations (randomly once per quarter of the stream length) and in 2012 once every two weeks at two locations (randomly within the first 5 m close to the inlet and outlet) in each channel. The abundance estimation of aquatic invertebrates was conducted with a metal frame (horizontal sampling area = 0.05 m^2), which was vertically covered with PES mesh (mesh size = 1 mm). The sampling frame was pushed to the substrate and the macrophytes, the leaf material and the upper substrate layer were removed from the horizontal sampling area. The macrophytes and leaf material were rinsed with tap water over a 500 µm mesh and checked for attached invertebrates. Species located in the stream water column and on the upper

substrate layer were removed individually using a hand net. Subsequently, known species were categorized immediately after sampling and returned to the respective sampling sites. Example specimens of unknown species were preserved in ethanol (70%) for subsequent determination in the laboratory.

Samplings of emerging adult insects were conducted in both years. During the whole sampling period in 2011, the emergence sampling was conducted at three locations once a week (sampling duration per sampling = 48 h). In 2012, emergence was assessed every two to three days from July 1 to August 10, 2012 at two locations (sampling duration per sampling = 48-72 h). The emergence traps consisted of pyramidal-shaped constructions (mesh size = 0.5 mm; sampling area = 0.25 m^2) and were equipped with a collecting chamber filled with water and a detergent. The emergence traps were, if possible, placed above sampling locations with a quantitatively comparable amount of macrophytes and they were installed in a manner as to cover the entire width of the respective stream mesocosm section. Further details concerning emerging insect sampling and taxonomic classification are given in Wieczorek et al. (2015).

Data analysis

Macrophyte coverage within the different experimental setups was evaluated with Definiens Professional 5.0 (Definiens AG, Munich, Germany) using digital photographs of the setups. In order to demonstrate individual retention capacity of the channels, the difference between the initial uranine concentration at the inlet and the concentration at the outlet was calculated. For data processing, the maximum value of the moving average of 5 consecutive values was used to account for scattering data. Furthermore, the residence time of the tracer in the water column was calculated for each sampling location (Table 1). A concentration threshold of 5% of the maximum moving average was used as a lower limit.

To evaluate the presence of taxa potentially sensitive to PPPs, all taxa were, if applicable, classified as (i) families with a mode specific sensitivity (MSS) according to Rico and van den Brink (2015), (ii) species being part of the orders Ephemeroptera, Plecoptera and Trichoptera (EPT) (Barbour et al. 1992), or (iii) SPEcies At Risk (SPEAR) (Liess and Beketov 2011). The MSS of the families occurring in 2011 and 2012 were taken from the supplementary dataset of Rico and van den Brink (2015). The calculation procedure of MSS values is described in Rico and van den Brink (2015).

As the present approach aims at demonstrating the overall potential of hosting potentially sensitive species, both the "field-focused" and the mesocosm SPEAR thresholds were used. For a SPEAR-classification in a "field-focused" manner ($SPEAR_{field}$), taxa had to fulfill the

following criteria: $S_{organic}$ value > -0.36, generation time ≥ 0.5 years, aquatic stages being not able to avoid PPP exposure during periods with intensive PPP usage patterns, and no migration ability (Liess and von der Ohe 2005; Liess and Beketov 2011). Except for generation time, which was taken from the Aquatic Guidance Document (EFSA 2013), all parameters were taken from the SPEAR Calculator (UFZ, Leipzig, Germany). As aerial migration from control streams to contaminated streams might distort the recovery potential of multivoltine and sensitive species, the generation time threshold was set to ≥ 1 year for species at risk for mesocosm approaches ($SPEAR_{mesocosm}$). As aerial recovery from non-exposed nearby areas of a stream might occur for some surface waterbodies in the environment, this assumption represents worst case conditions.

To demonstrate the variability of the population abundances over the whole sampling period, seasonal average coefficients of variation (ACV) of selected invertebrate taxa were calculated. Thus, an average value of the coefficients of variation (CV) was calculated for each taxon at each sampling date. In 2011, CV values were calculated using four sampling locations (in-stream variability), whereas in 2012 four replicates were used (between-stream variability).

The minimum detectable differences (EFSA 2013; Brock et al. 2014) were calculated for abundance data (2012) to check for the general "sensitivity" of the system to demonstrate potential PPP-related changes in taxon abundance. The available control data were used to simulate a PPP-treatment data set showing the same CV as the control data, but 30, 50 or 70% reduced abundance values compared to the abundance in the controls. Prior to analysis, each data set was log-transformed (ln(Ax+1)) with Ax = 2 for the lowest abundance above zero as suggested by van den Brink et al. (2000), an approach which was deemed suitable for the present study not looking at effect sizes. All calculations were performed on stream-wise summed-up values of the two sampling points per replicate. The data were checked for variance homogeneity (with residuals) and subsequently analyzed with ANOVA followed by the Williams' test procedure using ToxRat 2.10.05 (ToxRat Solutions GmbH, Alsdorf, Germany). The resulting MDD values for the transformed data (MDD_{ln}) were back-transformed to the MDD for the abundance (MDD_{abu}) using the equations given in Brock et al. (2014). MDD calculations were performed on data previously classified as robust data. The criteria of robustness were fulfilled when replicates include a minimum of three individuals of the respective species at each sampling date in three out of four replicates and in at least two consecutive samplings. According to the Aquatic Guidance Document (EFSA

2013), the MDD_{abu} values were classified in MDD classes (0 to IV) for each taxa and sampling date.

Results

The CV values of the four replicates for pH, oxygen saturation, temperature and conductivity measures ranged between 0.2 and 3.5% per sampling date in 2012. The average CV values for all sampling dates were ≤ 2% for all above-mentioned physico-chemical parameters and, thus, showed a low variability of these conditions between replicates. The average CV values for nitrate, nitrite, ammonium, phosphate and total hardness ranged between 10 and 63% with single CV values up to 200%. Occasionally high CVs can be referred to the fact that several values were close to the lower limit of the test kits and, thus, high CV values should be considered with caution. The average physico-chemical water parameters of the unreplicated approach in 2011 and replicated approach in 2012 are presented in Table 3.

Table 3: Average seasonal physico-chemical parameters for the period of June 15 to September 1, 2011 (± SD; n = 23) and June 4 to August 16, 2012 (± SD; n = 21). In 2012, measurements were displayed for two out of four streams.

Parameter	2011[a]			2012[b]	
	S-2011-A	S-2011-B	S-2011-C	S-2012-D2	S-2012-D4
pH	8.6 ± 0.3	9.4 ± 0.3	9.2 ± 0.3	8.4 ± 0.3	8.4 ± 0.2
Oxygen saturation (%)	100.8 ± 10.2	119.6 ± 11.9	118.2 ± 9.7	104.8 ± 8.2	105.3 ± 8.5
Temperature (°C)	19.4 ± 1.2	19.8 ± 1.6	19.7 ± 1.6	19.8 ± 1.4	20.0 ± 1.6
Conductivity (µS cm^{-1})	137.5 ± 5.9	126.1 ± 5.9	124.6 ± 7.1	138.7 ± 10.2	138.0 ± 9.4
Nitrate (mg L^{-1})	3	1	1	2.8 ± 0.5	2.8 ± 0.4
Nitrite (mg L^{-1})	0.01	0.00	0.00	0.02 ± 0.01	0.02 ± 0.01
Ammonium (mg L^{-1})	0.01	0.01	0.01	0.01 ± 0.02	0.01 ± 0.02
Phosphate (mg L^{-1})	0.00	0.00	0.15	0.12 ± 0.11	0.13 ± 0.09
Total hardness (°dH)	3.9	4.5	3.9	3.7 ± 0.6	3.7 ± 0.5

a = In 2011 nutrient parameters were measured once
b = In 2012 nutrient parameters were measured eight times

Exposure profile experiments

Specific experimental setups such as the presence/absence of macrophytes and different macrophyte species (Table 1) and durations of the applications (seconds-hours) were shown to influence the retention of the tracer uranine within the stream mesocosms. Thereby, the peak-scale exposures in 2010 and 2011 revealed concentration patterns with concentration dependent changes with increasing flow length which are expected to be typical for lotic edge-of-field waters. The peak-scale exposure scenarios in unvegetated stream mesocosms revealed that the maximum concentrations of uranine at the outlet were 65 and 26% lower than measured at the inlet in 2010 and 2011, respectively (Figure 1). The presence of aquatic macrophytes during peak-scale exposures revealed 80 and 49% decreased maximum

concentrations at the outlet compared to those measured at the inlet in 2010 and 2011, respectively. Thereby, residence times of uranine within vegetated systems were higher compared to those of the unvegetated stream mesocosms during peak-scale exposure scenarios in 2010 and 2011 (Table 1). For peak-scale exposures in vegetated stream mesocosms the residence times at the 40-m sampling sites were 3.5 times and 2 times longer compared to the residence times of uranine at 5-m sampling sites in 2010 and 2011, respectively.

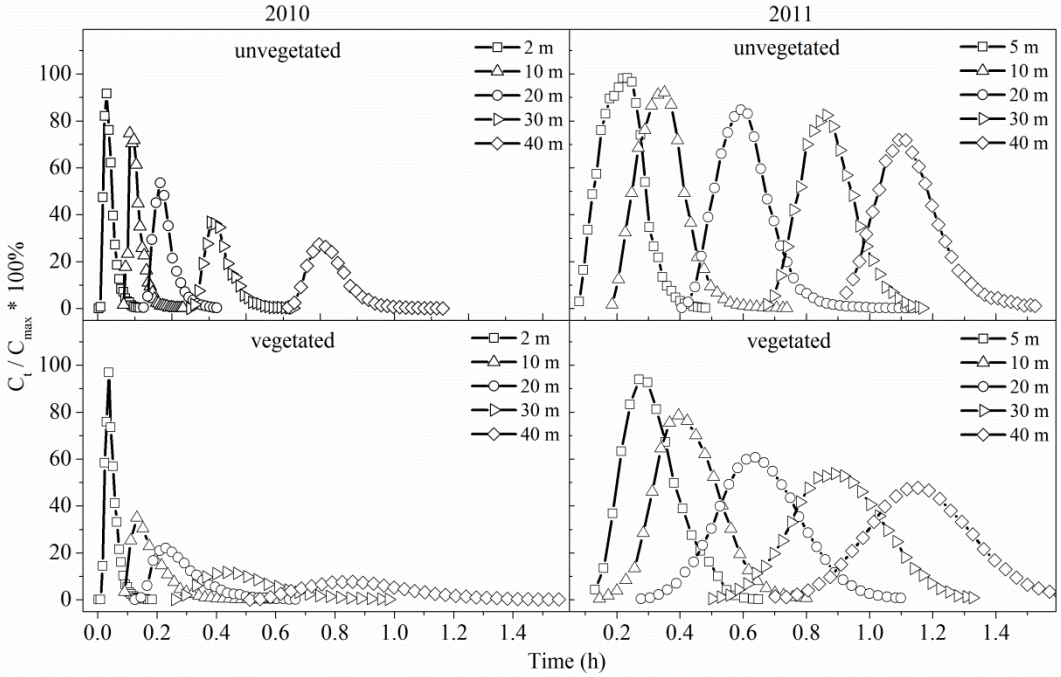

Figure 1: Tracer dynamics during two peak exposure scenarios with short tracer amendment (5 s in 2010 and 10 min in 2011). The time-dependent concentrations Ct are divided by the highest concentration C_{max} of each approach. Note, that in some cases ≤ 25 data points of the 5-second intervals were combined to average values. For reasons of comparability, all the recovery rates of the respective tracer test were normalized with the maximum recovery rate of the respective approach.

Generally, the hour-scale exposure scenarios in 2014 (Figure 2) resulted in constant concentrations over restricted periods of time (several hours). During the prolonged uranine amendment of 105 min (Figure 2), the difference in the intended uranine concentrations between the inlet and the outlet of the channels was in contrast to uranine amendment of 5 s and 10 min (peak-scale exposures) negligibly small, namely ≤ 2%. During the hour-scale exposure the residence time (Table 1) at the 42.5-m sampling site was 1.3 times higher compared to the 5-m sampling site.

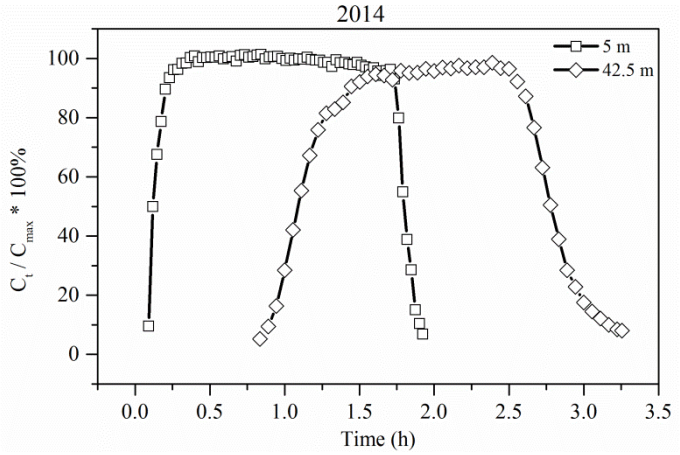

Figure 2: Hour-scale exposure scenario with a prolonged tracer amendment (105min). The time-dependent concentrations C_t are divided by the highest concentration C_{max} of the 5 m sampling site. Note, that in some cases ≤ 40 data points of the 5-second intervals were combined to average values. For reasons of comparability, all the recovery rates of the respective tracer test were normalized with the maximum recovery rate.

Different from peak- and hour-scale exposure scenarios using the flow-through mode, operating of the channels was changed to the recirculation mode in the case of a day-scale exposure to reproduce scenarios with constant concentrations of chemicals over time periods substantially extending 24 hours. Using the recirculation mode in combination with a stepwise reduction of the tracer dosage, a constant concentration with deviations as low as ± 2% (Figure 3 B) could be reached within 2 h after start of the tracer addition. Thereby, this application procedure prevented concentrations oscillating by > 12% above and below the nominal concentration level (Figure 3 A).

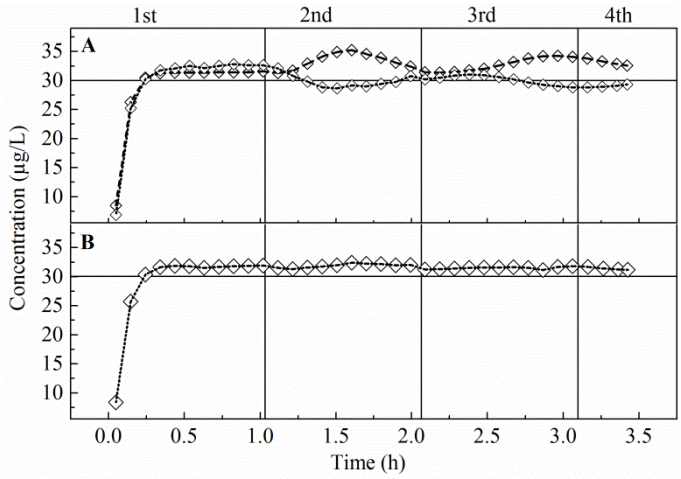

Figure 3: Day-scale exposure scenarios using recirculating mode with incompletely adapted (A) and successfully adapted (B) application technique. The figure displays the concentrations of uranine during and following the application phase. The concentrations are displayed for the application phase during which the water is recirculated once (1st), for the second cycle (2nd) with (B) and without (A) the adapted application technique and for two other recirculation cycles (3rd and 4th)

Invertebrate communities

In total, 28 and 44 different taxa were recorded in 2011 and 2012, respectively (Table 4). Thereof, 21 and 38 taxa were found in more than one stream in 2011 and 2012, respectively. In 2012, the average taxon richness was 35 with a between-stream CV of 5% and showed 27 taxa in common considering the whole sampling period. A detailed overview of the different taxa, the average population abundance of selected taxa and their relative occurrence over the whole sampling period is given in supporting information (Tables S1 and S2). The taxon richness of EPT and $SPEAR_{field}$ taxa is displayed in Table 4 and Table S1. Furthermore, the families occurring in 2011 and 2012 were ranked using the MSS (Figure S1). Thereby, the application of the MSS revealed a strong substance-dependent sensitivity of several families. The number of EPT taxa ranged between 3 and 5 in 2011 and between 10 and 13 in 2012. The number of $SPEAR_{field}$ taxa ranged between 4 and 6 in 2011 and 10 and 12 in 2012. In both years, the number of $SPEAR_{mesocosm}$ taxa was lower compared to $SPEAR_{field}$ taxa (Table 4).

In 2011, the median abundance (expressed as individuals m^{-2}) of the most abundant taxa was 3910 for *G. fossarum* (ACV = 58; S-2011-A) and 490 for *A. aquaticus* (ACV = 56; S-2011-C). The median abundance of *Cloeon simile* was 310 for S-2011-A (ACV = 58), 880 for S-2011-B (ACV = 43) and 1680 for S-2011-C (ACV = 38). The median abundance of *Ischnura elegans* was 20 for S-2011-A (ACV = 117), 410 for S-2011-B (ACV = 48) and 340 for S-2011-C (ACV = 43). In 2012, the median abundance of the most abundant taxa was 150 for Baetidae (ACV = 51.5), 690 for Chironomidae (ACV = 33), 60 for *Chaetopteryx* sp. (ACV = 40.4) and 60 for *G. fossarum* (ACV = 91).

Table 4: SPEAR and EPT taxa richness in 2011 and 2012

	S-2011-A†	S-2011-B†	S-2011-C†	S-2012††			
				D1	D2	D3	D4
Taxa per stream	23	18	20	33 (15)	33 (15)	36 (15)	36 (15)
$SPEAR_{field}$ taxa	6	4	4	10 (3)	11 (3)	12 (3)	12 (3)
$SPEAR_{mesocosm}$ taxa	4	3	3	7 (3)	9 (3)	9 (3)	9 (3)
EPT taxa	5	4	3	10 (4)	12 (4)	13 (4)	13 (4)
Sensitive taxa*	15	12	10	21 (9)	22 (9)	25 (9)	25 (9)

† The robustness of taxa could not be stated due to the non-replicated design
†† The included number of robust taxa is presented in brackets
* Potentially sensitive species to insecticides according to EFSA (2013)

In total, 9 out of 44 taxa showed increasing abundances during the experimental period of 12 weeks in 2012. These species were *Polycelis* spp., Naididae, *Physella acuta*, *Radix balthica*, *A. aquaticus* and predatory species such as *Erpobdella octoculata* and *Helobdella*

stagnalis. Furthermore, increasing abundances were shown for the merolimnic insects *I. elegans* and *Polycentropus flavomaculatus*.

Considerable differences in abundance and variability of invertebrate populations were observed between the different experimental setups in 2011 and 2012 (Figure 4). Considering the abundances of the damselfly larvae *I. elegans* and mayfly family Baetidae, differences between short (S-2011-A and S-2012-D) and long (S-2011-B/C) pre-experimental periods were visible. Higher median abundances of *I. elegans* (factor = 37.5) and Baetidae (factor = 5.6) were observed for the stream channels with a longer pre-experimental period (S-2011-B/C) compared to the streams S-2011-A and S-2012-D. This tendency was also visible for the abundance of the emerging imagines of *I. elegans*, showing no or occasional emergence for streams with short pre-experimental periods (S-2011-A and S-2012-D). Chironomidae were more abundant in 2012 compared to 2011. The in-stream variability of Chironomidae was generally rather high (ACV > 70%) in 2011, especially for S-2011-A (ACV = 117%). The taxa displayed in Figure 4 are representative for other taxa such as *Chaetopteryx* sp., Chironominae, Tanypodinae, Tanytarsini and *Sericostoma* sp. having similar abundances in 2012.

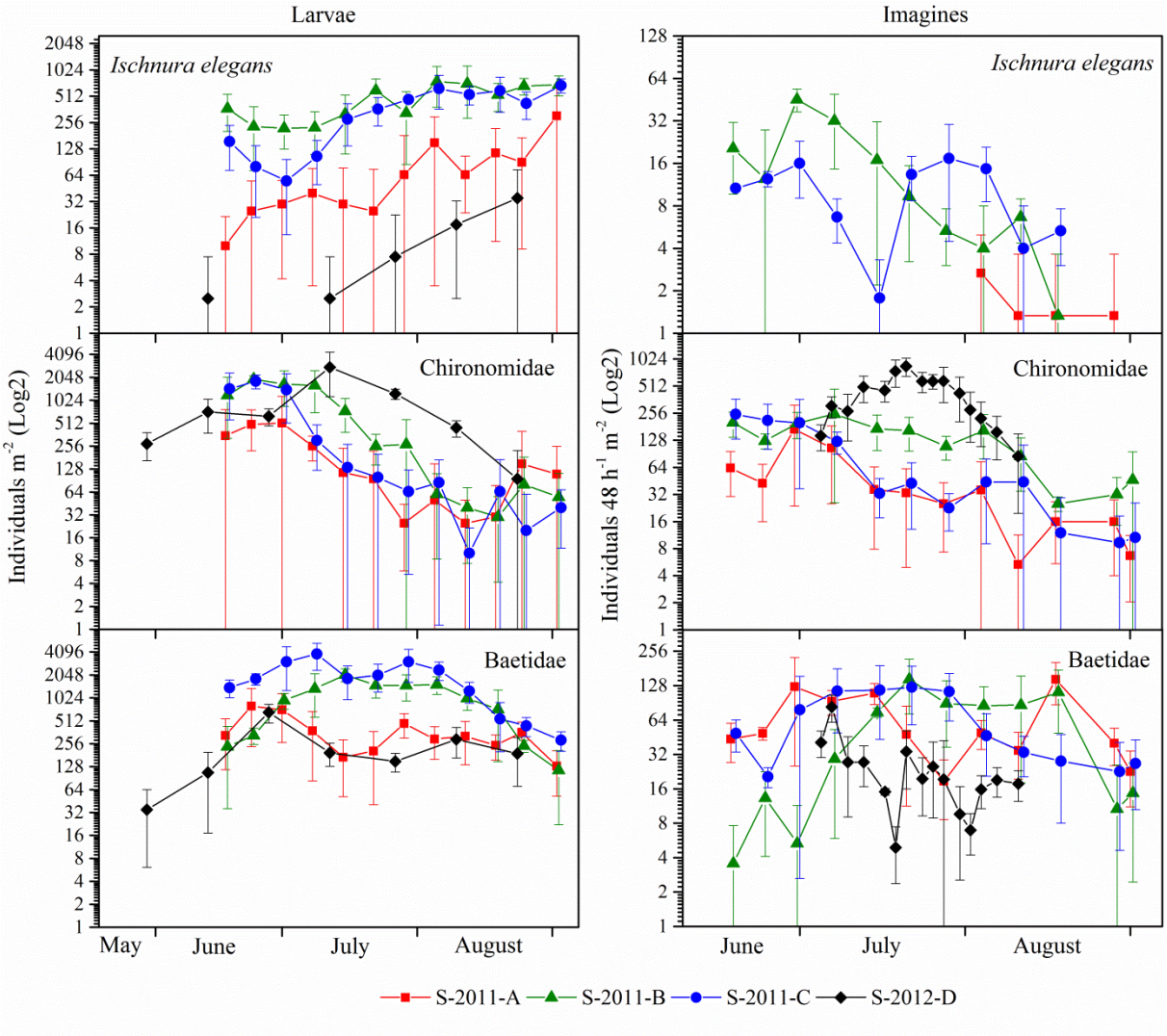

Fig. 4. Abundance of merolimnic insect larvae (number of individuals per square meter) and the emergence of the respective imagines (number of individuals per 48-h-interval and square meter). The displayed standard deviations refer to the four sampling location per stream in 2011 (in-stream variability) and the four streams in 2012 (between-stream variability).

For a minimum of two consecutive sampling dates, 15 out of 44 taxa fulfilled the criteria for robust taxa and were thus included in the MDD calculations. The different MDD classes according to EFSA (2013) are presented in Table 5. In total, 12 out of 15 taxa showed high MDD classes (III and IV) at least two consecutive sampling dates (Table 5). Taxa showing high MDD classes (III or IV) at the majority of the sampling dates were *C. dipterum*, *Chaetopteryx* sp., Chironominae, Tanypodinae and Tanytarsini. For *G. fossarum*, large to medium effects (MDD class II) were determined. Taxa showing robust abundances only at the beginning or at the end of the season with high MDD classes were *E. octoculata*, *P. acuta* and *S. ignita*. Varying robustness of data and MDD classes were shown for *Sericostoma* sp., *Polycelis* spp., Naididae and *H. stagnalis*.

Table 5: MDD classes for robust taxa at various sampling dates in 2012

	MDD class per sampling date						
Taxa	30.5.	13.6.	27.6.	11.7.	26.7.	09.8.	23.8.
C. dipterum	-	I	IV	IV	IV	IV	III
Chaetopterix sp.	III	IV	IV	IV	IV(III)	IV	-
Chironominae	-	III	III	III	III	III	IV
E. octoculata	-	-	-	-	IV	IV	IV
G. fossarum	-	II	II	II	II	II	II
H. stagnalis	-	-	-	-	II	II	IV
Naididae	-	-	III	-	III	III	II
Orthocladiinae	III	-	II	I	-	-	-
Physella acuta	-	-	-	II	III	IV	III
Polycelis spp.	-	-	II	IV	-	III	III
Radix balthica	-	-	-	-	I	III	III
S. ignita	IV	IV	-	-	-	-	-
Sericostoma sp.	-	-	IV	III	IV	-	III
Tanypodinae	III	III	III	III	IV	IV	III
Tanytarsini	-	II	IV	II	IV	III	IV

MDD classes were replaced by a minus in the case of not fulfilling the criteria for robust data. MDD classes according to the Aquatic Guidance Document: 0 = no effects can be determined (MDD > 100%); I = only large effects can be determined (MDD = 90-100%), II = large to medium effects can be determined (MDD = 70-90%); III = medium effects can be determined (MDD = 50-70%); IV = small effects can be determined (MDD < 50%).

Discussion

Exposure scenarios

The present study demonstrated the capabilities of the flow-through stream mesocosm system to mimic realistic dynamic exposure scenarios (peak-, hour- and day-scale) of flowing edge-of-field surface waters. Tier-3 risk assessment was mainly based on rather static pond-like systems, which is appropriate in the case of ERA for lentic systems or to represent worst case conditions for lotic systems. Nevertheless, as hour-scale exposures with stable compounds are hardly achievable in pond-like mesocosms, it is highly relevant to consider stream mesocosm based exposure techniques in ERA procedures. For both, exposure scenarios and the stream invertebrates, the present study was able to highlight the influence of aquatic macrophytes within the framework of stream mesocosm testing. The present uranine exposure scenarios on peak- and hour-scale demonstrated hydrological and physical aspects universally underlying transport scenarios of solutes and, thus, are highly relevant for the assessment of PPP exposure scenarios. The inclusion of such hydrological and physical aspects in stream mesocosm promotes typical fate processes of lotic systems which are technically difficult to realize in pond-like systems.

Peak-scale exposure scenarios in 2010 and 2011 (total residence time of a chemical ≤ 1 hour) are applicable in order to mimic exposure characteristics occurring in small lotic edge-of-field waters after spray drift or runoff events on an hourly scale as shown by Rabiet et al. (2010) and Leu et al. (2004). Furthermore, the application of such peak-scale exposure scenarios (Figures 1 and 2) in ecotoxicological stream mesocosm testing enables the integration of longitudinal stream gradients such as the pronounced decline of the maximum peak concentrations and the increased residence times with increasing flow length. For uranine exposures, the underlying processes of declining peak concentrations and increasing residence times can mainly be ascribed to longitudinal dispersion and transient storage (Nepf et al. 2007; Nepf 2012a; Nepf 2012b; Sukhodolova and Sukhodolov 2012; Stang et al. 2014). These mainly vegetation-dependent processes were related to the vertical coverage of the macrophytes (half of the water column) which is discussed in detail in Stang et al. 2014. A consideration of the vertical orientation of the macrophytes within the water column is, thus, essential for a comprehensive evaluation of peak-scale exposures in the frame work of ecotoxicological testing. The transferability of the presented uranine peak-scale exposure scenarios was demonstrated by a peak-scale exposure study with three PPPs (K_{oc} = 290 - 30,000 and log K_{ow} = 3.3 - 4.9) which was conducted also in the Landau stream mesocosm facility and had shown similar exposure dynamics as observed for uranine in 2011 but revealed an increased potential for peak reductions due to sorptive processes (Stang et al. 2014). Except Pusey et al. (1994) using a 6-hour application of chlorpyrifos and the studies conducted at the Landau stream mesocosm facility (e.g. Elsaesser et al., 2013; Stang et al., 2014), the majority of the recent studies used exposure times of ≥ 12 hours (e.g. Berghahn et al., 2012; Ippolito et al., 2012; Liess and Beketov, 2011) whereby dynamic concentration patterns and hydrological processes as observed for peak-scale exposures in 2010 and 2011 could not be observed. Nevertheless, the applicability in ERA might be limited as realistic to worst case conditions as requested by the Aquatic Guidance Document (EFSA 2013) might not be fulfilled below an hour-scale. Nevertheless, a general applicability in stream mesocosm testing might be valuable to enable an advanced process understanding of environmental fate (including longitudinal dispersion and transient storage) in peak-scale exposure events as occurring in the field.

The present hour-scale exposure scenario (total residence time of a chemical ≥ 1 hour) is applicable to stream mesocosm approaches with constant concentration levels on a multiple hour scale and, thus might fulfill the realistic to worst case conditions for insecticide exposures in ERA. The location-dependent increase of the residence time in flow direction

indicates that the above-mentioned physical processes are also of importance in prolonged exposure scenarios. Nevertheless, the decline of the maximum concentrations was negligible (< 2%) for the uranine exposure. The transferability to hour-scale PPP exposure scenarios was demonstrated using a highly sorptive insecticide (K_{oc} ≈ 18,000) which revealed a 34% decreased concentration level at the outlet (Wieczorek et al., unpublished data). This reveals location-dependent differences which are not detectable during applications while using recirculating conditions as demonstrated by e.g. Berghahn et al. (2012) and Liess and Beketov (2011b). Furthermore, the present hour-scale scenario using the flow-through mode has the advantage that it is comparable to laboratory test procedures by showing constant concentration levels over a restricted period of time (several hours). Flow-through exposure conditions for 30 days were presented in Bayona et al. (2015) which is quite long compared to the present study mainly focusing on short exposure characteristics. To achieve dynamic concentrations as displayed in the peak-scale exposure scenarios (Figure 1) and shown by Leu et al. (2004), a stepwise controlled application technique can be used instead of the constant application approach. Thus, the hour-scale exposure in vegetated stream mesocosms might be appropriate in ERA as realistic fate processes as location-dependent decrease of concentrations and enhanced residence times enable realistic to worst case exposure dynamics for lotic systems.

Day-scale exposure scenarios (Figure 3) using the recirculation mode are applicable to realistic worse case exposure scenarios on a daily scale (> 24 hours) comparable to those described in Leu et al. (2004). Generally, the day-scale exposure scenario might be appropriate for simulating long-lasting runoff events, subsurface drainage and any other sort of continuous exposures. The day-scale scenario is applicable to substances with a low tendency for sorption to organic components such as selected herbicides and insecticides (e.g. neonicotinoids). Thereby, step-wise reduced application technique prevented oscillating concentration peaks following the application in recirculating mode which might be relevant in the case of drift-initiating insecticides such as neonicotinoids (Beketov and Liess, 2008). Direct transferability was observed for a highly soluble herbicide (water solubility = 0.9 g l^{-1}) studied in the same mesocosm system in a continuous 24-hour exposure scenario (Wieczorek et al., unpublished data) comparable to the day-scale scenario (Figure 3). In the context of ERA, the subsequent dilution period (flow-through mode) following a day-scale exposure in the recirculation mode enhances the exposure realism compared to this in pond-like or solely recirculating systems. Furthermore, the present day-scale scenario can be utilized to simulate exposure scenarios resulting from models, such as FOCUS (FOCUS 2001).

With respect to the substance exposure routes (spray drift, runoff and drainage), the scenarios (Figures 1-3) may be used separately or in combination. For instance, day-scale concentrations might be supplemented by single or multiple peak-scale exposures. Furthermore, the peak characteristics differ whether they are driven by single input or multiple inputs. Thus, the application scenarios in stream mesocosms could be adapted in terms of multiple input scenarios and resulting effects of potential concentration addition.

The degree of longitudinal dispersion and transient storage may be altered by the targeted usage of aquatic macrophytes or alternative structures within the water column. Aquatic macrophyte species may be chosen considering their intrinsic morphology (to determine transient storage capacities), the vertical coverage within the water phase (emergent or submergent) (Stang et al. 2014) and the density of the macrophyte planting. Furthermore, macrophyte density and the vertical coverage may be standardized within the framework of ERA by manual macrophyte removal or cutting of macrophyte shoots to prevent a complete vertical coverage of the water column e.g. in the case of *Elodea*. The resulting alterations in the longitudinal dispersion and consequences for chemical sorption and solute transport of three PPPs following macrophyte removal was demonstrated for the present peak-scale exposure scenario (Figure 1) by Stang et al. (2014). The aspects of targeted use of macrophytes to enhance transient storage or dispersive processes, should be considered in a comprehensive manner as aquatic macrophytes as play an important role for habitat structures for macroinvertebrates. The choice of the macrophyte species and alteration of the latter may change abundance or composition of macroinvertebrates and, thus, should be considered with cation within ecotoxicological testing.

Invertebrate communities

Pond mesocosms have been frequently used in tier-3 risk assessment of PPPs. These systems sometimes lack the possibility to investigate effects on some sensitive and/or vulnerable species which typically do not occur in lentic water bodies. In particular, species occurring only in flowing water bodies cannot be investigated in pond mesocosms. To close the possible data gap on these species of interest, stream mesocosms appear as an appropriate tool.

The overall species richness was higher in 2012 compared to that in 2011 and was, thus, in the range of other stream mesocosm studies (Beketov et al. 2008; Liess and Beketov 2011; Ippolito et al. 2012). The enhanced taxon richness in 2012 might be attributed to the fact that higher numbers of taxa were passively introduced along with *B. erecta* and sediment in 2012

compared to the approaches in 2011. The low between-stream CV of taxon richness (S-2012-D1 to D4; Table 4) can be due to the very uniform and randomized introduction procedure of *B. erecta* and, thus, highlights the importance of standardized establishment procedures.

The enhanced presence of sensitive (EFSA 2013), EPT and SPEAR taxa in 2012 compared to 2011 might also be ascribed to the introduction of *B. erecta* which originated from an undisturbed headstream located in the Palatinate Forest. Nevertheless, except for *Sericostoma* sp., *Chaetopteryx* sp., *I. elegans* and occasionally *E. danica*, $SPEAR_{field}$ taxa were present in less than 50% of the samples. The ecotoxicological evaluation of families using the MSS according to Rico and van den Brink (2015) revealed that the number of sensitive families present in the stream mesocosms depends on the chemical class and, thus, on the definition of sensitivity. This approach might enable a more differentiated evaluation of ecotoxicological stream mesocosm data.

In general, targeted introduction of sensitive and/or vulnerable species is an option but it remains a challenge to find appropriate abundances which would fulfill the criteria of statistically evaluable populations for which at least medium effects can be demonstrated (MDD class III or IV). Furthermore, it remains a challenge to fulfill the regulatory requirements of 8 sensitive species using the environmental threshold option (ETO) and/or 8 vulnerable taxa (sensitive uni- or semivoltine taxa with low dispersal ability) in the case of the environmental recovery option (ERO) as suggested by the Aquatic Guidance Document (EFSA 2013). More research concerning the establishment and/or breeding of sensitive and vulnerable taxa is therefore needed to advance stream mesocosms in terms of costs and labor and enhance the ecological relevance and its applicability in ERA.

Generally, the between-stream variability (ACVs) in 2012 was in the range of several other mesocosm studies (Farmer et al. 1995; Wong et al. 2003; Mohr et al. 2012). In a stream mesocosm study, comparable CVs between 26 and 105% were shown for several aquatic invertebrate taxa by Wong et al. (2003). Mohr et al. (2012) reported CVs between 14 and 141% for abundant gammarids, ephemerids, Tanypodinae and total insects found within straw bags in control stream mesocosms. Farmer et al. (1995) reported pond-specific CVs of 31 to 42% for Asellidae, Gammaridae, Chironomidae and Baetidae which were in the range or slightly below the values reported in the present study for 2012. Compared to the values of Farmer et al. (1995), *G. fossarum* showed high CV values in 2012.

The question arises, which aspects might positively enhance the abundance and reduce the variability of desired invertebrates within the stream mesocosms. Overall, the abundance and variability (ACVs) of populations were associated with the successional time of the system

(pre-experimental period). The present study indicates beneficial aspects of a prolonged pre-experimental period considering invertebrate abundance and population development. The observation that 9 out of 44 taxa showed increasing abundance and in several cases sufficient MDDs only at the end of the season in 2012 is in accordance with the guidance documents suggesting establishment periods of several months or longer for structurally complex systems (Giddings et al. 2002, EFSA 2013). Especially, the implementation of taxa with uni- or semivoltine life cycles would benefit from a pre-experimental period sufficient to support natural reproduction of already introduced taxa or a repeated introduction procedure. The reduction of the pre-experimental period by introducing population at natural densities (EFSA 2013) might be associated with the above-mentioned problems of the need of introducing approx. 10^5 individuals per taxon to reach evaluable densities at the population level. The question arises how stream mesocosm approaches can be designed to deal with the trade-off between the beneficial effect of a prolonged pre-experimental period and the issues in terms of effort and time related with a total experimental duration of more than one year (pre-experimental and experimental period). Furthermore, longer pre-experimental time periods may lead to increasing variability between the replicates and, thus, to decreasing MDD classes. Here, pragmatic approaches are needed which are accepted by scientists and regulators.

Invertebrate abundance and variability were also explainable by the habitat structure. For instance, the setups S-2011-B/C with high macrophyte densities and complex structures hosted the highest abundances of epiphytic Ephemeroptera species (Figure 4). The higher ACVs and lower abundances of Baetidae and *I. elegans* in S-2011-A might be attributed to the low and rather heterogeneously distributed macrophyte density or the short pre-experimental period of S-2011-A and, thus, the reduced successional time allocated to macrophyte development. The importance of macrophyte structures is supported by findings of Lalonde and Downing (1991) which indicate that high total epiphyton biomass is associated with complex (whorled) macrophyte structures such as those of *Elodea* sp. or *Myriophyllum* sp. Therefore, a high macrophyte density with complex structures might be beneficial for scrapers in general and, thus, for potential sensitive and/or vulnerable taxa. Furthermore, the intrinsic complexity of macrophytes was visually observed to be valuable for net-spinning caddisfly species (such as *P. flavomaculatus*) if alternative structures such as rocky substrate (Georgian and Thorp 1992) are not present. Moreover, aquatic macrophytes provide structures for oviposition of merolimnic taxa. For instance, the distribution and abundance of emergent aquatic macrophytes was shown to be a determining factor for habitat

selection and the location for the oviposition of damselflies (Guillermo-Ferreira and Del-Claro 2011). Favoring these taxa, the stream mesocosms replicates should be provided with equal amounts of emergent macrophytes or comparable structures to provide the same possibilities of oviposition for dragonflies.

Overall, the presence of aquatic macrophytes revealed an integrative aspect in stream mesocosm testing between the exposure-determining influence and the beneficial aspects on the establishment of invertebrates. Within the scope of the hour-scale scenario, aquatic macrophytes enhanced on the one hand the realism of PPP exposures in stream mesocosms by favoring location-dependent changes in hydraulic retention times and maximum peak concentration reflecting realistic lotic conditions and on the other hand the ecological studies in 2011 and 2012 demonstrated beneficial influence of macrophytes for macroinvertebrate establishment. To include these comprehensive findings in future ERA, integration of such location-dependent aspects within the scope of hour-scale exposures, invertebrates should be sampled at least at the inlet and outlet of the streams and, where applicable, at intermediate sampling points. Furthermore, submerged aquatic macrophytes should be introduced to stream mesocosms in a comprehensive manner to provide beneficial habitat structures for potentially sensitive and/or vulnerable species, prevent potentially adverse effects of enhanced pH values which might result out of too high plant densities (Veeningen, 1982) and thereby maintain plant densities capable to enable macrophyte-related storage and dispersive processes. Thus, more research is needed to advance the stream mesocosm design in general and in order to enhance the amount of potentially sensitive and vulnerable species.

Considering the MDD classes (Table 5), small to medium ecotoxicological adverse effects (MDD class III or IV) on population level, following a manipulation of the stream mesocosms with aquatic contaminants, are generally detectable for 12 out of 15 taxa at least for two sampling dates. Especially high MDD classes (III and IV) of univoltine, rheophilic and potentially sensitive and vulnerable taxa such as *Chaetopteryx* sp. and *Sericostoma* sp. demonstrate possibilities of the present stream mesocosm approach. The recommended amount of 8 potentially sensitive species (insects) with high MDD classes (MDD_{abu} < 100%) was reached within this study as required using the definition of sensitivity to insecticides of EFSA (2013) and the environmental threshold option (EFSA 2013) (Tables 4 and 5). The environmental recovery option with the recommendation of 8 vulnerable species with acceptable MDD classes < 100% (EFSA 2013) was, however, not met within this study. Nevertheless, at least the temporary presence of ≥ 10 vulnerable and long-lived taxa, according to the definition of EFSA (2013), was demonstrated for each channel in 2012.

Overall, the evaluation of MDD values highlights the need of reducing the between-stream variability of invertebrates which is one of the driving factors to detect even small ecological effects with statistical methods. Concerning this matter, suggestions to reduce the system-inherent variability and general sampling techniques have been made by Brock et al. (2014).

Concluding remarks

Considering the representation of peak- and hour-scale exposure scenarios with the aim to reflect realistic conditions of flowing surface waters, the present study highlighted the hour-scale scenarios as applicable in ERA using stream mesocosms. In future ecotoxicological applications within the scope of ERA, the location-dependent invertebrate sampling within the scope of hour-scale exposures enables a specific evaluation of potentially adverse effects of PPP on macroinvertebrates to account for changing maximum concentrations and prolonged residence times with increasing flow length. The influence of the aquatic macrophytes on the underlying hydrological processes and chemical sorption was highlighted, but more research is necessary to optimize the stream mesocosm setup for the establishment of sensitive and vulnerable species. Nevertheless, the present study demonstrated the successful establishment of several potentially sensitive invertebrate populations (insects) with MDD classes III and IV for at least two sampling days. With regards to cost and labor and the resulting applicability in ERA, stream mesocosms guidance should be increased to facilitate and standardize stream mesocosm setup including the use of macrophytes and the establishment procedures of sensitive and vulnerable species to enhance the comparability of stream mesocosms to lotic surface waters.

The day-scale exposure scenario using recirculating conditions and a post-exposure flushing period with unpolluted water as presented in this study advances the realism of long lasting exposure events in stream mesocosms in a replicated design with up to 16 stream replicates. The day-scale scenario thus promotes an application in ERA for herbicides (e.g. acetolactate synthase inhibiting herbicides) and insecticides with a high solubility. Thus, this day-scale approach to evaluate exposure in lotic systems might complement the ERA of lentic systems using pond-like mesocosm.

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Supporting information

Table S1: Taxonomic overview of the aquatic invertebrate larvae in 2011 and 2012

Taxa		S-2011			S-2012			
		A	B	C	D1	D2	D3	D4
Amphipoda	*Gammarus fossarum*	+++	++	-	++	++	++	+++
Arachnida	Hydracarina	-	-	-	-	-	-	r
Clitellata	*Erpobdella octoculata*	-	-	-	++	++	++	++
	Glossiphonia complanata	-	-	-	+	+	+	r
	Glossiphonia nebulosa	-	-	-	r	+	+	-
	Helobdella stagnalis	r	+	+	++	++	+	++
	Hirundinea	r	-	+	r	-	r	-
	Lumbricidae	-	-	-	r	r	-	-
	Naididae	-	-	-	++	++	++	++
	Oligochaeta	++	+	+++	-	-	-	-
Coleoptera	Elmidae	-	-	-	-	-	r	-
Diptera	Ceratopogonidae	-	-	-	+	r	+	+
	Chironominae	++	++	++	+++	++	++	++
	Culicidae	-	-	-	r	-	-	-
	Empididae	-	-	-	-	-	r	-
	Orthocladiinae	r	r	r	+	++	++	++
	Simuliidae	r	-	r	r	+	+	+
	Tanypodinae	++	++	++	+++	+++	++	+++
	Tanytarsini	-	-	-	++	+++	++	++
	Tipula	-	-	-	-	r	-	-
Ephemeroptera	*Baetis* sp.*	-	-	-	+	r	+	+
	*Centroptilum luteolum**	-	-	-	r	-	+	+
	Cloeon dipterum	-	-	-	++	++	++	+++
	Cloeon simile	+++	+++	+++	-	+	+	+
	Ecdyonurus sp.*	r	-	-	-	-	-	-
	*Ephemera danica***	-	-	-	+	++	+	+
	*Serratella ignita***	-	-	-	+	+	+	+
	Leptophlebiidae**	-	-	-	-	r	r	r
Gastropoda	*Acroloxus lacustris*	r	-	-	-	-	-	-
	Bathyomphalus contortus	r	-	+	-	-	-	+
	Bithynia tentaculata	-	-	+	-	-	-	-
	Gyraulus albus	-	-	-	+	+	+	+
	Physella acuta	++	+	+	++	++	++	++
	Planorbarius corneus	r	r	r	-	-	-	-
	Radix balthica	-	-	-	+	++	+	++
	Stagnicola corvus	-	r	r	-	-	-	-
	Valvata piscinalis	+	-	-	-	-	-	-
Hemiptera	*Hesperocorixa* sp.	+	-	-	-	-	-	-
	Notonecta maculata	+	+	-	-	-	-	-
Isopoda	*Asellus aquaticus*	-	+	+++	+	+	+	++
Megaloptera	*Sialis* sp.**	r	-	r	-	-	-	-
Odonata	*Aeshna* sp.	-	-	-	r	-	-	r
	Calopteryx virgo	-	-	-	+	r	r	+
	Cordulegaster sp.	-	-	-	-	-	r	r
	*Ischnura elegans**	++	+++	+++	+	r	+	+
	Libellula quadrimaculata	-	r	-	-	-	-	+
	Sympetrum striolatum	r	+	r	-	-	-	-
Plecoptera	Leuctridae**	-	-	-	+	+	+	r
	Nemurella**	-	-	-	-	-	-	r
	Plecoptera**	-	-	-	-	r	r	-
Trichoptera	*Chaetopteryx* sp.**	-	-	-	+++	++	+++	++
	*Halesus radiatus***	-	-	-	-	r	r	-
	Limnephilidae**	+	r	-	-	-	-	-
	*Oecetis lacustris***	++	++	++	r	+	r	r
	*Polycentropus flavomaculatus***	-	-	-	+	+	+	+
	Sericostoma sp.**	r	r	r	++	++	++	++
	Trichoptera**	-	-	-	+	+	+	+
Turbellaria	*Polycelis* spp.	-	-	r	++	++	++	++

Individuals of the respective taxa are present in none (-), 7 (2011) or 6 (2012) % (r), 7/6 to 50% (+), 50 to 90% (++) and > 90% (+++) of the samples. $SPEAR_{field}$ taxa are marked with asterisks (*) and $SPEAR_{mesocosm}$ taxa with a second asterisk (**).

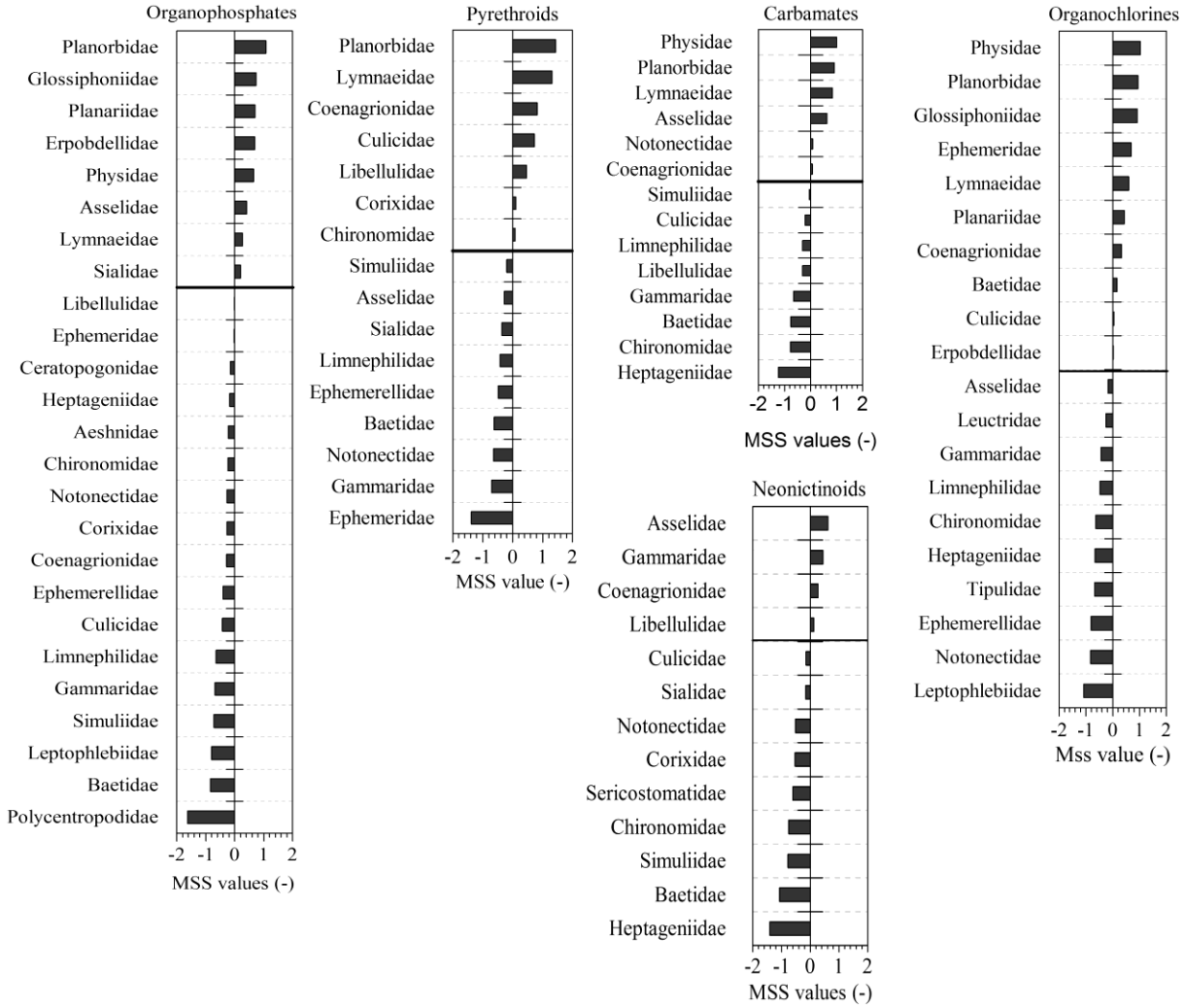

Figure S14: Mode-specific sensitivity (MSS) ranking of all invertebrate families in 2011 and 2012 for the different chemical classes as presented in the SI of Rico and Van den Brink (2015). Invertebrate families which are not listed in Rico and Van den Brink (2015) but are present in 2011 and 2012 could not be presented here. Low MSS values correspond to higher relative sensitivity.

Table S2: Presentation of the test results for the MDD calculation using the control data of 2012 with a simulated reduction of 30, 50 and 70% per taxon: Range of control data (Co_min and Co_max) and the geometric means of the abundance data of 2012 (1/10 m^2). The MDD values are presented as $\%MDD_{ln}$ values for ln-transformed data and $\%MDD_{abu}$ for back transformed MDD values according to Brock et al. (2014) (one-sided Williams test, alpha = 0.05). MDD values replaced by a minus did not fulfill the criteria for robust data.

C. dipterum	Ln-transformed values (range of controls and geometric means)							Abundance 1/10 m^2 (range of controls and geometric means)							MDD
Date	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{ln}$	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{abu}$	class
30.05.2012	0.0	2.0	1.0	0.9	0.7	0.5	-	0.0	1.0	0.5	0.4	0.3	0.2	-	-
13.06.2012	0.0	4.9	3.1	2.8	2.6	2.2	-79.8	0.0	20.0	8.8	6.1	4.4	2.6	-96	I
27.06.2012	5.8	6.3	6.0	5.7	5.4	4.8	-5.9	48.0	84.0	65.0	45.5	32.5	19.5	-30	IV
11.07.2012	4.4	5.0	4.8	4.4	4.1	3.6	-7.1	12.0	23.0	18.0	12.6	9.0	5.4	-29	IV
26.07.2012	4.0	4.8	4.5	4.1	3.8	3.3	-10.0	8.0	19.0	13.8	9.6	6.9	4.1	-37	IV
09.08.2012	4.4	5.5	5.1	4.8	4.4	3.9	-12.1	12.0	35.0	27.0	18.9	13.5	8.1	-46	IV
23.08.2012	3.5	5.1	4.4	4.1	3.8	3.3	-19.0	5.0	24.0	14.5	10.2	7.3	4.4	-58	III

Chaetopteryx sp.	Ln-transformed values (range of controls and geometric means)							Abundance 1/10 m^2 (range of controls and geometric means)							MDD
Date	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{ln}$	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{abu}$	class
30.05.2012	3.3	4.7	4.0	3.6	3.3	2.8	-17.5	4.0	16.0	9.0	6.3	4.5	2.7	-51	III
13.06.2012	4.7	5.1	5.0	4.6	4.3	3.8	-4.8	16.0	24.0	21.5	15.1	10.8	6.5	-21	IV
27.06.2012	4.0	4.6	4.4	4.0	3.7	3.2	-7.6	8.0	15.0	12.0	8.4	6.0	3.6	-29	IV
11.07.2012	4.2	4.7	4.4	4.1	3.7	3.2	-5.7	10.0	16.0	12.5	8.8	6.3	3.8	-23	IV
26.07.2012	2.7	3.5	3.3	3.0	2.7	2.2	-15.9	2.0	5.0	4.3	3.0	2.1	1.3	-43	IV
09.08.2012	3.0	3.5	3.3	2.9	2.6	2.2	-10.5	3.0	5.0	4.0	2.8	2.0	1.2	-30	IV
23.08.2012	0.0	3.5	2.2	1.9	1.7	1.4	-	0.0	5.0	2.3	1.6	1.1	0.7	-	-

Chironominae	Ln-transformed values (range of controls and geometric means)							Abundance 1/10 m^2 (range of controls and geometric means)							MDD
Date	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{ln}$	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{abu}$	class
30.05.2012	0.0	3.3	1.8	1.6	1.4	1.1	-	0.0	4.0	1.5	1.1	0.8	0.5	-	-
13.06.2012	4.1	5.2	4.6	4.3	4.0	3.5	-15.3	9.0	28.0	17.5	12.3	8.8	5.3	-51	III
27.06.2012	2.7	4.0	3.4	3.1	2.7	2.3	-23.1	2.0	8.0	5.0	3.5	2.5	1.5	-56	III
11.07.2012	2.7	4.5	3.9	3.5	3.2	2.7	-26.2	2.0	14.0	8.5	6.0	4.3	2.6	-65	III
26.07.2012	3.9	5.1	4.5	4.1	3.8	3.3	-16.8	7.0	25.0	15.3	10.7	7.6	4.6	-54	III
09.08.2012	3.3	5.0	3.8	3.5	3.2	2.7	-26.3	4.0	23.0	9.3	6.5	4.6	2.8	-65	III
23.08.2012	3.7	4.9	4.3	3.9	3.6	3.1	-15.1	6.0	21.0	12.0	8.4	6.0	3.6	-48	IV

E. octoculata	Ln-transformed values (range of controls and geometric means)							Abundance 1/10 m^2 (range of controls and geometric means)							MDD
Date	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{ln}$	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{abu}$	class
30.05.2012	0.0	2.0	0.5	0.4	0.4	0.3	-	0.0	1.0	0.3	0.2	0.1	0.1	-	-
13.06.2012	0.0	2.0	1.5	1.3	1.1	0.8	-	0.0	1.0	0.8	0.5	0.4	0.2	-	-
27.06.2012	0.0	3.0	2.1	1.8	1.6	1.3	-	0.0	3.0	1.8	1.2	0.9	0.5	-	-
11.07.2012	0.0	3.3	2.1	1.9	1.6	1.3	-	0.0	4.0	2.0	1.4	1.0	0.6	-	-
26.07.2012	3.3	3.7	3.6	3.2	2.9	2.4	-6.4	4.0	6.0	5.3	3.7	2.6	1.6	-21	IV
09.08.2012	3.9	4.9	4.5	4.1	3.8	3.3	-12.4	7.0	20.0	14.3	10.0	7.1	4.3	-43	IV
23.08.2012	4.2	4.8	4.6	4.3	3.9	3.4	-7.8	10.0	19.0	15.5	10.9	7.8	4.7	-30	IV

G. fossarum	Ln-transformed values (range of controls and geometric means)							Abundance 1/10 m^2 (range of controls and geometric means)							MDD
Date	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{ln}$	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{abu}$	class
30.05.2012	0.0	2.7	1.7	1.5	1.2	1.0	-	0.0	2.0	1.0	0.7	0.5	0.3	-	-
13.06.2012	3.3	5.9	4.4	4.0	3.7	3.2	-31.1	4.0	54.0	19.0	13.3	9.5	5.7	-75	II
27.06.2012	2.0	5.3	3.8	3.5	3.2	2.7	-44.7	1.0	31.0	12.3	8.6	6.1	3.7	-84	II
11.07.2012	3.5	7.3	5.9	5.5	5.2	4.7	-34.6	5.0	218.0	99.5	69.7	49.8	29.9	-87	II
26.07.2012	4.5	7.2	6.5	6.1	5.8	5.3	-26.1	13.0	207.0	150.0	105.0	75.0	45.0	-82	II
09.08.2012	3.3	6.3	5.1	4.7	4.4	3.9	-31.2	4.0	84.0	38.0	26.6	19.0	11.4	-80	II
23.08.2012	2.0	4.8	3.7	3.4	3.0	2.6	-42.3	1.0	19.0	9.5	6.7	4.8	2.9	-81	II

H. stagnalis	Ln-transformed values (range of controls and geometric means)							Abundance 1/10 m^2 (range of controls and geometric means)							MDD
Date	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{ln}$	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{abu}$	class
30.05.2012	0.0	2.0	0.5	0.4	0.4	0.3	-	0.0	1.0	0.3	0.2	0.1	0.1	-	-
13.06.2012	0.0	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0	0.0	0.0	0.0	0.0	-	-
27.06.2012	2.0	3.0	2.4	2.1	1.8	1.4	-	1.0	3.0	1.8	1.2	0.9	0.5	-	-
11.07.2012	0.0	3.3	2.0	1.8	1.5	1.2	-	0.0	4.0	1.8	1.2	0.9	0.5	-	-
26.07.2012	3.3	5.4	4.5	4.2	3.9	3.4	-27.8	4.0	34.0	19.5	13.7	9.8	5.9	-72	II
09.08.2012	4.5	6.6	5.2	4.8	4.5	4.0	-24.3	13.0	112.0	40.3	28.2	20.1	12.1	-72	II
23.08.2012	4.0	5.0	4.5	4.1	3.8	3.3	-11.5	8.0	22.0	13.8	9.6	6.9	4.1	-41	IV

Naididae	Ln-transformed values (range of controls and geometric means)							Abundance 1/10 m^2 (range of controls and geometric means)							MDD
Date	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{ln}$	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{abu}$	class
30.05.2012	2.0	3.5	2.7	2.4	2.1	1.7	-	1.0	5.0	2.5	1.8	1.3	0.8	-	-
13.06.2012	0.0	4.0	1.7	1.5	1.3	1.1	-	0.0	8.0	2.5	1.8	1.3	0.8	-	-
27.06.2012	2.7	4.0	3.4	3.0	2.7	2.3	-20.0	2.0	8.0	4.8	3.3	2.4	1.4	-51	III
11.07.2012	2.0	4.5	3.3	3.0	2.7	2.3	-	1.0	13.0	6.5	4.6	3.3	2.0	-	-
26.07.2012	3.5	5.2	4.2	3.9	3.6	3.1	-22.8	5.0	28.0	13.0	9.1	6.5	3.9	-63	III
09.08.2012	4.2	5.5	5.0	4.6	4.3	3.8	-14.9	10.0	37.0	24.8	17.3	12.4	7.4	-53	III
23.08.2012	4.7	7.4	6.2	5.9	5.5	5.0	-23.0	16.0	251.0	110.5	77.4	55.3	33.2	-76	II

Orthocladiinae	Ln-transformed values (range of controls and geometric means)							Abundance 1/10 m^2 (range of controls and geometric means)							MDD
Date	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{ln}$	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{abu}$	class
30.05.2012	3.7	5.3	4.7	4.4	4.0	3.5	-18.5	6.0	31.0	19.5	13.7	9.8	5.9	-59	III
13.06.2012	0.0	2.7	1.2	1.0	0.9	0.7	-	0.0	2.0	0.8	0.5	0.4	0.2	-	-
27.06.2012	2.7	4.7	3.7	3.4	3.0	2.6	-32.9	2.0	16.0	8.3	5.8	4.1	2.5	-72	II
11.07.2012	0.0	6.0	3.5	3.2	3.0	2.6	-82.7	0.0	59.0	19.3	13.5	9.6	5.8	-97	I
26.07.2012	2.0	4.3	2.9	2.5	2.2	1.8	-	1.0	11.0	4.0	2.8	2.0	1.2	-	-
09.08.2012	0.0	2.0	1.0	0.9	0.7	0.5	-	0.0	1.0	0.5	0.4	0.3	0.2	-	-
23.08.2012	0.0	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0	0.0	0.0	0.0	0.0	-	-

Physella acuta	Ln-transformed values (range of controls and geometric means)							Abundance 1/10 m² (range of controls and geometric means)							MDD
Date	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{ln}$	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{abu}$	class
30.05.2012	0.0	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0	0.0	0.0	0.0	0.0	-	-
13.06.2012	0.0	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0	0.0	0.0	0.0	0.0	-	-
27.06.2012	0.0	5.0	2.7	2.5	2.3	1.9	-	0.0	46.0	15.5	10.9	7.8	4.7	-	-
11.07.2012	4.5	7.2	5.7	5.4	5.0	4.5	-26.6	28.0	386.0	151.0	105.7	75.5	45.3	-78	II
26.07.2012	4.9	6.7	6.0	5.7	5.3	4.8	-16.6	40.0	249.0	148.5	104.0	74.3	44.6	-63	III
09.08.2012	5.9	6.6	6.4	6.0	5.7	5.2	-5.8	113.0	214.0	178.0	124.6	89.0	53.4	-31	IV
23.08.2012	4.8	6.9	6.0	5.7	5.3	4.8	-18.8	37.0	284.0	160.5	112.4	80.3	48.2	-68	III

Polycelis spp.	Ln-transformed values (range of controls and geometric means)							Abundance 1/10 m² (range of controls and geometric means)							MDD
Date	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{ln}$	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{abu}$	class
30.05.2012	0.0	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0	0.0	0.0	0.0	0.0	-	-
13.06.2012	0.0	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0	0.0	0.0	0.0	0.0	-	-
27.06.2012	2.0	4.6	3.4	3.0	2.7	2.3	-37.9	1.0	15.0	6.3	4.4	3.1	1.9	-75	II
11.07.2012	3.5	4.5	4.0	3.6	3.3	2.8	-16.3	5.0	14.0	8.8	6.1	4.4	2.6	-49	IV
26.07.2012	0.0	4.3	2.6	2.4	2.1	1.8	-	0.0	11.0	4.5	3.2	2.3	1.4	-	-
09.08.2012	4.1	5.6	4.7	4.3	4.0	3.5	-18.2	9.0	42.0	19.3	13.5	9.6	5.8	-58	III
23.08.2012	5.1	6.8	6.0	5.6	5.3	4.8	-16.1	24.0	131.0	72.0	50.4	36.0	21.6	-62	III

Radix balthica	Ln-transformed values (range of controls and geometric means)							Abundance 1/10 m² (range of controls and geometric means)							MDD
Date	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{ln}$	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{abu}$	class
30.05.2012	0.0	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0	0.0	0.0	0.0	0.0	-	-
13.06.2012	0.0	3.0	2.0	1.8	1.6	1.2	-	0.0	3.0	1.8	1.2	0.9	0.5	-	-
27.06.2012	0.0	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0	0.0	0.0	0.0	0.0	-	-
11.07.2012	0.0	2.0	0.5	0.4	0.4	0.3	-	0.0	1.0	0.3	0.2	0.1	0.1	-	-
26.07.2012	0.0	4.5	3.2	2.9	2.7	2.3	-74.9	0.0	14.0	8.0	5.6	4.0	2.4	-95	I
09.08.2012	4.8	6.4	5.7	5.4	5.0	4.5	-15.1	19.0	86.0	53.0	37.1	26.5	15.9	-58	III
23.08.2012	5.6	6.7	6.2	5.8	5.5	5.0	-11.5	42.0	122.0	82.3	57.6	41.1	24.7	-51	III

S. ignita	Ln-transformed values (range of controls and geometric means)							Abundance 1/10 m² (range of controls and geometric means)							MDD
Date	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{ln}$	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{abu}$	class
30.05.2012	2.2	3.0	2.6	2.3	2.0	1.6	-15.8	7.0	17.0	12.3	8.6	6.1	3.7	-37	IV
13.06.2012	2.0	3.0	2.7	2.4	2.1	1.7	-20.2	6.0	18.0	13.8	9.6	6.9	4.1	-45	IV
27.06.2012	0.7	1.5	1.0	0.8	0.7	0.4	-	1.0	3.0	1.8	1.2	0.9	0.5	-	-
11.07.2012	0.0	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0	0.0	0.0	0.0	0.0	-	-
26.07.2012	0.0	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0	0.0	0.0	0.0	0.0	-	-
09.08.2012	0.0	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0	0.0	0.0	0.0	0.0	-	-
23.08.2012	0.0	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0	0.0	0.0	0.0	0.0	-	-

Sericostoma sp.	Ln-transformed values (range of controls and geometric means)							Abundance 1/10 m² (range of controls and geometric means)							MDD
Date	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{ln}$	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{abu}$	class
30.05.2012	0.0	2.7	1.2	1.0	0.9	0.7	-	0.0	2.0	0.8	0.5	0.4	0.2	-	-
13.06.2012	0.0	2.7	1.8	1.6	1.4	1.1	-	0.0	2.0	1.3	0.9	0.6	0.4	-	-
27.06.2012	2.7	3.9	3.2	2.9	2.6	2.1	-19.1	2.0	7.0	4.0	2.8	2.0	1.2	-48	IV
11.07.2012	3.3	4.5	4.0	3.6	3.3	2.8	-17.8	4.0	13.0	9.0	6.3	4.5	2.7	-52	III
26.07.2012	3.9	4.5	4.1	3.8	3.4	2.9	-7.9	7.0	13.0	9.3	6.5	4.6	2.8	-28	IV
09.08.2012	2.0	3.3	2.7	2.3	2.1	1.6	-	1.0	4.0	2.3	1.6	1.1	0.7	-	-
23.08.2012	2.0	3.9	3.2	2.8	2.5	2.1	-31.5	1.0	7.0	4.3	3.0	2.1	1.3	-66	III

Tanypodinae	Ln-transformed values (range of controls and geometric means)							Abundance 1/10 m² (range of controls and geometric means)							MDD
Date	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{ln}$	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{abu}$	class
30.05.2012	2.0	3.9	3.2	2.9	2.6	2.1	-30.7	1.0	7.0	4.5	3.2	2.3	1.4	-66	III
13.06.2012	3.9	5.1	4.2	3.9	3.5	3.0	-17.5	7.0	24.0	11.5	8.1	5.8	3.5	-53	III
27.06.2012	4.5	5.9	5.2	4.9	4.5	4.0	-13.0	14.0	53.0	30.8	21.5	15.4	9.2	-50	III
11.07.2012	4.9	6.4	5.5	5.1	4.8	4.3	-14.5	21.0	91.0	43.3	30.3	21.6	13.0	-55	III
26.07.2012	5.2	6.0	5.5	5.2	4.8	4.3	-8.3	27.0	58.0	39.0	27.3	19.5	11.7	-37	IV
09.08.2012	4.9	5.7	5.2	4.9	4.5	4.0	-8.0	20.0	44.0	28.8	20.1	14.4	8.6	-34	IV
23.08.2012	3.9	5.3	4.8	4.5	4.2	3.7	-17.3	7.0	30.0	21.8	15.2	10.9	6.5	-57	III

Tanytarsini	Ln-transformed values (range of controls and geometric means)							Abundance 1/10 m² (range of controls and geometric means)							MDD
Date	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{ln}$	Co_min	Co_max	Control	30%	50%	70%	$\%MDD_{abu}$	class
30.05.2012	0.0	3.5	2.1	1.8	1.6	1.3	-	0.0	5.0	2.0	1.4	1.0	0.6	-	-
13.06.2012	3.7	6.2	5.3	4.9	4.6	4.1	-26.7	6.0	73.0	42.0	29.4	21.0	12.6	-76	II
27.06.2012	4.1	5.4	4.8	4.4	4.1	3.6	-13.8	9.0	33.0	19.3	13.5	9.6	5.8	-49	IV
11.07.2012	5.3	7.7	6.9	6.6	6.2	5.7	-19.6	31.0	338.0	205.5	143.9	102.8	61.7	-74	II
26.07.2012	5.7	6.5	6.1	5.7	5.4	4.9	-6.7	45.0	98.0	66.3	46.4	33.1	19.9	-33	IV
09.08.2012	2.7	4.4	3.5	3.2	2.9	2.4	-25.2	2.0	12.0	6.0	4.2	3.0	1.8	-61	III
23.08.2012	4.6	5.7	5.3	4.9	4.6	4.1	-11.1	15.0	43.0	32.3	22.6	16.1	9.7	-45	IV

Appendix II: **Scientific publication 2**

Response and recovery of the macrophytes *Elodea canadensis* and *Myriophyllum spicatum* following a pulse exposure to the herbicide iofensulfuron-sodium in outdoor stream mesocosms

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ABSTRACT

Recently, stream mesocosms have regained interest for higher tier aquatic macrophyte risk assessment of plant protection products mainly because (i) the highest predicted environmental concentrations for the assessment of effects are frequently derived from stream scenarios and (ii) they enable an effect assessment using stream-typical pulse exposures. Therefore, the present stream mesocosm study used an herbicide pulse exposure and evaluated the responses of *E. canadensis* and *M. spicatum*. Macrophytes were exposed for 24 hours to 1, 3, 10 and 30 µg/L of the herbicide iofensulfuron-sodium with a subsequent recovery period of 42 days. Biological endpoints were growth rates of the main, side and total (TSL) shoot length, the shoot number, the maximum root length and the dry weight. The TSL was identified as the most sensitive endpoint; the growth rate of TSL was inhibited up to 66 and 45% in *M. spicatum* and *E. canadensis*, respectively. The lowest No Observed Effect Concentrations (NOECs) were observed at day 7 and/or 14 after herbicide treatment and were 1 µg/L for *M. spicatum* and 3 µg/L for *E. canadensis.* The No Observed Ecologically Adverse Effect Concentrations (NOEAECs) were 10 and 30 µg/L for *M. spicatum* and *E. canadensis*, respectively. Such or similar mesocosm designs are useful to simulate typical stream exposures and estimate herbicide effects on aquatic macrophytes in stream systems.

INTRODUCTION

Aquatic macrophytes play an essential role as structural and functional features of lotic surface waters [1]. For the aquatic fauna, aquatic macrophytes serve as a food source [2], provide shelter from stream currents and predators and supply habitats for reproduction [3]. Freshwater macrophytes may influence a stream's nutrient cycle and physico-chemical parameters such as oxygen and pH [1,4]. They may also function as ecological engineers, influencing the hydrological conditions by e.g. reducing the flow velocity and retaining sediment particles [2,5,6]. The ecotoxicological value of aquatic macrophytes in lotic ecosystems can also be related to the mitigation of exposure-related adverse effects through sorption and phytoremediation of plant protection products (PPPs) [7–9].

In the field aquatic macrophyte populations and/or communities may be adversely affected by exposure to unintentionally released herbicides present in edge-of-field streams [10,11]. Depending on the entry pathways such as runoff and/or leaching, edge-of-field stream exposure to herbicides was shown to last from hours to days [12,13]. Therefore, these stream exposure patterns should be specifically addressed during risk assessment of pesticides for aquatic macrophytes to protect them from potentially adverse effects of herbicides.

In the EU the higher tier risk assessment for herbicides is carried out in accordance with the recommendations of two guidance documents, the Aquatic Macrophyte Risk Assessment for Pesticides document (AMRAP; [14]) edited by the Society of Environmental Toxicology and Chemistry (SETAC) Europe and the Aquatic Guidance Document (AGD; [15]) provided by the European Food Safety Authority (EFSA). The AGD accepts negligible effects (ecological threshold option) on aquatic macrophytes or acceptable effects on vulnerable species within a recovery period (ecological recovery option, ERO) at population level. However, most of the higher tier risk assessment studies are carried out in lentic systems (e.g., pond mesocosms). As pond mesocosms are usually not designed to simulate runoff induced pulse exposure events, which frequently occur in edge-of-field streams [12,13], typical exposure conditions of stagnant (pond) systems often represent worst-case scenarios for stream ecosystems. Here, we intended to test the effect of a transient (24 h) herbicide exposure as typical for streams.

Up to now, stream mesocosms have mainly been used to study exposure dynamics and fate of pesticides in the presence of aquatic macrophytes [16,17] and/or to evaluate effects of insecticides on aquatic fauna [18–21]. Studies on the response of submerged macrophytes to herbicide pulse exposures in stream mesocosms are however rare. Mohr et al. [10] showed, in

an unreplicated design of indoor streams mesocosms, negative effects on biomass of the macrophytes *Myriophyllum verticillatum* and *Potamogeton natans* exposed to metazachlor. However, the exposure profile established in their study was similar to conditions obtained in pond systems as no subsequent flushing with clean water was provided. More recently, King et al. [22] used stream mesocosms with a triplicated design to evaluate the response of periphyton as well as the macrophyte species *Ceratophyllum demersum* to multiple 4-day pulses of atrazine; short-term effects on the biomass of *C. demersum* were shown. In the study by King et al. [22] the pulsed atrazine exposure with a successive exchange of water resembled typical stream exposure conditions.

To specifically address stream exposure conditions in the present study, a 24-hour pulse exposure with the herbicide iofensulfuron-sodium was established in the stream mesocosms. In particular, the clean-water-flushing period subsequent to the herbicide exposure provided a promising opportunity to simulate realistic and worst case exposures observed in the field [15] as well as durations comparable to FOCUS (FOrum for Co-ordination of pesticide fate models and their Use; [23]) stream scenarios, which are used in the EU to model Predicted Environmental Concentrations (PECs).

The first goal of the present study was to evaluate the effects of a 24-hour pulse exposure to the sulfonylurea herbicide iofensulfuron-sodium on the growth of two potentially sensitive and vulnerable macrophytes *E. canadensis* and *M. spicatum* in a replicated stream mesocosm design. The macrophyte response was assessed using the endpoints of growth of shoots and roots as suggested by the AMRAP document. The second goal of the present study was to discuss the applicability of AMRAP macrophyte endpoints and AGD requirements such as the ERO in a replicated stream mesocosm design.

MATERIALS AND METHODS

Stream mesocosm setup

The present study was conducted at the stream mesocosm facility at the University of Koblenz-Landau, Campus Landau, Germany [24,25]. The study design comprised 16 independent stream mesocosms (S1 – S16; Figure 1) consisting of concrete channels (length = 45 m; width = 0.4 m; average water depth = 0.26 m). The mesocosms were equipped with sieved topsoil (silty loamy sand; height = 0.09 m; Markus Wolf, Kieswerk & Transporte) and two submerged freshwater macrophyte species, the Canadian waterweed (*Elodea canadensis* Michx.) and the Eurasian watermilfoil (*Myriophyllum spicatum* L.). The grain size

distribution of the top soil was 11% clay, 40% silt, and 49% sand; the organic carbon content was 0.31% in dry mass. The plant relevant nutrients phosphorus pentoxide (P_2O_5), potassium oxide (K_2O), and magnesium (Mg) were measured [26] in the soil at concentrations of 9, 4, and 7 mg/100g dry mass, respectively. *E. canadensis* was collected in the river Ammer (Baden-Württemberg, Germany) in a stretch close to the source, upstream of any sewage treatment plant or agriculture and thus assumed to be unpolluted, whereas *M. spicatum* was taken from the in-house plant culture (Campus Landau, Germany). Macrophyte material of both species was planted in the stream mesocosms in a patchy design in autumn 2012 and spring 2013. Both macrophyte species established vertical and horizontal vegetation coverage of up to 100% during the following months. Inside each of the streams, three locations (L1, L2 and L3; 2 m each) were kept free of planted macrophytes and contained pots with macrophytes used for endpoint assessment (Figure 1).

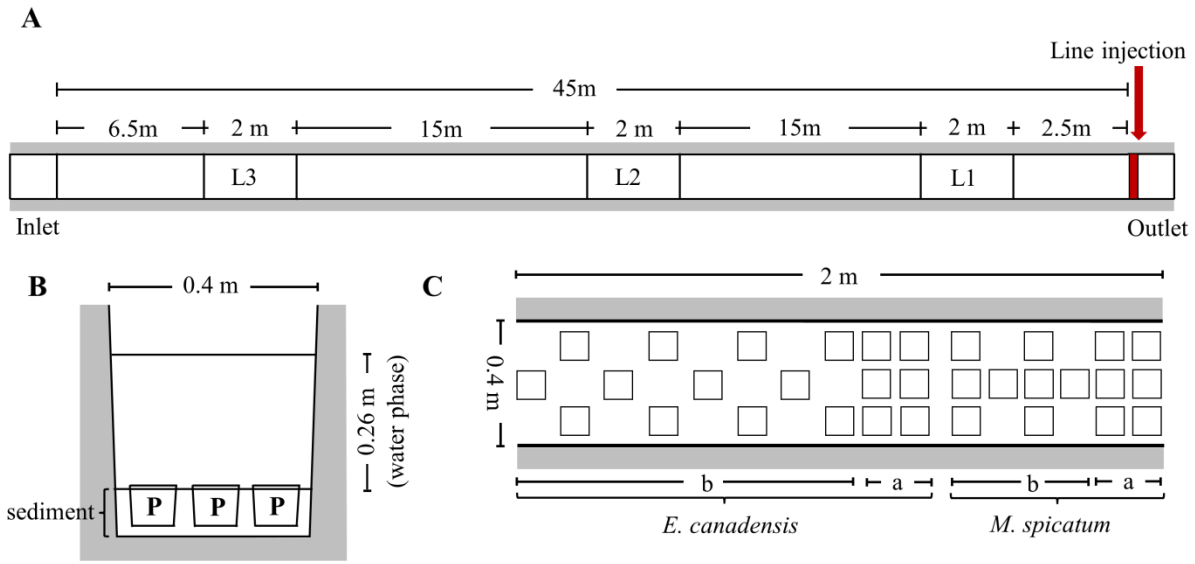


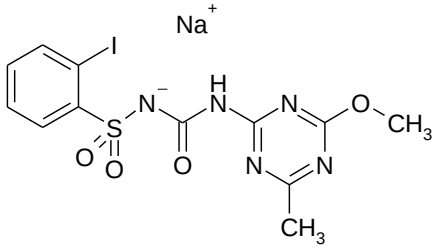
Figure 1: Design of the mesocosms. (A) Schematic overview of the stream mesocosm design including the sampling locations 1-3, (B) side view (ratio 1:13) of the stream channels and the pot (P) arrangement within the water column, and (C) top view of the pot arrangement (ratio 1:23) at each sampling location for *M. spicatum* and *E. canadensis*. Due to identical construction of the channels, only one exemplary scheme of the 16 flow-through stream mesocosms is shown. The grey colored areas indicate the concrete channels.

Herbicide application

The herbicide iofensulfuron-sodium (Table 1) belongs to the class of sulfonylurea herbicides, which act as inhibitors of acetolactate synthase and, therefore, usually induces the inhibition of plant shoot and root growth [27]. Iofensulfuron-sodium (BCS-AA 10579) was provided by Bayer CropScience AG. Prior to the preparation of the aqueous stock solutions, the technical grade (92% purity) iofensulfuron-sodium was dissolved in methanol (LC-MS grade, Merck). Subsequently, defined volumes of the methanolic stock solution were spiked

into 14 L tap water (total methanol volume ≤ 0.02%) in dosing vessels and homogenized thoroughly. The exposure period was comprised of 24 hours (July 20 to 21, 2013), and was referred to as the plateau phase with nominal herbicide concentrations of 1 µg/L, 3 µg/L, 10 µg/L and 30 µg/L (n = 3).

Table 3: Chemical and physical properties of iofensulfuron-sodium

	iofensulfuron-sodium[a]
Structural formula:	
CAS name:	1-(2-iodophenylsulfonyl)-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)urea, sodium salt
Empirical formula:	$C_{12}H_{11}IN_5O_4SNa$
Molecular weight:	471.21 g/mole
Water solubility (20°C):	0.9 g/L
Log K_{ow} (pH 9)	-1.4

[a] Internal data of Bayer CropScience

The range of concentrations was selected on the basis of previous tests. Four streams remained untreated as controls to assess macrophyte growth without any influence of the herbicide. The 24-hour plateau phase using recirculating water flow conditions was followed by a subsequent flushing period with tap water in all 16 streams using flow-through conditions. After the flushing period herbicide low residues were still present in two treatments (Table 2). Recovery of macrophytes was assessed for 42 days after herbicide exposure using recirculating water flow conditions with a weekly flushing period of 1 hour using unchlorinated tap water at flow rates of approximately 5 L/s. The physico-chemical parameters of the tap water are displayed in Table S1 (Supplementary Data). The aqueous stock solutions were injected into the streams using three 24-channel peristaltic pumps (Ismatec IPC 24, IDEX Health & Science GmbH). For each treated stream, six peristaltic tubes were arranged at equal distances to each other in order to enable an injection over the whole stream width and to reach a homogenous distribution of the herbicide in the water column of the stream mesocosms. To enhance the mixing of the herbicide in the water column, stock solution was injected at the outlet (Figure 1) to enable additional mixing during the pumping process. Stable concentration levels during the plateau phase were achieved within approximately 2 hours with a two-phased application scheme established in preliminary tracer experiments [28]. An exemplary injection scheme is displayed in the Supplemental Data (Table S2). While the injected volumes of the stock solutions (mean = 99

mL/min) were held constant during the first injection phase (≈ 1 hour), they were reduced in a stepwise manner during the second injection phase to avoid cumulative dosing effects (≈ 1 hour; Supplemental Data, Table S2). Taking into account slight variations in water volumes of the stream systems (mean volume = 5,050 L; coefficient of variation (CV) = 3.8%) due to differing densities of macrophytes and heights of the sediment layers, three different application schemes (one scheme per peristaltic pump) were established. The injection of herbicide ended after the second injection phase. During the 24-hour plateau phase, at which constant concentrations of the herbicide were maintained, streams were run in recirculating mode with a flow rate of 1 L/s. Following the 24-hour plateau phase, the entire water volume of treatment and control stream mesocosms and the tubing system was completely discarded and replaced within approximately 2 hours at flow rates of up to 15 L/s by herbicide-free tap water.

Table 2: Average concentrations of iofensulfuron-sodium in the water phase (± SD; n=6 per stream) prior to (T0), at 11 (T1), and at 23 (T2) hours within the 24-hour exposure and at 8 (T3) and 12 (T4) hours after the end of the 24-hour exposure

Stream no.	Nominal concentration (µg/L)	Mean measured concentration (µg/L) [a, b]				
		T0	T1	T2	T3	T4
5	0	nd	nd	nd	nd	nd
3	1	nd	1.1 ± 0.12	1.1 ± 0.04	nd	nd
15	1	nd	1.0 ± 0.10	1.2 ± 0.03	nd	nd
8	3	nd	2.8 ± 0.26	2.6 ± 0.08	nd	nd
13	3	nd	3.2 ± 0.37	3.5 ± 0.17	nd	nd
1	10	nd	9.1 ± 0.70	8.9 ± 0.29	0.02[c]	0.02[c]
9	10	nd	9.1 ± 1.14	10.9 ± 0.10	0.02[c]	0.02[c]
7	30	nd	31.9 ± 1.87	33.6 ± 1.47	0.05 ± 0.01	0.09 ± 0.01
16	30	nd	28.5 ± 3.66	35.1 ± 0.52	0.02[c]	0.04 ± 0.01

[a] Limit of quantification = LOQ (0.03 µg/L); limit of detection = LOD (0.01 µg/L)

[b] nd indicates values below the LOD; values below the LOQ but above the LOD are set at LOQ/2 for calculation purposes.

[c] Values mainly consisting of values < LOQ. No standard deviation was calculated.

Stream water quality

The physico-chemical parameters, oxygen saturation (%), pH, conductivity (µS/cm) and temperature (°C) were measured hourly with a data logger (ProfiLux 3.1N) in two streams per treatment and control (at least once per treatment or control in case of technical problems) at approximately 1 m prior to the outlet. Mean physico-chemical parameters (± SD) are displayed for the period following the herbicide injection (July 21 – September 1, 2013) in Table 3. Detailed pH and specific conductivity values of the stream water are displayed in Table S3 and S4 (Supplemental Data). In individual cases, data were not recorded for up to

6% of the total time due to technical problems. Due to device-inherent limits (pH values > 10.5 and oxygen saturation > 150%), data were interpolated occasionally using nonlinear curve fits.

Table 3: Average of physico-chemical parameters (± SD) measured hourly in stream mesocosm water between July 21 and September 1, 2013 in the different herbicide treatments

	Control	1 µg/L	3 µg/L	10 µg/L	30 µg/L
Oxygen saturation (%)	107 ± 25	112 ± 26	116 ± 27	125 ± 30	114 ± 21
pH	10.1 ± 0.8	9.1 ± 0.7	10.0 ± 0.5	9.2 ± 0.6	9.6 ± 0.5
Conductivity (µS cm^{-1})	129 ± 20	129 ± 11	133 ± 14	134 ± 17	127 ± 11
Temperature (°C)	23.0 ± 2.5	22.5 ± 2.7	22.6 ± 2.5	22.0 ± 2.3	22.3 ± 2.5

Water and macrophyte sampling for chemical analyses

Water and macrophyte samples were collected (1) to control the uniform distribution of the herbicide in the water phase during the 24-hours plateau phase and (2) to assess the macrophyte-associated herbicide concentrations. Water and macrophyte samples were taken at three sampling locations (L1, L2, L3), 6, 23 and 40 m below the inlet of each stream (Figure 1). The samplings consisted of one pre-injection sampling (T_0), two samplings at the herbicide concentration plateau (T_1 and T_2 = 11 and 23 hours after the start of injection), and two samplings during the post-plateau phase (T_3 and T_4 = 8 and 12 hours after the end of the plateau phase).

Water samples (sample volume = 20 mL) were collected from the middle of the water column using plastic pipettes and, if possible, directly measured with LC-MS or stored at –18°C until LC-MS analysis. In total 270 water samples (2 replicates x 3 locations x 9 streams x 5 samplings) were taken. To identify the maximum herbicide residues in macrophytes and to show the differences in herbicide sorption over time, we examined herbicide partitioning to macrophytes in streams with the highest herbicide water phase concentration of 30 µg/L (S2, S7 and S16). *E. canadensis* samples were taken from additional pots, which were added to each location, and *M. spicatum* samples were taken directly from the in-stream vegetation due to the dense populations of this species. Approximately 15 g (fresh weight) of *Elodea* and *Myriophyllum* shoots were cut from the middle of the water column, transferred to aluminum cups, covered with plastic lids (Carl Roth GmbH) and stored at –18°C until further sample preparation and analysis.

Sample processing for chemical analysis

The extraction of the herbicide residues from the macrophyte samples was performed using accelerated solvent extraction (ASE 350, Dionex). Prior to the extraction, frozen macrophyte samples were lyophilized (Alpha 1-2 LD Plus vacuum freeze dryer, Martin Christ). Stainless steel extraction cells (34 mL) were filled with 1 g of the lyophilized sample, then completely covered with cindered sea sand (Merck) and extracted according to the settings provided in the Supplemental Data (Table S5). Macrophyte extracts of approximately 40-50 mL were collected in glass vials (60 mL) and subsequently evaporated until completely dry under a gentle nitrogen stream and redissolved in 1 mL of MeOH (LC-MS grade; Merck). To avoid potential matrix effects during chemical analysis, methanolic ASE extracts were diluted (1/100) before analysis with MeOH (LC-MS grade; Merck) and Milli-Q water (Millipore, Bedford) (20/80; v/v).

For method validation, 1 g of lyophilized uncontaminated macrophyte material of each species was spiked with 50 µL of a methanolic stock solution (2 ng/µL) at the top of the extraction cell. Each macrophyte species was run in triplicate and processed following the same ASE method as described above. Recovery rates (± %SD) of the sulfonylurea herbicide were 90 ± 1.9% and 76 ± 6.0% for *E. canadensis* and *M. spicatum*, respectively. Concentrations of the herbicide in the aqueous phase were determined by direct injection of the aqueous samples to the LC-MS without any previous processing.

Chemical analyses

Pesticide concentrations in aqueous and macrophyte samples (ASE extracts) were performed using a U-HPLC-MS system. The LC separation was done by a U-HPLC system equipped with two LC pumps, a C18 column and a PAL autosampler (Combi PAL, CTC Analytics). Separation of the herbicide was carried out with a solvent gradient consisting of solvent A (Milli-Q/MeOH/formic acid; 900/100/0.12; v/v/v) and solvent B (MeOH/Milli-Q/formic acid; 900/100/0.12; v/v/v) with 10 mM NH_4 formate (Sigma-Aldrich; puriss. p.a. grade), added as a buffer to both solvent A and solvent B. Without any previous processing, aqueous samples (injection volume, 20 µL) were directly injected and delivered to the analytical column. To determine the target compound at lower sub-ppb levels, a large-volume injection of 1.0 mL was applied. This volume was transferred by the loading pump (Surveyor LC pump; Thermo Fisher Scientific) onto the in-line preconcentration column (Thermo Hypersil Gold aQ, 20 x 2.1 mm, 12 µm; Thermo Fisher Scientific) for enrichment. After the enrichment step, the loaded compound was eluted from the preconcentration column to the analytical column (Thermo Hypersil Gold C18, 50 x 2.1 mm, 1.9 µm; Thermo Fisher

Scientific) by a back flush. The gradient programs for the LC pumps are presented in the Supplemental Data (Table S6 and S7).

The detection and quantification of the herbicide was executed on the Exactive Orbitrap MS system (Thermo Fischer Scientific) equipped with an electrospray ionization (ESI) probe. The MS detection was performed in the positive ionization mode at a scan range of 100-2000 m/z. The spray voltage was set up at 3.0 kV and the capillary temperature at 450°C. The herbicide was identified using the accurate ion mass $[M+H]^+$ of m/z = 449.9727. For detection and quantification of the herbicide in ASE extracts, matrix-matched standard solutions that were prepared out of uncontaminated blank macrophyte extracts, extracted according to the ASE-method described above, were used for external calibration. The limits of quantification (LOQ) and the limits of detection (LOD) were calculated according to DIN standard 32645.

Macrophyte response to herbicide treatment

The effects of the herbicide on growth and morphological development of the macrophytes were investigated on the basis of morphological endpoints, namely, (1) main shoot length (MSL), (2) side shoot length (SSL), (3) total shoot length (TSL; main shoot plus side shoots), (4) dry weight of total shoots (DW), (5) maximum length of roots (RL) and (6) side shoot number (SSN).

On 6 July 2013 (exposure -15 days), nine days prior to the pre-exposure sampling at day -6, non-branched macrophyte shoot tips (12 cm) of *M. spicatum* and *E. canadensis* were planted into plastic pots (length = 9 cm; width = 9 cm; height = 8 cm; Pöppelmann GmbH & Co. KG) containing OECD sediment [29] without the addition of supplemental nutrients and placed into the streams at three sampling locations L1, L2, L3 (Figure 1). At each of the three sampling locations, pots were arranged using the design as described in Figure 1. Each of the three locations per stream received 12 pots with 2 shoots each of *E. canadensis* and *M. spicatum* (Figure 1). As a backup for unintentionally damaged shoots, 2 and 6 additional pots with 2 shoots each were respectively used for *E. canadensis* and *M. spicatum* at each sampling location (Figure 1).

To assess the morphological endpoints, 2 pots per macrophyte species and location were sampled via destructive sampling on each sampling day; on day 6 prior to injection and on days 1, 3, 7, 14 and 42 post plateau phase resulting in 192 shoots (4 subsamples x 3 locations x 16 streams) per macrophyte species and sampling day. The macrophyte shoots were carefully washed with tap water to remove adherent sediment, algae, and snails from shoots and roots. *E. canadensis* established lateral shoots mainly being located outside of the pots

and showed root development at those side shoots. To avoid breaking off side shoots during the sampling procedure, roots of *Elodea* side shoots were cut off and not considered during the assessment. The longest macrophyte shoots were defined as main shoots, the other shoots were defined as side shoots and the longest root was recorded for the root length. Prior to shoot measurement, the first two centimeters of macrophyte shoots were cut off since those parts were located in the sediment and for this reason not defined as being part of the main shoot. For evaluation of dry weight, macrophyte shoots were put in individual paper bags and dried until they reached a constant weight in an oven at 60°C (for at least 48 hours) and subsequently weighed [30]. As the macrophyte and invertebrate community samplings caused turbidity due to resuspended sediment particles, the streams were flushed with clean water on average once per week. This was done to minimize deposit layers of sediment particles on the macrophytes.

Data analyses

According to recommendations from experts (AMRAP) [14], p. 52 and 53, the effects on the growth of MSL, SSL, TSL, RL, SSN and DW were evaluated using the specific growth rates over the entire test period; from day -6 onwards until the respective sampling day (day -6 to +1, -6 to +3, -6 to +7, -6 to +14, -6 to +42). In order to demonstrate potential recovery effects at the end of the experimental period, the interim growth rates were calculated for the time interval between day 14 and 42 (MSL_{14-42}, SSL_{14-42}, TSL_{14-42} and DW_{14-42}).

Specific growth rates were calculated according to the equation given in the AMRAP document [14]; p. 52 and 53: $\mu_{i,j} = (\ln (N_j) - \ln (N_i))/t$. Thereby, $\mu_{i,j}$ is the specific growth rate from time *i* (day -6) to time *j*, N_i and N_j are the endpoints at time *i* and *j*, respectively, and *t* is the time period from *i* to *j*. The percent inhibition growth rate $\%I_r$ was calculated as follows: $\%I_r = ((\mu_C - \mu_T)/\mu_C) \times 100$. The percent inhibition is determined by the average specific growth rate based of the respective endpoint, μ_C is the mean value of the control, and μ_T is the mean value of the treatments.

The macrophyte sampling was destructive and resulted occasionally in negative growth rates, due to macrophyte shoots being shorter on a later sampling day compared to the preceding sampling. No causal link of the negative growth rates to the herbicide injection was observed. Because of this, for the statistically evaluated endpoints, negative growth rates were replaced by zero values in 4 – 43 and 6 – 56 out of 960 values per species for *E. canadensis* and *M. spicatum*, respectively. As side shoots or roots (*M. spicatum*) were occasionally not

yet present at the first sampling day, growth rates of the respective endpoints were calculated with a modified version of Eq. 1 using $\ln(N_{i,j}+1)$ instead of $\ln(N_{i,j})$.

On each sampling date, statistically significant differences between treated and control streams (alpha-level = 0.05), and the resulting NOECs were determined with linear mixed effect models [31] with a nested design and random effects using the R statistical software [32]. In order to include the subsamples without pseudo replication, the three locations per stream were nested within streams (random factor); concentration was used as fixed factor. A group-wise comparison of each treatment to the control was done with the pairwise multiple comparisons of Dunnett's (two-sided) *post-hoc* test (package *multcomp*). To ensure that the model assumptions were fulfilled, they were visually tested using diagnostic plots of residuals [33].

As the Aquatic Guidance Document requests the calculation of the minimum detectable difference (MDD) for micro-/mesocosm studies [15], the present study used an additional statistical evaluation for this purpose. Thus, the MDDs were determined by the standardized statistical evaluation using ANOVAs and a subsequent Dunnett's post-hoc procedure (two-sided) of ToxRat 2.10.05 (ToxRat Solutions GmbH). For the calculation of MDD values, average values of all 12 macrophyte subsamples per stream were used.

RESULTS

Environmental fate of iofensulfuron-sodium

During the 24-hour plateau phase, the aqueous herbicide concentrations remained stable and deviated from the nominal concentrations by less than 20% (Table 2). Therefore, endpoints (i.e. NOEC) are expressed hereafter as nominal concentrations. In treatments with the two highest herbicide concentrations (i.e.10 µg/L and 30 µg/L), trace amounts of ≤ 0.3% of the maximum measured herbicide concentrations were still present in the water phase after the 24-hour plateau phase (T3 and T4, Table 2).

During the plateau phase, the average concentrations of the herbicide were in the range of 13.2 to 19.3 ng/g dry weight in *E. canadensis* samples and 31.1 to 71.1 ng/g dry weight in *M. spicatum* samples (Table 4). Average herbicide concentrations in macrophytes were 4.2 and 3.3 times higher for *M. spicatum* than for *E. canadensis* at T1 and T2, respectively (Table 4). At T3 after the 24-hour plateau phase, trace residues of the herbicide were also present in treatments with the two highest herbicide concentrations (10 µg/L and 30 µg/L; Table 4) for both species. At T4, herbicide residues were detected solely in *M. spicatum*.

General macrophyte growth patterns

On day -6 prior to the herbicide injection, macrophyte shoots of subsequently treated streams were not statistically significantly different compared to the control streams considering their TSL ($p > 0.4$) and DW ($p > 0.17$). On day -6, the coefficients of variations of the 16 streams were 8.0 and 9.5% for average TSL and DW of *E. canadensis* and 7.1 and 13% for average TSL and DW of *M. spicatum*, respectively. In total, ≥ 94% of the 192 macrophyte shoots of both species were rooted in the sediment on the sampling day -6. For *M. spicatum* only 4 out of 192 shoots had not formed any side shoots. Hence, the starting conditions (Supplemental Data, Table S8) were considered as sufficiently stable. Average values of the endpoints (Table S9), growth rates (Table S10), and major statistical results (Table S11) are displayed in Supplemental Data.

Table 4: Average concentrations of iofensulfuron-sodium in samples of *M. spicatum* and *E. canadensis* (± SD; n=9 per stream) prior to (T0), during (T1 and T2) and following (T3 and T4) the 24-hour plateau phase

Stream no.	Measured concentration (ng/g) [a, b]				
	T0	T1	T2	T3	T4
M. spicatum					
S2	nd	71.1 ± 31.0	66.4 ± 20.4	20.7 ± 8.5	13.3 ± 11.0 [c]
S7	nd	68.8 ±11.0	62.7 ± 24.9	22.9 ± 21.6	20.1 ± 6.2
S16	nd	43.8 ± 7.5	31.1 ± 8.6	12.9 ± 9.9 [c]	7.5 [c]
E. canadensis					
S2	nd	15.9 ± 9.9	13.2 ± 4.6	1.9 [c]	nd
S7	nd	13.8 ± 6.1	15.8 ± 5.2	nd	nd
S16	nd	13.6 ± 9.1	19.3 ± 3.1	1.6 [c]	nd

[a] Limit of quantification = LOQ (8 ng/g); limit of detection = LOD (3 ng/g)

[b] nd indicates all values below the LOD

[c] For calculation of average values, data points being < LOD were set to zero. For average values mainly consisting of values < LOD, no SD was calculated. Values below the LOQ but above the LOD are set at LOQ/2 for calculation purposes.

Considering the whole sampling period, both macrophytes showed a linear or exponential growth in the controls. The evaluation of average total shoots and dry weights of the controls using regressions analysis revealed the highest R^2 values for the linear model ($R^2 \geq 0.972$) for TSL of both macrophytes and for DW of *M. spicatum* ($R^2 = 0.995$). For DW of *E. canadensis*, the R^2 was highest for the exponential model ($R^2 = 0.986$).

On day -6, the composition of TSL was similar between the two macrophyte species, with 75% MSL for *M. spicatum* and 67% MSL for *E. canadensis* (calculation based on control streams; Supplemental Data, Table S12). From day -6 until day 42, the MSL decreased

progressively to 30% MSL for *M. spicatum* and 12% MSL for *E. canadensis* (Supplemental Data, Table S12) while the SSL increased. On day 42 after the plateau phase, total and side shoots of *E. canadensis* were on average 3.7 and 4.7 times larger compared to those of *M. spicatum*, respectively. These differences were less pronounced at the start of the sampling (15 July, 2013) and increased during the 42 day recovery period (Supplemental Data, Table S13). The highest difference between the two species was observed for the endpoint SSN, which was 6.8 times higher in *E. canadensis* compared to *M. spicatum* (Supplemental Data, Table S13). For the sampling interval day -6 to 42, the ranges of the growth rates of MSL, SSL, TSL and DW were 0.018 to 0.055 and 0.026 to 0.081 for *M. spicatum* and *E. canadensis*, respectively (Table S10). The growth rates of SSL were on average 3.1 fold higher than those of MSL for both *M. spicatum* and *E. canadensis*. Generally, controls of *E. canadensis* showed a significantly larger growth rate over the entire test period compared to *M. spicatum* ($p = 0.001$).

Variability of macrophyte endpoints and minimum detectable differences

The CVs of growth rate endpoints (MSL, TSL; SSL, and DW), separately calculated for each treatment and sampling day, were in the range of 1.4 to 37.3% and 1.9 to 46.0% for *E. canadensis* and *M. spicatum*, respectively. The MDD values resulting from the statistical endpoint evaluation based on the growth rates were mainly below 50% (Table 5). Values below 50% are classified as class IV effects which indicates small effects could be determined statistically according to the Aquatic Guidance Document [15].

Table 5: NOEC values of the growth rate-based macrophyte endpoints as well as the respective MDD values (two-sided Dunnett post-hoc)

		NOEC growth rate (µg/L)					MDD (%)				
	Days after the end of the plateau phase	+1	+3	+7	+14	+42	+1	+3	+7	+14	+42
M. spicatum	Main shoot length	30	30	10	1	10	36	39	45	30	24
	Side shoot length	10	3	3	3[a]	30	29	19	14	20	22
	Total shoot length	10	3	1	1	30	31	22	16	23	29
	Dry weight of total shoots	30	30	30	30[a]	10	46	54	34	36	30
	Root length	30	30	30	10	30	57	43	34	32	30
E. canadensis	Main shoot length	30	30	10[a]	10	1[b]	50	47	31	26	14
	Side shoot length	30	10	10	10	30	47	35	18	24	23
	Total shoot length	30	10	3	10	30	38	32	19	27	22
	Dry weight of total shoots	30	30	30	30	30	39	46	35	39	26
	Side shoot number	30	3	3	10	30	61	28	20	26	17

[a] In accordance with recommendations of OECD 211 [42] the NOEC was determined solely including treatments with a concentration-response relationship. Lower concentrations without a clear concentration-response relationship were not included.

[b] For *E. canadensis* the endpoint main shoot length was excluded from NOEAEC calculation due to ecological reasons as discussed in the following.

Macrophyte response to herbicide treatment

After exposure to the herbicide, the inhibition of macrophyte growth was observed to differ between macrophyte species and at different time intervals (Table 5). Considering both macrophyte species, growth inhibition of the TSL was highest for *E. canadensis* at day 7 (45%) and for *M. spicatum* at day 14 (66%). On day 1 after the 24-hour plateau phase, significant effects were determined for SSL and TSL of *M. spicatum* treated with 30 µg/L (Table 5). A concentration-response relationship could be demonstrated for *M. spicatum* shoot endpoints at several sampling dates. For *E. canadensis* shoot endpoints and for the dry weight endpoints of both macrophytes, no concentration-response relationships were observed. However, significant differences between the control and the highest herbicide concentration could be demonstrated for the DW of *M. spicatum* at day 42 (Figure 2). The inhibition of root length growth in *M. spicatum* was found to be lower (29% inhibition; day 14) than the growth inhibition of TSL and SSL.

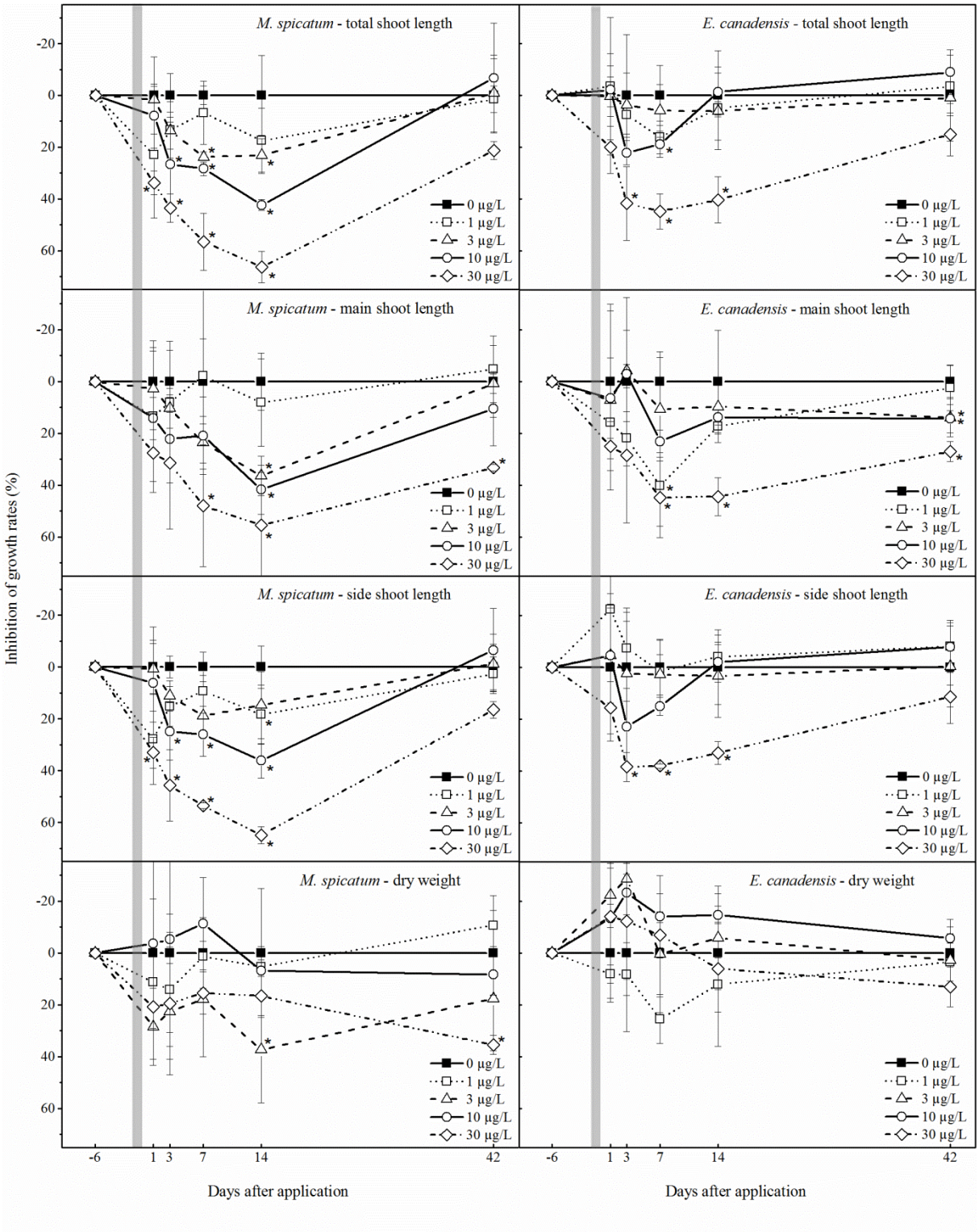


Figure 2: Percent inhibition of growth rates of *M. spicatum* and *E. canadensis*. The growth rates are presented as average percent inhibitions of growth rates (± SD) compared to the control. The asterisks indicate statistically significant differences between growth rates of the control and the treatments. The grey vertical bar marks the 24-h exposure phase to a sulfonylurea herbicide.

On day 42, the previously inhibited TSL and SSL growth of both macrophyte species recovered; both endpoints were not significantly different from controls ($p > 0.12$) at the last sampling date. In contrast, the MSL growth showed no recovery for *M. spicatum* from the

highest herbicide treatment. For *E. canadensis* at the same sampling date, the MSL growth did not recovered for any of the herbicide treatment levels, except for the 1 µg/L herbicide treatment.

The MSL_{14-42} interim growth rates, which were calculated based on the sampling interval day +14 to +42, indicated in contrast to the entire period growth rates, a recovery of the MSL growth rates for both macrophyte species on day 42 (Supplemental Data, Figure S1). In addition, the interim growth of the TSL_{14-42} and the $SSL_{14-42\ at}$ at 10 µg/L and the SSL_{14-42} at 30 µg/L was significantly higher compared to the control for *M. spicatum*, which was not observed for growth rates over the entire period. Nevertheless, it should be stated that recovery occurred mainly during the time interval between sampling days 14 and 42. As no samplings were available within this period, we cannot provide more detailed information on the exact starting point of recovery.

Considering the whole sampling period, the lowest NOECs for *M. spicatum* were determined on days 7 and 14 after the herbicide plateau phase (Table 5). *E. canadensis* showed the lowest NOECs on days 3 and 7 after the plateau phase. The lowest NOEC of 1 µg/L was determined for growth rates of *M. spicatum* MSL on day 14, and TSL on days 7 and 14. The lowest NOECs for *E. canadensis* were 3 µg/L for the endpoints TSL at day 7 and SSN at day 3 and 7. For the TSL and SSL, recovery was demonstrated on day 42 for all treatments. Recovery for DW and MSL of *M. spicatum* was not seen on day 42. Hence, the NOEAEC was determined at 10 µg/L and 30 µg/L for *M. spicatum* and *E. canadensis*, respectively.

DISCUSSION

Macrophyte response to iofensulfuron-sodium

In the present study, a standardized approach was used to simulate an herbicide pulse exposure scenario relevant for edge-of-field streams exposed to PPPs during the growing period of field crops [12,13]. Although pulse series may occur in the field, the present exposure scenario using a single pulse was in line with the exposure duration of a FOCUS stream scenario simulating a single runoff event. All this considered, the 24-hour herbicide exposure reflects realistic exposure scenarios with PPPs as suggested by the EFSA for edge-of-field streams.

The 24-hour herbicide exposure resulted in short-term effects on macrophyte growth that differed over time and between the two macrophyte species tested. During the entire recovery period of 42 days, water pH values recorded during the present study were higher than those

usually observed in European natural lotic surface waters [34]. Nevertheless, pH-associated alteration of iofensulfuron-sodium stability is unlikely since the concentrations remained stable during the 24-hour exposure period (Table 2). Although macrophyte growth might be lower under the observed pH conditions than under normal stream conditions [35], continuous growth of the control macrophytes over the duration of the study indicates that growth was not severely limited in space or by nutrients over the entire experimental period. The apparently higher oxygen saturation (e.g. at 10 µg/L herbicide concentration) could be caused by algae being resistant towards sulfonylurea herbicides [36]. Though macrophyte growth is highly dependent on environmental parameters as nutrient availability and light intensity, and due to this varies among test systems and study approaches, the growth of the TSL observed in the present study was in the range of laboratory studies and pond micro- and mesocosm experiments reviewed by Knauer et al. [37].

Taking into account all these factors, the inhibition of macrophyte growth as observed in the present study can be mainly attributed to the herbicide treatment. Generally, the inhibition of macrophyte growth seemed more pronounced in *M. spicatum* than in *E. canadensis*. This may be partly explained by the presence of herbicide residues in the macrophytes during the 24-hour plateau phase and in the subsequent hours; the amount of the herbicide residues was 4.5-fold higher in *M. spicatum* than in *E. canadensis*. These contrasting amounts of herbicide residues can generally be explained with species-specific differences in (*i*) ad- and/or absorption, (*ii*) distribution within the macrophytes, (*iii*) the metabolic transformation such as the inactivation of the acetolactate synthase inhibitors [27] and/or (*iv*) excretion of the herbicide. In literature the tolerance of plants to sulfonylurea herbicides was positively correlated to the plant's metabolic response time for herbicide breakdown and transformation [27]. However, these processes could not be sufficiently verified with our study design.

For the regulatory assessment of the effects of PPPs on growth and development of macrophytes, several endpoints are suggested in the AMRAP document. Under the present experimental approach, some of these endpoints were shown not to be sensitive for the assessment of adverse effects of the sulfonylurea herbicide iofensulfuron-sodium on *E. canadensis* and *M. spicatum* in stream mesocosms.

Total shoot length – Showing the earliest growth effects only one day after the 24-hour herbicide exposure, and being the most highly inhibited among all endpoints, TSL was considered to be the most sensitive endpoint in the assessment of adverse effects of iofensulfuron-sodium on the growth of *E. canadensis* and *M. spicatum*. Especially for *M.*

spicatum, TSL was sensitive enough to demonstrate inhibition of growth at the highest herbicide treatment within the first 24 hours after herbicide exposure. These early effects may be explained by the species-specific sensitivity of *M. spicatum* to acetolactate synthase inhibitors, such as iofensulfuron-sodium, which may induce a stop of the cell division within hours after exposure due to the rapid mode-of-action [38]. The early growth inhibition of TSL in *M. spicatum* could also be explained by the macrophyte-associated herbicide residues found at low-ppt levels 8 and 12 hours after the herbicide exposure. Furthermore, at the same sampling times of 8 and 12 hours post-exposure, herbicide residues above the LOD were found in the water phase of the 10 µg/L and 30 µg/L treatments, and they might have contributed to the early macrophyte effects in the highest treatment. In general, the trace residues in the post exposure phase might be explained by processes such as desorption of previously adsorbed residues, as well as the presence of transient storage locations [39]. As outlined by Stang et al. [17], both of these processes are typical for stream systems. In the present experimental setup the stream-inherent vegetation, filamentous algae and *M. spicatum*, situated between the sampling sites provided herbicide-retaining structures.

Side and main shoot length – Along with TSL, SSL was found to be an important endpoint to indicate herbicide-induced growth inhibition, as early effects were visible on day 1. According to Cedergreen [40], first effects of sulfonylurea herbicides are likely to occur for endpoints with high growth rates. However, high growth rates might however also accelerate recovery from an adverse impact. This was particularly true for *E. canadensis*, which tended to recover faster than *M. spicatum*. Furthermore, as SSL had a greater contribution to TSL than MSL the ecological relevance of SSL seems to be more pronounced in terms of recovery than that of MSL.

Side shoot number– SSN was shown to be another sensitive endpoint to evaluate herbicide-related adverse effects on macrophyte growth, especially on *E. canadensis*. On day 3, an even lower NOEC was demonstrated for SSN than for both MSL and TSL. This might be explained by the MDD values for this sampling date. On day 3, the MDD value for SSN was 28%, which was low enough to statistically confirm the inhibition of growth of 31.6%. On the contrary, the MDD of TSL was 32% on day 3, and due to this the TSL growth inhibition of 22.2% could not be confirmed statistically. This highlights the need for procedures to lower the variability of growth endpoints to achieve MDD values low enough to differentiate small effects.

In general, *E. canadensis* developed an excessive growth of side shoots over the experimental period, making the assessment of SSL and SSN very labor-intensive, especially

on sampling days 14 and 42. In contrast, *M. spicatum* showed low side shoot numbers and, for this reason, the statistical evaluation of SSN was not possible.

Root-associated endpoints – The endpoint maximum RL was not very sensitive for *M. spicatum* compared to TSL and SSL. Generally, the present study and other studies that used root endpoints of *Myriophyllum* [37] revealed that measuring the length of all roots is extremely time consuming and therefore not practical. Furthermore, the maximum RL of *E. canadensis* could not be used within the present study due to the fact that it was hardly possible to recover the roots from the sediment without breaking off at least parts of them. Furthermore, to remove the side shoots of *E. canadensis* without breaking them, it was frequently necessary to cut off some roots, which were fixed in the sediment between the pots.

Dry weight – The limited change of DW compared to those of length-related endpoints and the absence of a concentration-response relationship showed a limited sensitivity of the DW to the sulfonylurea herbicide in the stream mesocosms. This observation is in line with other studies that reported decreased growth rates of shoot length while no significant effects were shown on the basis of shoot dry weight following exposure to a sulfonylurea herbicide [36,41]. In the present study, coatings on the macrophyte leaf surfaces were observed, which could not be removed prior to weighing the dried macrophytes. Although the composition of this coating cannot be determined within the present study, it might have biased dry weight measurements. These observations are partially in line with Wendt-Rasch et al. [36] indicating that dry weights might have been biased due to an increase of a leave-associated biomass of periphytic algae following exposure to a sulfonylurea herbicide. Furthermore, the macrophyte tissue might have been physiologically altered in terms of increased shoot tissue density [36] or herbicide-induced starch accumulation in the shoots might have increased dry weight [41].

Recovery

Within a period of 42 days, the growth of TSL, as the ecologically most relevant endpoint of macrophyte growth and development, recovered from the herbicide-induced inhibition. The MDD values for both species were lower than 30%, therefore the variability of TSL was low enough to determine even small statistical differences. For *E. canadensis* the growth of MSL did not recover after herbicide exposure even by day 42. However, on day 42 MSL contributed only 30 and 12% to TSL of *M. spicatum* and *E. canadensis*, respectively. This

indicates a low ecological importance of MSL for the assessment of the recovery of macrophyte species with growth patterns comparable to those used in the present study. Due to its rather low ecological importance, MSL was excluded from the calculation of the NOEAEC for *E. canadensis* but not for *M. spicatum*. This resulted in NOEAEC values of 10 µg/L and 30 µg/L for *M. spicatum* and *E. canadensis*, respectively. Recovery of both macrophytes within 42 days might have been supported by the weekly flushing events with clean water, which might have provided nutrients for the compensation of inhibited growth in the treated macrophytes.

The recovery of *E. canadensis* and *M. spicatum* during the 42 days following the 24-hour herbicide exposure demonstrated the general suitability of stream mesocosms to apply the ERO for macrophytes as suggested by the AGD [15]. However, further assessments of the ERO in stream mesocosms using other vulnerable macrophytes and PPPs with different modes of action are needed.

In order to evaluate specific aspects of macrophyte recovery within the last sampling interval (i.e., between day 14 and 42), the interim growth was used as an alternative method for the assessment of macrophyte growth rates. Interestingly, the interim growth of $TSL_{14\text{-}42}$ and/or $SSL_{14\text{-}42}$ in the two highest herbicide treatments was found to be significantly increased compared to the control, suggesting that an overcompensated growth of TSL and SSL after a period of growth inhibition occurred in *M. spicatum* during the last sampling interval. However, it should be mentioned, that the period between day 14 and 42 does not necessarily represent the complete recovery period, as the exact start of recovery is unclear due to the absence of samplings between day 14 and 42. The increased interim growth of TSL and SSL in the last sampling interval can, therefore, only be used as an indicator for recovery of normal growth.

GENERAL RECOMMENDATIONS AND CONCLUDING REMARKS

To our knowledge the present study is the first attempt to assess herbicide-induced growth effects on the two submerged macrophyte species *E. canadensis* and *M. spicatum* in stream mesocosms using replicated designs. Though the response to a sulfonylurea herbicide exposure was assessed with two out of eight macrophyte species, as suggested by the AGD [15], some general recommendations can be summarized for stream mesocosms users and regulatory agencies such as the EFSA.

As typical exposure dynamics of edge-of-field streams can be simulated, the dynamic short-term exposure appears to be a useful supplement to existing pond studies within the

scope of the environmental risk assessment of selected herbicide groups. Furthermore, this study's design enables the simulation of typical FOCUS stream scenarios and therefore enables the simulation of realistic and worst-case conditions as suggested by the AGD [15].

In the present study, TSL and SSL were demonstrated to be the two most relevant endpoints to assess herbicide-induced growth inhibition after a single-pulse exposure of 24 hours and recovery from the herbicide impact after 42 days. However, if multiple applications of PPPs are predicted to occur in the field, an experimental approach consisting of a multiple-pulse exposure profile of a PPP should be taken into account, as consecutive exposure pulses may delay the recovery in macrophytes. In the present study the highest growth inhibition of TSL for *E. canadensis* was demonstrated on day 7 (45% inhibition of TSL growth) and for *M. spicatum* on day 14 (66% inhibition of TSL growth) after the 24-hour herbicide exposure. If the system would have received a second exposure pulse with the sulfonylurea herbicide on day 7 or 14, the inhibition of macrophyte growth could possibly be more pronounced and the recovery period prolonged.

In the present study, root endpoints were not sensitive enough to demonstrate growth effects in response to the sulfonylurea herbicide exposure. This could be explained by the fact that roots were not directly exposed to the herbicide, due to its relatively high solubility in the water phase. However, if effects of a PPP on roots are expected, due to a more pronounced sediment exposure, or a translocation of the PPP from the shoots to the roots, sampling locations without any surrounding sediment should be considered to facilitate the root sampling of macrophytes such as *E. canadensis*.

The recovery within 42 days especially demonstrated the applicability of the two macrophytes within the ERO of the AGD [15]. Special precautions should also be taken to reduce the amount of labor and the costs of a stream mesocosm approach to aquatic macrophyte assessment. Hence, the choice of the species and macrophyte endpoints should be considered with regard to time-saving handling procedures.

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SUPPLEMENTAL DATA

Tables S1-S13

Figure S1

Table S1: Specific conditions of the tap water. The sample was taken on June 19, 2013 and analyzed by the DVGW-Technologiezentrum Wasser (Karlsruhe)

Physico-chemical parameter	
pH	8.1
Oxygen saturation (mg/L)	10.3
TOC (mg/L)	0.72
Ammonium (NH_4^+; mg/L)	< 0.01
Nitrite (NO_2^-; mg/L)	< 0.01
Nitrate (NO_3^-; mg/L)	3.7

Table S2: Injection scheme of the peristaltic pumps during the exposure and the tracer experiments

	Percent of maximum flow volume (%)	Duration (min)
First injection phase	100.0	62
Second injection phase	93.0	6
	86.9	3
	81.0	4
	73.1	3
	64.9	3.5
	56.0	2.5
	47.0	5
	35.8	5
	25.0	5
	14.9	8
	7.1	10

Table S3: Mean pH values at the sampling dates. The values were measured hourly (n = 24; ± SD).

		pH (± SD)									
Sampling days	date	control		1 µg/L		3 µg/L		10 µg/L		30 µg/L	
-6	17.07.2013	9.4	± 0.6	8.6	± 0.5	9.4	± 0.6	8.5	± 0.4	9.1	± 0.7
+1	22.07.2013	9.2	± 0.7	9.0	± 0.7	9.1	± 0.9	9.0	± 0.7	9.1	± 0.7
+3	24.07.2013	9.4	± 0.3	8.9	± 0.4	9.5	± 0.3	9.0	± 0.3	9.1	± 0.3
+7	29.07.2013	10.0	± 0.3	9.1	± 0.6	9.9	± 1.0	9.1	± 0.6	9.4	± 0.4
+14	05.08.2013	9.8	± 0.5	9.5	± 0.6	10.3	± 0.3	9.4	± 0.5	9.8	± 0.4
+42	31.08.2013	-[a]	-[a]	8.7	± 0.7	10.0	± 0.4	9.4	± 0.6	9.4	± 0.5

[a] the measurement was not possible due to a technical error

Table S4: Mean specific conductivity values at the sampling dates. The values were measured hourly (n = 24; ± SD)

		Conductivity (µS/cm; ± SD)				
Sampling day	date	control	1 µg /L	3 µg/L	10 µg/L	30 µg/L
-6	17.07.2013	147.0 ± 6.8	216.2 ± 28.4	177.7 ± 20.4	207.2 ± 27.5	231.9 ± 54.2
+1	22.07.2013	162.5 ± 26.1	151.8 ± 20.6	153.8 ± 24.5	153.6 ± 12.9	144.5 ± 24.2
+3	24.07.2013	132.1 ± 9.4	126.8 ± 8.2	133.5 ± 14.6	180.2 ± 36.3	131.3 ± 11.8
+7	29.07.2013	117.9 ± 8.1	120.9 ± 6.0	127.3 ± 3.9	134.4 ± 6.6	120.1 ± 5.4
+14	05.08.2013	137.9 ± 9.6	126.7 ± 11.3	136.6 ± 16.6	126.5 ± 8.1	123.0 ± 5.0
+42	31.08.2013	113.3 ± 8.2	128.5 ± 5.2	127.7 ± 5.9	130.6 ± 5.0	118.7 ± 4.6

Table S5: Accelerated solvent extraction settings

		ASE stetting	
		E. canadensis	*M. spicatum*
Eluent		Acetone	Methanol
Temperature (°C)		40	40
Equilibration time (min)		5	5
Extraction cycles	number	2	3
	duration (min)	5	5
Rinse volume (%)		30	30
Purge time (s)		30	60

Table S6: Gradient program for Accela pump

Time (min)	Eluents		Flow (µL/min)
	A%	B%	
0.00	98	2	200
2.00	98	2	200
4.00	5	95	200
10.00	5	95	200
10.01	98	2	200
12.00	98	2	200

Table S7: Gradient program for Surveyor pump

Time (min)	Eluents		Flow (µL/min)
	A%	B%	
0.00	98	2	1000
2.00	98	2	1000
2.01	98	2	100
10.00	98	2	100
10.01	98	2	1000
12.00	98	2	1000

Table S8: Starting conditions of the macrophytes at the sampling day -6 prior to the exposure. Mean maximum root length, root number and dry weight of total shoots (n=4; 12 subsamples each; ±SD) of *Myriophyllum spicatum* and *Elodea canadensis*

	BCS-AA10579 (µg /L)				
	Control	1	3	10	30
Myriophyllum spicatum					
Maxiumum root length (cm)	6.9 ± 0.4	5.7 ± 1.0	6.0 ± 1.2	6.3 ± 1.1	6.9 ± 0.6
Root number	4.0 ± 0.7	3.4 ± 0.3	3.7 ± 0.4	3.4 ± 0.8	3.8 ± 0.6
Dry weight of total shoots (mg)	164.5 ± 28.5	146.6 ± 16.6	185.7 ± 23.5	179.4 ± 10.0	165.7 ± 8.8
Elodea canadensis					
Maxiumum root length (cm)	9.8 ± 0.7	7.7 ± 4.5	8.6 ± 2.2	9.4 ± 1.1	9.0 ± 1.8
Root number	3.4 ± 0.4	2.7 ± 1.9	3.1 ± 0.0	3.4 ± 0.3	2.9 ± 1.2
Dry weight of total shoots (mg)	61.4 ± 6.6	61.6 ± 5.3	60.8 ± 9.5	60.3 ± 7.1	58.8 ± 2.7

Table S9: Endpoints of *Myriophyllum spicatum* and *Elodea canadensis* (mean values ± SD). The mean values consisted of 3 and 4 replicates for treatments and controls respectively; each replicate consisted of a mean value of the 12 subsamples per stream

	Myriophyllum spicatum								*Elodea canadensis*							
			BCS-AA10579 (µg /L)								BCS-AA10579 (µg /L)					
	Control		1		3		10		30		Control		1		3		10		30	
Average main shoot length (cm)																				
Day -6	13.8	± 0.6	13.3	± 0.9	13.3	± 0.1	13.6	± 0.2	13.3	± 0.6	14.2	± 0.7	13.6	± 0.9	14.0	± 1.0	14.3	± 1.0	13.8	± 0.7
Day 1	17.7	± 1.0	16.7	± 1.4	16.9	± 0.8	16.8	± 0.1	15.9	± 0.6	19.0	± 1.0	17.3	± 1.6	17.9	± 1.4	18.5	± 0.4	17.0	± 1.3
Day 3	20.1	± 1.2	18.8	± 0.6	18.2	± 0.9	18.2	± 1.6	17.2	± 1.2	19.4	± 1.4	17.5	± 1.3	19.3	± 0.3	19.8	± 1.3	17.2	± 2.1
Day 7	21.8	± 1.8	21.2	± 2.8	18.6	± 0.9	19.4	± 1.7	16.8	± 1.4	24.2	± 0.9	18.8	± 2.0	22.2	± 1.1	21.6	± 2.1	18.5	± 2.2
Day 14	24.9	± 2.5	22.7	± 0.8	19.3	± 0.9	19.1	± 1.0	17.3	± 1.9	28.5	± 3.4	24.6	± 2.0	26.3	± 2.8	25.9	± 2.3	20.2	± 0.9
Day 42	33.50	± 3.2	33.4	± 1.7	32.0	± 0.4	30.0	± 4.2	23.9	± 1.2	49.8	± 4.9	47.4	± 4.1	40.6	± 2.1	41.2	± 0.7	34.3	± 1.2
Average side shoot length (cm)																				
Day -6	4.6	± 1.0	4.6	± 1.4	3.9	± 1.0	4.9	± 0.5	4.5	± 1.0	6.9	± 0.3	5.7	± 1.8	6.9	± 0.8	7.4	± 0.8	6.9	± 1.5
Day 1	13.8	± 2.4	10.5	± 3.2	12.5	± 2.0	13.5	± 0.7	9.4	± 1.5	20.7	± 4.9	19.4	± 1.8	20.5	± 2.0	22.2	± 1.6	16.7	± 2.0
Day 3	17.4	± 3.3	14.1	± 3.4	13.3	± 3.2	13.0	± 3.5	9.2	± 0.6	31.6	± 8.1	25.8	± 3.1	28.9	± 5.3	22.8	± 3.3	17.3	± 4.4
Day 7	25.1	± 5.0	21.3	± 4.7	16.3	± 1.8	16.2	± 0.5	10.1	± 2.2	52.0	± 8.3	37.8	± 6.5	46.4	± 5.4	39.1	± 8.6	23.5	± 4.9
Day 14	37.7	± 16.0	25.3	± 7.2	24.0	± 2.7	17.5	± 0.8	9.6	± 1.2	98.8	± 31.9	83.0	± 10.7	85.6	± 21.7	104.2	± 30.0	39.7	± 11.7
Day 42	78.1	± 24.2	70.2	± 8.9	74.7	± 29.6	102.6	± 51.2	48.7	± 9.4	365.9	± 93.6	349.2	± 21.7	363.7	± 194.8	488.4	± 133.6	217.8	± 38.2
Average total shoot length (cm)																				
Day -6	18.5	± 1.5	17.8	± 2.2	17.2	± 0.9	18.5	± 0.7	17.8	± 0.8	21.2	± 0.9	19.3	± 2.6	20.8	± 0.7	21.7	± 1.8	20.7	± 1.9
Day 1	31.5	± 3.2	27.2	± 4.6	29.4	± 1.7	30.3	± 0.8	25.3	± 0.9	39.7	± 5.8	36.7	± 3.4	38.4	± 0.9	40.8	± 1.4	33.6	± 3.0
Day 3	37.5	± 4.4	32.9	± 3.5	31.5	± 3.0	31.2	± 4.9	26.4	± 0.9	51.1	± 8.6	43.4	± 4.2	48.2	± 5.4	42.7	± 4.6	34.5	± 6.4
Day 7	46.8	± 4.6	42.5	± 6.4	34.9	± 2.6	35.6	± 1.8	26.8	± 3.6	76.2	± 8.9	56.6	± 6.8	68.6	± 6.3	60.7	± 10.5	41.9	± 7.1
Day 14	62.7	± 17.9	48.1	± 7.4	43.3	± 3.3	36.6	± 0.5	26.9	± 3.1	127.2	± 34.8	107.6	± 12.7	111.9	± 24.2	130.0	± 32.3	59.9	± 12.4
Day 42	111.6	± 26.2	103.6	± 8.4	106.6	± 30.0	132.5	± 55.3	72.6	± 8.8	415.7	± 98.2	396.5	± 24.5	404.3	± 196.9	530.0	± 134.3	252.1	± 38.9
Average side shoot number																				
Day -6	2.6	± 0.6	2.6	± 0.5	2.6	± 0.5	2.7	± 0.5	2.8	± 0.6	3.1	± 0.2	3.0	± 0.1	3.1	± 0.4	3.4	± 0.2	3.0	± 0.2
Day 1	3.0	± 0.4	3.0	± 0.6	3.2	± 0.6	3.3	± 0.7	2.7	± 0.1	6.5	± 1.9	6.9	± 0.2	7.0	± 0.3	7.0	± 1.8	5.8	± 0.4
Day 3	3.4	± 0.4	3.0	± 0.1	3.4	± 0.6	2.9	± 0.5	2.4	± 0.1	9.4	± 2.7	8.6	± 0.6	9.6	± 1.6	7.1	± 0.5	6.2	± 0.6
Day 7	4.4	± 0.5	4.1	± 0.3	3.6	± 0.4	3.8	± 0.4	3.2	± 0.4	13.9	± 2.4	11.8	± 0.6	12.7	± 1.4	10.2	± 1.7	7.5	± 1.2
Day 14	5.8	± 1.9	4.9	± 1.0	5.4	± 0.5	4.2	± 0.1	2.8	± 0.1	23.6	± 7.4	22.6	± 3.3	24.3	± 8.1	29.9	± 7.0	14.1	± 2.9
Day 42	10.6	± 3.1	9.4	± 2.2	10.5	± 2.9	9.9	± 1.8	7.7	± 0.7	72.0	± 13.6	77.9	± 12.7	70.3	± 33.2	95.1	± 23.8	46.4	± 6.0
Dry weight of total shoots (g)																				
Day -6	0.16	± 0.03	0.15	± 0.02	0.19	± 0.02	0.18	± 0.01	0.17	± 0.01	0.06	± 0.01	0.06	± 0.01	0.06	± 0.01	0.06	± 0.01	0.06	± 0.00
Day 1	0.25	± 0.05	0.22	± 0.03	0.25	± 0.02	0.28	± 0.04	0.24	± 0.02	0.10	± 0.02	0.10	± 0.01	0.12	± 0.01	0.11	± 0.01	0.11	± 0.01
Day 3	0.29	± 0.05	0.24	± 0.05	0.30	± 0.04	0.33	± 0.08	0.27	± 0.03	0.11	± 0.03	0.11	± 0.01	0.13	± 0.03	0.13	± 0.01	0.12	± 0.02
Day 7	0.38	± 0.05	0.34	± 0.07	0.39	± 0.09	0.46	± 0.06	0.34	± 0.02	0.19	± 0.06	0.14	± 0.02	0.18	± 0.05	0.20	± 0.02	0.18	± 0.01
Day 14	0.43	± 0.10	0.36	± 0.05	0.35	± 0.08	0.44	± 0.05	0.36	± 0.04	0.27	± 0.10	0.23	± 0.09	0.30	± 0.12	0.31	± 0.04	0.23	± 0.02
Day 42	0.87	± 0.25	0.92	± 0.09	0.75	± 0.22	0.81	± 0.08	0.47	± 0.06	1.33	± 0.48	1.09	± 0.28	1.18	± 0.53	1.38	± 0.19	0.79	± 0.16

Table S10: Average growth rates of the main, side, and total shoot length, the side shoot number and the dry weight of total shoots (± SD) for the respective intervals

Interval (days)	*Myriophyllum spicatum*					*Elodea canadensis*				
		BCS-AA10579 (µg /L)					BCS-AA10579 (µg /L)			
	Control	1	3	10	30	Control	1	3	10	30
	Average main shoot length (cm)									
-6 to +1	0.037 ± 0.006	0.032 ± 0.009	0.036 ± 0.006	0.031 ± 0.003	0.027 ± 0.006	0.041 ± 0.012	0.034 ± 0.004	0.038 ± 0.014	0.038 ± 0.006	0.031 ± 0.004
-6 to +3	0.041 ± 0.005	0.038 ± 0.010	0.037 ± 0.003	0.032 ± 0.007	0.028 ± 0.010	0.035 ± 0.011	0.027 ± 0.002	0.036 ± 0.005	0.036 ± 0.001	0.025 ± 0.009
-6 to +7	0.034 ± 0.006	0.035 ± 0.011	0.026 ± 0.003	0.027 ± 0.005	0.018 ± 0.008	0.040 ± 0.005	0.024 ± 0.006	0.036 ± 0.008	0.031 ± 0.002	0.022 ± 0.006
-6 to +14	0.029 ± 0.003	0.027 ± 0.005	0.019 ± 0.002	0.017 ± 0.003	0.013 ± 0.006	0.034 ± 0.007	0.028 ± 0.002	0.031 ± 0.004	0.029 ± 0.001	0.019 ± 0.003
-6 to + 42	0.018 ± 0.003	0.019 ± 0.002	0.018 ± 0.001	0.016 ± 0.003	0.012 ± 0.000	0.026 ± 0.002	0.025 ± 0.002	0.022 ± 0.002	0.022 ± 0.001	0.019 ± 0.001
	Average side shoot length (cm)									
-6 to +1	0.147 ± 0.015	0.106 ± 0.025	0.145 ± 0.024	0.138 ± 0.022	0.098 ± 0.009	0.156 ± 0.044	0.191 ± 0.034	0.163 ± 0.029	0.163 ± 0.031	0.131 ± 0.016
-6 to +3	0.136 ± 0.006	0.115 ± 0.010	0.121 ± 0.008	0.102 ± 0.015	0.074 ± 0.019	0.166 ± 0.035	0.178 ± 0.026	0.162 ± 0.034	0.128 ± 0.017	0.102 ± 0.009
-6 to +7	0.121 ± 0.007	0.110 ± 0.007	0.098 ± 0.010	0.090 ± 0.010	0.056 ± 0.001	0.152 ± 0.016	0.150 ± 0.018	0.148 ± 0.012	0.129 ± 0.005	0.094 ± 0.001
-6 to +14	0.097 ± 0.008	0.079 ± 0.011	0.083 ± 0.013	0.062 ± 0.006	0.034 ± 0.003	0.130 ± 0.019	0.135 ± 0.006	0.126 ± 0.021	0.133 ± 0.010	0.087 ± 0.006
-6 to + 42	0.055 ± 0.005	0.054 ± 0.004	0.056 ± 0.006	0.059 ± 0.009	0.046 ± 0.002	0.081 ± 0.006	0.088 ± 0.008	0.082 ± 0.013	0.088 ± 0.008	0.072 ± 0.008
	Average total shoot length (cm)									
-6 to +1	0.076 ± 0.011	0.059 ± 0.019	0.075 ± 0.004	0.070 ± 0.009	0.051 ± 0.004	0.088 ± 0.027	0.091 ± 0.007	0.088 ± 0.007	0.090 ± 0.012	0.071 ± 0.003
-6 to +3	0.078 ± 0.007	0.067 ± 0.008	0.067 ± 0.006	0.057 ± 0.013	0.044 ± 0.004	0.095 ± 0.022	0.088 ± 0.007	0.092 ± 0.012	0.074 ± 0.005	0.056 ± 0.013
-6 to +7	0.071 ± 0.003	0.066 ± 0.009	0.054 ± 0.001	0.051 ± 0.002	0.031 ± 0.008	0.096 ± 0.011	0.080 ± 0.006	0.090 ± 0.010	0.078 ± 0.005	0.053 ± 0.006
-6 to +14	0.060 ± 0.009	0.049 ± 0.008	0.046 ± 0.004	0.034 ± 0.001	0.020 ± 0.004	0.087 ± 0.015	0.082 ± 0.002	0.081 ± 0.013	0.088 ± 0.008	0.052 ± 0.008
-6 to + 42	0.037 ± 0.005	0.036 ± 0.002	0.037 ± 0.005	0.039 ± 0.008	0.029 ± 0.001	0.060 ± 0.005	0.063 ± 0.004	0.060± 0.010	0.066 ± 0.005	0.051 ± 0.005
	Average side shoot number									
-6 to +1	0.033 ± 0.013	0.032 ± 0.015	0.029 ± 0.007	0.046 ± 0.028	0.027 ± 0.014	0.100 ± 0.037	0.123 ± 0.009	0.115 ± 0.023	0.100 ± 0.040	0.097 ± 0.003
-6 to +3	0.034 ± 0.017	0.023 ± 0.009	0.032 ± 0.005	0.025 ± 0.013	0.018 ± 0.007	0.118 ± 0.027	0.118 ± 0.006	0.123 ± 0.008	0.081 ± 0.005	0.082 ± 0.004
-6 to +7	0.035 ± 0.008	0.030 ± 0.005	0.024 ± 0.007	0.029 ± 0.013	0.021 ± 0.004	0.112 ± 0.010	0.105 ± 0.004	0.107 ± 0.015	0.083 ± 0.010	0.069 ± 0.009
-6 to +14	0.033 ± 0.009	0.025 ± 0.010	0.030 ± 0.007	0.020 ± 0.008	0.009 ± 0.003	0.099 ± 0.014	0.099 ± 0.008	0.100 ± 0.015	0.107 ± 0.012	0.076 ± 0.007
-6 to + 42	0.024 ± 0.003	0.022 ± 0.003	0.024 ± 0.003	0.023 ± 0.006	0.018 ± 0.002	0.064 ± 0.003	0.068 ± 0.004	0.063 ± 0.008	0.069 ± 0.004	0.057 ± 0.003
	Dry weight of total shoots (g)									
-6 to +1	0.065 ± 0.013	0.057 ± 0.007	0.046 ± 0.010	0.067 ± 0.021	0.051 ± 0.013	0.076 ± 0.014	0.070 ± 0.007	0.093 ± 0.009	0.087 ± 0.019	0.087 ± 0.014
-6 to +3	0.066 ± 0.010	0.057 ± 0.011	0.051 ± 0.012	0.069 ± 0.027	0.053 ± 0.018	0.067 ± 0.020	0.061 ± 0.005	0.086 ± 0.009	0.082 ± 0.013	0.075 ± 0.015
-6 to +7	0.066 ± 0.009	0.065 ± 0.009	0.054 ± 0.015	0.073 ± 0.012	0.055 ± 0.005	0.082 ± 0.019	0.061 ± 0.008	0.081 ± 0.013	0.093 ± 0.013	0.087 ± 0.004
-6 to +14	0.047 ± 0.012	0.045 ± 0.001	0.030 ± 0.010	0.044 ± 0.004	0.040 ± 0.004	0.072 ± 0.016	0.063 ± 0.017	0.076 ± 0.014	0.082 ± 0.002	0.067 ± 0.003
-6 to + 42	0.034 ± 0.006	0.038 ± 0.004	0.028 ± 0.007	0.031 ± 0.003	0.022 ± 0.001	0.062 ± 0.008	0.059 ± 0.004	0.060 ± 0.006	0.065 ± 0.003	0.054 ± 0.005

Table S11: *P* values of the group-wise comparisons between the treatments and the control based on growth rates of the main, side, and total shoot length, the dry weight of the total shoots for the respective time intervals. The linear mixed effect model and a Dunnett's *post-hoc* test were used as described in the materials and methods section

Interval (days)	*M. spicatum*				*E. canadensis*			
	control - T1	control - T3	control - T10	control - T30	control - T1	control - T3	control - T10	control - T30
Average main shoot length (cm)								
-6 to +1	0.772	0.999	0.733	0.173	0.809	0.986	0.991	0.443
-6 to +3	0.940	0.871	0.319	0.076	0.500	0.997	0.999	0.258
-6 to +7	0.999	0.399	0.507	0.010*	<0.001*	0.733	0.107	< 0.001*
-6 to +14	0.864	0.002**	< 0.001***	< 0.001***	0.190	0.692	0.381	< 0.001*
-6 to + 42	0.960	0.999	0.527	< 0.001***	0.986	0.043*	0.031 *	< 0.001*
Average side shoot length (cm)								
-6 to +1	0.100	1.000	0.974	0.036*	0.472	0.996	0.996	0.759
-6 to +3	0.353	0.629	0.040*	< 0.001*	0.944	0.999	0.208	0.008*
-6 to +7	0.600	0.056	0.003*	< 0.001*	0.998	0.983	0.095	< 0.001*
-6 to +14	0.029*	0.112	< 0.001*	< 0.001*	0.973	0.986	0.998	< 0.001*
-6 to + 42	0.994	1.000	0.828	0.115	0.738	1.000	0.752	0.435
Average total shoot length (cm)								
-6 to +1	0.116	0.999	0.891	0.007*	0.998	1.000	1.000	0.389
-6 to +3	0.279	0.256	0.003*	< 0.001*	0.924	0.994	0.181	< 0.001*
-6 to +7	0.639	< 0.001*	< 0.001*	< 0.001*	0.061	0.821	0.02*	< 0.001*
-6 to +14	0.087	0.012*	< 0.001*	< 0.001*	0.963	0.923	1.000	< 0.001*
-6 to + 42	1.000	1.000	0.926	0.141	0.980	1.000	0.617	0.169
Dry weight (g)								
-6 to +1	0.904	0.249	0.998	0.535	0.943	0.304	0.746	0.715
-6 to +3	0.884	0.602	0.996	0.713	0.967	0.251	0.434	0.886
-6 to +7	1.000	0.411	0.768	0.532	0.129	1.000	0.630	0.952
-6 to +14	0.985	0.012*	0.961	0.513	0.816	0.981	0.684	0.982
-6 to + 42	0.702	0.283	0.870	0.003*	0.967	0.985	0.858	0.217

Table S12: Percent composition of TSL for all sampling dates

Sampling day	*M. spicatum*		*E. canadensis*	
	MSL (%)	SSL (%)	MSL (%)	SSL (%)
-6	75	25	67	33
1	56	44	48	52
3	54	46	38	62
7	46	54	32	68
14	40	60	22	78
42	30	70	12	88

Table S13: Quotient of average *E. canadensis* and *M. spicatum* endpoints of the control streams

Sampling day	Quotient of *E. canadensis* and *M. spicatum*				
	MSL	SSL	TSL	SSN	DW
-6	1.03	1.50	1.15	1.19	0.38
1	1.07	1.50	1.26	2.17	0.40
3	0.97	1.82	1.36	2.76	0.41
7	1.11	2.07	1.63	3.16	0.50
14	1.14	2.62	2.03	4.07	0.63
42	1.44	4.70	3.72	6.79	1.53

MSL = main shoot length; SSL = side shoot length; TSL = total shoot length; SSN = side shoot number; DW = dry weight

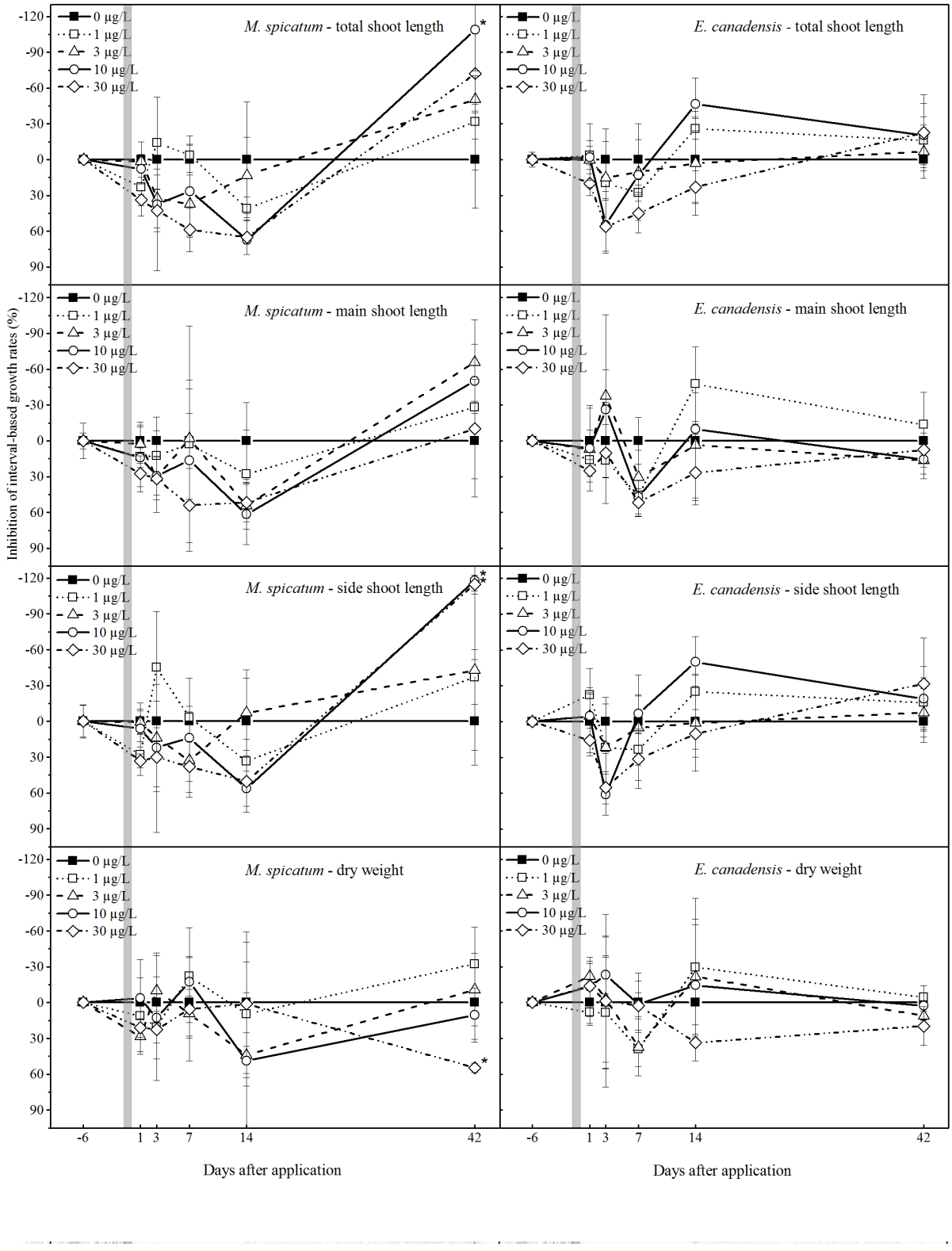

Figure S1: Percent inhibition of interval-based growth rates of *M. spicatum* and *E. canadensis*. The interim growth rates are presented as average percent inhibitions of the treatments (± SD) compared to the control samples. The asterisks indicate statistically significant differences between interim growth rates of the control and the treatments. The grey vertical bar marks the 24-h exposure phase to a sulfonylurea herbicide. The statistical analysis was restricted to the last time interval (day 14 to day 42).

Appendix III: **Scientific publication 3**

Structural and functional effects of a short-term pyrethroid pulse exposure on invertebrates in outdoor stream mesocosms

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Environmental Pollution

(submitted)

Abstract

Agricultural land-use frequently results in short pulse exposures of insecticides such as pyrethroids in small streams, adversely affecting local invertebrate communities. In order to mimic these scenarios, stream mesocosms can be used during higher tier aquatic risk assessment. To assess for the usefulness of such mesocosm studies, the present study used a 6-h pulse exposure with concentrations of 0.05, 0.5 and 5 µg/L of the pyrethroid etofenprox as a model scenario. As structural endpoints the present study used abundance, drift and emergence of invertebrates and as functional endpoint the *in situ*-measured feeding rates of *Asellus aquaticus*. Overall, 5 µg/L etofenprox caused adverse effects at the population and community level. Transient effects were observed for invertebrate drift (effect duration ≤ 24 h) and for the invertebrate community (9 days after exposure) at 0.5 µg/L etofenprox. Furthermore, 0.05 µg/L etofenprox affected the abundance of the mayfly *Cloeon simile* and the feeding rate of *A. aquaticus* (decrease by 44%). These effects at the structural level of the populations and the feeding rate as functional endpoint thus occurred at concentrations only slightly above the predicted environmental (0.024 µg/L) and below field concentrations. The present study supports the hypothesis that short pyrethroid pulse exposures may adversely affect invertebrate populations and communities. Moreover, implications in functional properties (i.e. the leaf litter breakdown) of heterotrophic ecosystems can be expected. A hypothetical regulatory acceptable concentration (RAC) derived from the present mesocosm study is in line with the official tier-1 RAC (0.0044 µg/L).

Introduction

Agricultural insecticides enter small edge-of-field streams typically via spray drift or rainfall-induced runoff events (Neumann et al. 2002; Schulz 2004; Rabiet et al. 2010), with subsequent in-stream exposure lasting only for a few hours (Spurlock et al. 2005; Rasmussen et al. 2013b; Stehle et al. 2013; Stehle and Schulz 2015). Despite short pulse durations, exposures with insecticides can adversely affect the integrity of aquatic ecosystems (Schulz and Liess 1999; Schulz 2004; Rasmussen et al. 2013b). For instance, pulse exposures induce catastrophic drift (Lauridsen and Friberg 2005; Heckmann and Friberg 2005; Beketov and Liess 2008), as well as mortality of invertebrates in experimental studies and under field conditions (Jergentz et al. 2004; Bereswill et al. 2013). Furthermore, ecosystem functions such as leaf breakdown, which are the basis of heterotrophic food webs, may be adversely affected as a consequence of pesticide exposure (Schäfer et al. 2007). Among different

insecticide classes, particularly pyrethroid insecticides have been detected at ecologically relevant concentrations in agricultural surface waters worldwide (Stehle and Schulz 2015).

In order to prospectively assess adverse effects of insecticides on aquatic ecosystems a tiered approach using laboratory standard tests (tier 1) and micro- and mesocosm tests (higher tier risk assessment) is used. The current higher tier risk assessment mainly uses pond mesocosms with static test conditions and rather long exposure durations, which is typical for lentic surface waters and considered as worst case exposure scenario. However, such pond systems are – in contrast to stream mesocosms – not designed to mimic stream-typical pulse exposures of few hours. Especially for sorptive insecticides, such as pyrethroids, pulse exposure events are long enough to cause adverse effects (Schulz and Liess 2000) due to rapid substance uptake by the organism (Tang and Siegfried 1995) and fast mode of action (Farmer et al. 1995).

Up to now, knowledge of effects on invertebrates following pulse exposures was mainly based on laboratory and microcosm approaches (Rasmussen et al. 2013a). Although stream mesocosm were generally used more frequently in the recent years, most setups focused on low or moderately lipophilic insecticides (Liess and Beketov 2011; Mohr et al. 2012), fungicides (Bayona et al. 2015b; Bayona et al. 2015a) or herbicides (Mohr et al. 2007; Wieczorek et al. 2016b) while exposure durations of ≥ 12 hours were used. Up to now experiments assessing effects of sorptive insecticides on invertebrates using pulse exposures are scarce for stream mesocosms.

This study simulated a field relevant pulse exposure representative for moderate to highly lipophilic insecticides using the pyrethroid ether etofenprox as model insecticide. This model insecticide was measured in the field at concentrations between 0.04 and 0.2 µg/L (Tanabe et al. 2001; Añasco et al. 2010) over up to 7 h (Tanabe and Kawata 2009). Since the first tier RAC of the EU regulatory risk assessment of 0.0044 µg active substance (a.s.)/L (based on *Daphnia* 48-h EC_{50} for the formulation Trebon 30EC; EFSA (2008)) is up to two orders of magnitude below the measured field concentrations, adverse effects cannot be excluded. A logical next step during the EU risk assessment would be a refinement of the RAC using higher tier studies. However, the existing higher-tier mesocosm study could not be used due to lacking information on population recovery and high uncertainty (EFSA 2008). Thus, the present study aims at providing additional data on the ecological effects of etofenprox under semi-field conditions for a future regulatory assessment using a 6-h pulse exposure with concentrations between 0.05 and 5 µg/L etofenprox. The structural endpoints abundance, drift and emergence of invertebrates were complemented by the functional endpoint of *in situ*-

measured feeding rates of *Asellus aquaticus*, to uncover potential effects on the invertebrate mediated decomposition of allochthonous organic matter.

Material and methods

Experimental design

The study was conducted at the Landau Stream Mesocosm Facility (LSMF) at the University of Koblenz-Landau, Campus Landau (Germany). The test facility consists of 16 independent channels (length = 45 m, width = 0.4 m and average water depth 0.26 - 0.27 m). Further information about the LSMF is described elsewhere (e.g. Elsaesser et al. 2013).

The experimental period started in October 2013 through September 2014. Artificial sediment and aquatic macrophytes were introduced at the beginning of October 2013. The sediment (height approx. 0.08 m) consisted of medium to coarse sand (grain size = 50% 0 - 0.5 mm, 50% 0.2 – 1.0 mm) and in total 5 % vol. white peat. Two sampling areas SA1 (5 m below the water inlet) and SA2 (35 m below the water inlet), each with a length of 7.5 m were planted with both western waterweed (*Elodea nuttallii* (Planch.) H. St. John) and Eurasian watermilfoil (*Myriophyllum spicatum* L.) (Figure 1). The stream sections between the sampling areas were also planted with *M. spicatum* and *E. nuttallii* (Figure 1). The vegetation coverage of the sampling areas was in the range of 50 to 100%. The first 5 m below the inlet were kept free of macrophytes in order to enable a homogeneous distribution of etofenprox in the water phase within all channels.

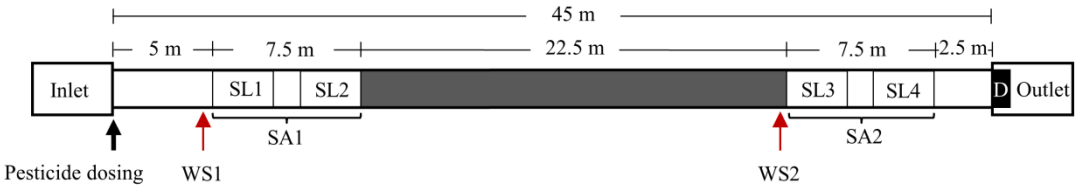

Figure 15: Exemplary scheme of one stream mesocosm channel with two sampling areas (SA1 and SA2) comprising in total four sampling locations (SL1-4) for invertebrate and emergence sampling each. The stream sections between the sampling areas were planted with *M. spicatum* and *E. nuttallii* (grey color). Etofenprox injection took place at the inlet. Red arrows indicate water sampling locations (WS1 and WS2). The location of the drift net is displayed by the black bar (D).

In order to provide organic material as food for introduced shredders, dried leaf material of *Alnus glutinosa* was added to the streams over the entire experimental period. The organic material was restocked once a month to the initial density of 105 ± 22 leaves/m^2.

Stream water quality

The water quality parameters temperature, pH, oxygen saturation, and conductivity were measured once a week at 9 a.m. and 4 p.m. with the WTW Multi 340i (WTW GmbH, Weilheim, Germany) in all 16 channels. Mean values are presented in the supplemental data for the sampling dates (Figure S1). Additionally, nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+), phosphate (PO_4^{3-}), sulfate (SO_4^{2-}) and total hardness (°dH) were measured twice on the day of the etofenprox application and one week after the last invertebrate sampling (Table S1) using Visocolor test-kits (Macherey-Nagel, Düren, Germany).

Etofenprox application and monitoring

Etofenprox (IUPAC name 2-(4-ethoxyphenyl)-2-methylpropyl 3-phenoxybenzyl ether, CAS: 80844-07-1) is an insecticide belonging to the pyrethroid ethers. Due to the very low solubility of the active substance the commercial etofenprox formulation Trebon 30EC (287.5 g (a.s.)/L; Mitsui Chemicals Agro, Inc.) was used. Etofenprox was injected to the streams for 6 hours on June 8, 2014 with nominal concentrations of 0, 0.05, 0.5 and 5 µg (a.s.)/L (n=4 each). The two highest concentrations (0.5 and 5 µg/L) were above the 48 hour EC_{50} for *Daphnia magna* (0.44 µg (a.s.)/L; Trebon 30EC) and the lowest concentration (0.05 µg/L) was below the chronic 21 day no observed effect concentration (NOEC) for the same species (0.054 µg/L) (EFSA 2008). For each treatment, 140 L of stock solution (6 L/h/channel x 6 h exposure x 4 replicates) was prepared in stainless steel tanks and stirred throughout the whole injection period. The stock was injected to the streams at the inlet (Figure 1) of the channels using peristaltic pumps (Ismatec IPC 24, IDEX Health & Science GmbH). To reach a homogeneous distribution of etofenprox in the water column, 6 polyvinyl chloride tubes were distributed equally over the channels' width. To minimize etofenprox loss as a consequence of sorption to the tube material, tubes were conditioned before the experiment for 1 h with etofenprox solutions at the same concentration levels as used for injection in the stream mesocosms. During the pesticide injection and the following 48 hours the stream mesocosms were run in flow-through mode (1 L/s) using water from an adjacent storage reservoir.

For accurate water sampling times, a preliminary tracer experiment with the non-sorptive fluorescent tracer uranine (Sigma–Aldrich, Steinheim, Germany) was performed one week prior to etofenprox injection. Uranine was measured via fiber-optic fluorometers (FOFs, Hermess Messtechnik, Stuttgart, Germany) at two water sampling locations WS1 and WS2

(Figure 1) using the same settings as used for etofenprox injection (see also Wieczorek et al. 2016).

Water samples for etofenprox analysis were taken at WS1 and WS2 (Figure 1) in all 16 channels prior to (T0) and at the end of the 6-h injection period, namely 5.25 h ($T1_{WS1}$) at location WS1 and 5.45 h ($T1_{WS2}$) at location WS2 after the start of the application. Additional water sampling was done 12 (T2), 24 (T3) and 48 (T4) hours after each T1-water sampling of WS1 and 2 to detect etofenprox residues in the water phase following the injection. For each sample, a water volume of approximately 10 ml was taken from the middle of the water phase using glass pipettes and stored in 20 ml glass vials at -20°C until chemical analyses.

Chemical analyses

The concentrations of etofenprox were analyzed using high performance liquid chromatography with mass spectrometry (HPLC-MS; Thermo Orbitrap Exactive; Thermo Fisher Scientific, Dreieich, Germany) according to the settings shown in Table S2. Briefly, after injection water samples were first transferred to the in-line preconcentration column for enrichment and then eluted by back flash to the analytical column for separation. For volume correction and to compensate for instrumental drift each calibration standard and aqueous samples contained the deuterated internal standard etofenprox-D5 (purchased from Dr. Ehrenstorfer GmbH, Augsburg, Germany) at a concentration of 5 ng/mL. The calibration range of at least 6 standards was 0.02 – 10 ng/mL. The limit of detection (LOD) and quantification (LOQ) was 0.006 and 0.012 µg/L, respectively.

Invertebrate response to etofenprox exposure

In order to establish invertebrate populations and communities covering essential functional feeding groups (shredders, grazers and predators), several aquatic species were added to the mesocosms in October 2013 and from March to April 2014. Passive introduction was associated with (i) the addition of leaf material from the pristine Hainbach stream (49°14′N; 08°03′E), (ii) the addition of the aquatic macrophyte species and (iii) the root-associated sediment of the macrophytes (Wieczorek et al. 2015; Wieczorek et al. 2016a). The passive introduction of invertebrate taxa was assumed to be quantitatively and qualitatively equal since equal amounts of the plant material was introduced into each stream. The selective introduction of invertebrate taxa focused on EPT species, amphipods and isopods (*Chaetopteryx fusca/villosa*, *Cloeon* spp., *Gammarus fossarum* and *A. aquaticus*). These

species were transferred in an 80-L container, whereby the water was stirred gently for an even distribution of individuals in the water phase. Subsequently, even volumes of the water phase were randomly introduced to the streams. The presence of additional taxa, such as dragonflies and damselflies, is related to colonization from surrounding habitats.

Changes in community structure in response to etofenprox exposure were assessed using drift, abundance and emergence of aquatic invertebrates as structural endpoints. Furthermore, leaf breakdown as functional endpoint was assessed using the feeding rate of *Asellus aquaticus* in *in situ* bioassays.

Abundance of invertebrates – Aquatic invertebrates were sampled semi-destructively using a metal frame cage (0.16 x 0.3m, hereafter referred to as frame sampling) open at the bottom and top panel and with side panels lined with Polyester netting (surface area 0.05 m²; mesh size 1 mm). Frame sampling was performed nine times, namely 33, 19 and 5 days prior to and 2, 9, 23, 44, 72 and 107 days after etofenprox injection. At each sampling date, invertebrate hatching was assessed at the four sampling sections situated at the inlet (S1-S4) of the 16 streams (Figure 1). Invertebrate sampling inside these sections was done randomly but without using of the same spot twice. More details on the practical sampling procedure are described in Wieczorek et al. (2016). Known taxa were counted and transferred back to the respective sampling site. Unknown taxa and up to 10 individuals of families consisting of several species were preserved in ethanol (70%) for further determination. The proportions of these 10 determined species were assigned to the additionally counted individuals of the same family in the same sample. Where possible, taxa were determined to species, otherwise to the lowest, practically taxonomic level possible.

Abundance of emerged merolimnic insects – Emerging merolimnic insects were assessed in each stream at four randomly selected locations within SA 1 and 2 using pyramidal-shaped emergence traps (mesh size = 0.5 mm; sampling area = 0.25 m^2). The emergence traps were placed above stream sections considered as equal in terms of habitat structure such as the macrophyte coverage. The emerged insects were collected for a period of 48 hours 3 days prior to and 4, 11, 18, 39, 53, 67 and 95 days after the etofenprox injection. The sampling dates were set in the same week before and after frame samplings (Figure S2) to differentiate etofenprox effects on invertebrates abundance from effects related to invertebrate emergence. Emerged insects were aspirated after 24 and 48 hours, frozen at -20 °C, preserved in ethanol (70%) and determined to the lowest, practically level possible.

Invertebrate drift – Drift of invertebrates during and following the pyrethroid peak exposure was assessed with drift nets (mesh size = 1 mm) at the outlet of the streams. In total, drifting invertebrates were assessed at six sampling intervals; 3 hours prior to and 3, 6, 9, 24 and 48 hours following the start of the etofenprox injection. After each of the sampling intervals (-3-0, 0-3, 3-6, 6-9, 9-24 and 24-48 hours after start of the etofenprox injection), drift nets were emptied and returned to the respective stream. The collected invertebrates were preserved in ethanol (70%) and determined. The 3-h interval prior to the start of the pesticide injection was used as control.

Feeding rate of A. aquaticus – Effects of etofenprox on survival and feeding rate of *A. aquaticus* were assessed by *in situ* bioassays according to Bundschuh et al. (2011). Briefly, leaf discs with a diameter of 2.0 cm were cut from frozen (-20°C) leaves of *A. glutinosa*. In order to establish a natural microbial community consisting of fungi and bacteria, leaf discs were conditioned in a nutrient medium (for 10 days together with leaves of *A. glutinosa* previously inoculated by microbes in the near natural stream Rodenbach, Germany (49°33`N, 8°02`E)). To ensure an accurate measurement of the asselids' feeding rate, leaf discs were subsequently dried at 60°C, weighed to the nearest 0.01 mg and re-soaked in tap water for 48 h before being transferred to *in situ* bioassay. One week before etofenprox exposure, individuals of *A. aquaticus* were collected in the stream Linnebach near Landau (49°7' N, 8°6'E). Subsequently, the organisms were cultured at 16 ± 1°C in stream water from the Linnebach and sufficiently fed with preconditioned black alder leaves. By increasing the share of mesocosm water in the culturing water from the sampling site, test organisms were adapted stepwise to the experimental conditions in the mesocosm facilities. The *in situ* bioassays consisted of ten cages (top and bottom sides lined with a mesh screen; mesh size = 1 mm) each containing one individual of *A. aquaticus* (size 6 - 8 mm) as well as two pre-weighed and conditioned leaf discs and were placed into each of the 16 stream channels. In addition, five similar cages containing only leaf discs were placed in the streams to account for microbial decomposition and abiotic leaf mass losses. The bioassays remained in the stream channels for a total of 35 h, namely 5 h prior to, 6 h during and 24 h following the 6-h etofenprox pulse exposure. Subsequently, the bioassays were cultured under more controllable laboratory conditions at 16 ± 1 °C in mesocosm water under aeration. After a total experimental duration of 7 days, individuals of *A. aquaticus* and any remaining leaf tissue were removed, dried at 60°C and weighed to the nearest 0.01 mg. The feeding rate was expressed in mg per mg dry weight per day.

Data analysis

Because of different taxonomic resolutions, we harmonized ambiguous taxa before statistical analyses. If the sum of abundance of lower level taxa (e.g. species level) was greater than the abundance of higher level taxa (e.g. family level) higher level taxa have been removed and lower level taxa kept. Otherwise, lower level taxa abundances have been assigned to their higher level (Cuffney et al. 2007). We discarded taxa that were not present in control treatments. To down weight highly abundant taxa and to approximate a normal distribution, macroinvertebrate abundances were ln(Ax +1) transformed, where x is the abundance and A was chosen to be equal to 2 for the lowest abundance value (x) greater than zero (Van den Brink et al. 2000).

Effects at community level were analyzed using Principal Response Curves (PRC; (Van den Brink and Ter Braak 1998)). To further scrutinize the interaction between treatment and time, we performed a Redundancy Analysis (RDA) at each sampling date. We tested for a treatment effect using 1000 restricted permutations, taking the nested structure of sampling locations within channels into account (Legendre et al. 2011). To determine LOEC at the community level we fitted Linear Mixed Models (LMM, (Bates et al. 2015)) with channel as random intercept to the sample scores of a Principal Components Analysis (PCA) for each sampling date (Van den Brink et al. 2009).

Similarly, effects at population level were analyzed using LMM with channel as random intercept. For population level analyses most of the taxa occurred at very low abundances (Figure 3) and therefore, we considered only taxa with a mean abundance (all sampling dates) in control greater than one as robust for analyses (Figure S3).

Effects on drift were analyzed using Linear Models employing the same transformation as for PRC. We considered taxa with a mean drift rate greater than 0.1 animals per hour in the highest treatment as robust and performed analyses only on these. All multiple comparisons have been performed using Dunnett contrasts (Hothorn 2014). The mortality and feeding rate of *A. aquaticus* was compared among treatments using chi square testing and nested analysis of variance (ANOVA; each individual was nested in the respective stream), respectively.

All computations were performed using R (version 3.2.5 on Linux, 64-bit (Team 2016)). PRCs were calculated using the vegan package (Oksanen et al. 2016). Linear mixed effect models were fitted using the lme4 package (Bates et al. 2015) and multiple comparison were performed using the lsmeans package (Lenth 2016).

Results

Exposure of etofenprox

Maximum etofenprox concentrations (Table 1) were detected during the 6-h injection period at the sampling location near the inlet (WS1; Figure 1). Although the average measured etofenprox concentrations per stream deviated by more than 20% from the nominal concentration, nominal concentrations were used in the following to enhance the clarity of the result section and the discussion. Maximum etofenprox concentrations in the water phase at the outlet (WS2) were on average approximately 25% (0.05 µg/L), 20% (0.5 µg/L) and 35% (5 µg/L) lower compared to those at the inlet (WS1; Figure 1). During the 24 hours after T1 sampling, etofenprox concentrations decreased in most cases below the limit of detection (LOD). In the highest treatment, average residues of up to 0.03 µg/L etofenprox were present in 4 out of 8 samples after 24 hours (T3) and 1 out of 8 samples after 48 hours (T4). No etofenprox was detected in samples taken prior to pesticide application and samples from control treatments.

Table 4: Mean etofenprox concentrations ± SD (n = 4) in the water phase over time. Water samples were taken prior to (T0) and at the end of the injection period, namely 5.25 h ($T1_{WS1}$) and 5.45 h ($T1_{WS2}$) after the start of the application. Additional water samples were taken 12 (T2), 24 (T3) and 48 (T4) hours after T1-samples.

Nominal concentrations (µg/L)	Mean measured concentrations (µg/L) per sampling time (T1-4; ± SD)[A]								
	T0	$T1_{WS1}$	$T1_{WS2}$	$T2_{WS1}$	$T2_{WS2}$	$T3_{WS1}$	$T3_{WS2}$	$T4_{WS1}$	$T4_{WS2}$
0.05	< LOD	0.04 ± 0.02	0.03 ± 0.01	0.06 ± 0.002	0.06 ± 0.01	< LOD	< LOD	< LOD	< LOD
0.05	< LOD	0.32 ± 0.06	0.26 ± 0.04	0.05 ± 0.003	0.05 ± 0.004	< LOD	LOQ	< LOD	< LOD
0.5	< LOD	6.50 ± 1.34	4.12 ± 0.54	0.06 ± 0.01	0.08 ± 0.02	< LOD	0.03 ± 0.02	< LOD	0.01 ± 0.02

Limit of detection (LOD) = 0.006 µg/L; limit of quantification (LOQ) = 0.012 µg/L

[A] For the calculation of mean concentrations including single values of < LOQ and < LOD, values of LOQ/2 and LOD/2, respectively, were used

Invertebrate response to etofenprox exposure

Structural endpoints

Abundance of invertebrates - In total, 57 taxa (Tables S3-5) were found during the entire experiment of 142 days. Out of the 57 taxa, 11 were found in control frame samples (0.05 m^2) at mean abundances of ≥ 1 individuals over the whole sampling period (Figure 3). Table 2 and Table S6 give an overview of statistically significant effects and resulting lowest-observed-effect concentrations (LOEC) values.

Table 2: Overview of LOECs of the structural and functional endpoints

Endpoint	LOEC (µg/L)
Structural	
Abundance	0.05
Drift	0.5
Emergence	na
Community	0.5
Functional	
Feeding rate	0.05

[na] the endpoint was not statistically evaluated

Directly after etofenprox injection (i.e. on day 2 and 9) effects were visible for *C. simile* and *C. dipterum*, showing significantly lower abundances in the 5 µg/L treatments compared to the control (Figure 2). From day 23 onwards, effects were observed for the damselfly *P. nymphula* and on day 44 and 72 also for the caddisfly *C. fusca/villosa* in the 5 µg/L treatment. On day 23 and 44, abundances of Aeshnidae in the 0.5 and 5 µg/L treatment were up to 9 and 5 fold increased relative to the control, respectively. At the end of the sampling period (day 107) LOECs were 0.5 µg/L and 0.05 µg/L for *P. nymphula* and *C. simile*, respectively (see also Supplemental Table S6).

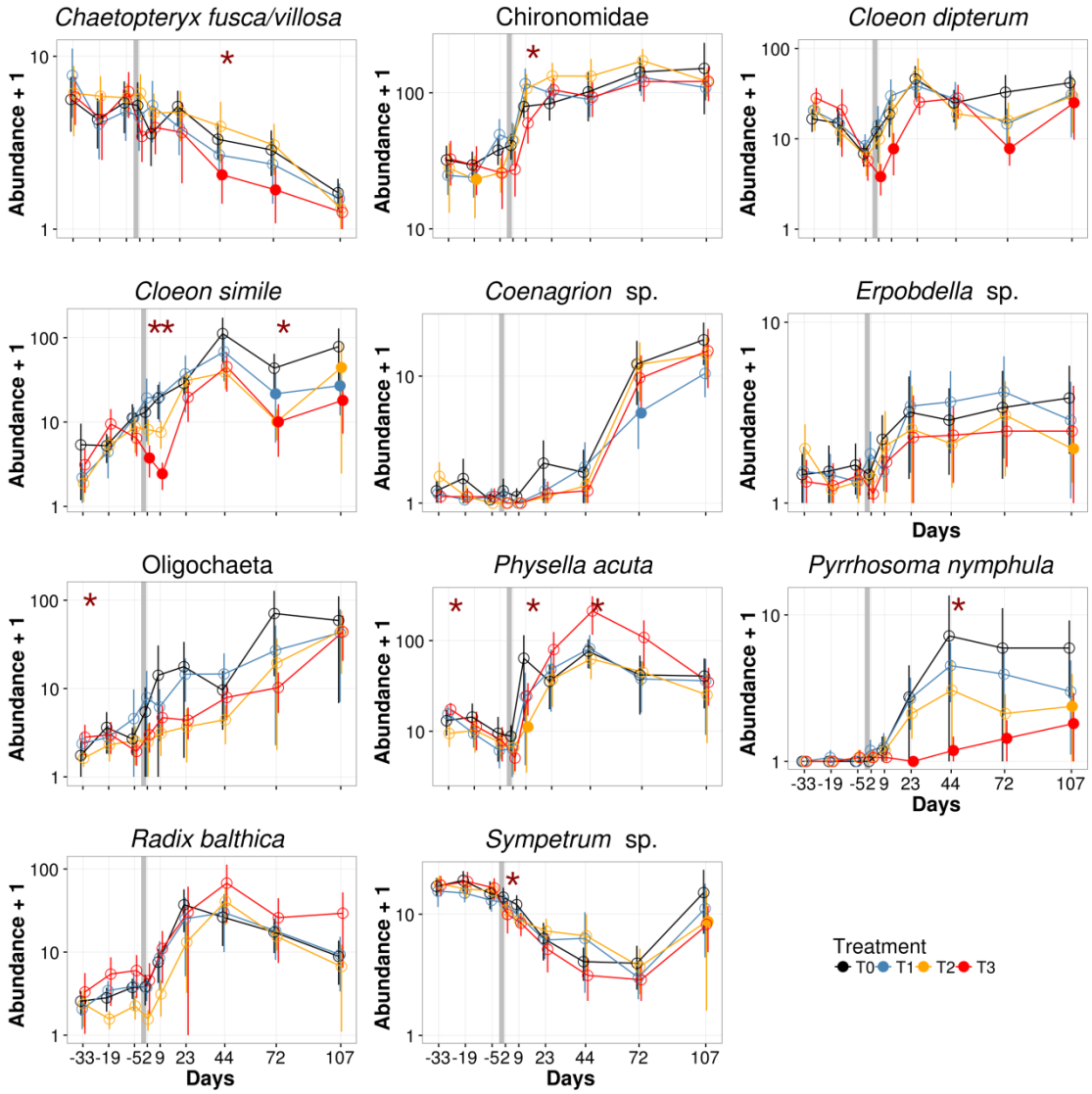

Figure 2: Trajectories of eleven taxa with mean abundance in controls greater than 1 (based on all assessed control samples). Displayed are mean abundances per 0.05 m^2 with 95% confidence intervals (CI) on a logarithmic scale. Negative CIs have been truncated to zero. Stars on top show sampling dates with statistically significant treatment effect (Likelihood-Ration Test), filled circles show statistically different treatments ($p < 0.05$) compared to control (Dunnett-contrasts). The vertical grey bar indicates treatment application.

PRCs revealed only a slight effect of etofenprox on the invertebrate communities following the pesticide application and at the end of the experiment (Figure 3, left). Most of the variance remained unexplained (62%) with the etofenprox treatment explaining 6% and time 32% of variation. On day 9 after pesticide application, statistically significant treatment effects on the community were found in the 0.5 and 5 µg/L treatment. These effects were present in the 5 µg/L treatment until day 23. For several taxa, particular mayflies and damselflies, positive species scores indicated treatment related declines in abundance. Contrary, treatment related increased abundances were indicated by negative species scores

(Figure 3, right) for the dragonfly *Anax* sp. and the snails *P. acuta* and *Radix balthica*. At the end of the experiment (day 107), the invertebrate community composition of the 5 µg/L treatment revealed significant differences relative to the community composition of the control and, thus, PRC did not indicate any recovery.

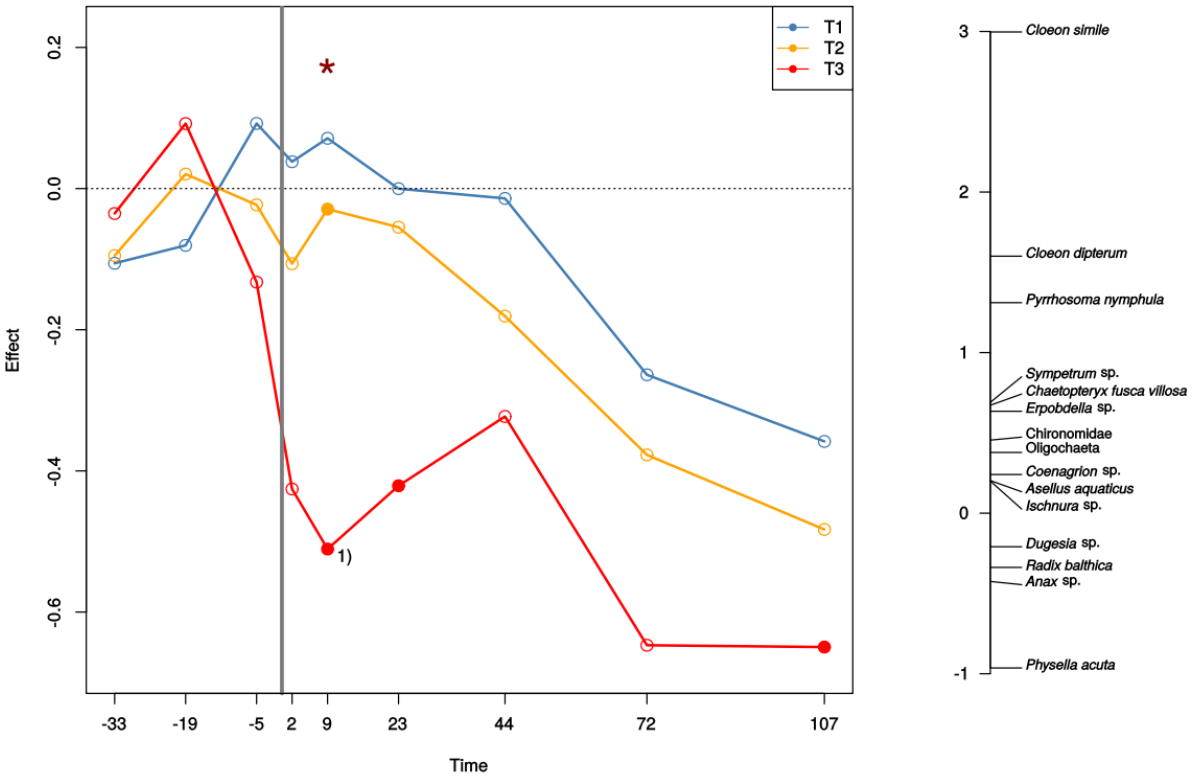

Figure 3: Principal Response Curves (PRC) indicating the effect of etofenprox on macroinvertebrate communities (left part). The dotted horizontal line represents the control and the vertical line represents the peak exposure at day 0. The first axis displays 3.3% of variation. The star on top shows the sampling date with a statistically significant treatment effect. Filled circles indicate treatments with statistically different PCA scores at each date compared to control. 1) Significant on the second PCA axis. The right part of Figure 3 display species weights with an absolute weight greater than 0.2.

Invertebrate drift - The etofenprox exposure increased significantly the drift of *C. simile*, *C. dipterum*, *Sympetrum* sp., *Notonecta maculata*, and *Polycentropus flavomaculatus* at 0.5 or 5 µg/L within the first 24 hours after application (Figure 4; Supplement Table S7). We observed higher drift of Simuliidae at the lowest treatment of 0.05 µg/L, however, variation was high.

Emergence of merolimnic insects - Most of emerged insects belonged to the families Libellulidae, Chironomidae and Baetidae. Other taxa showed rather low and heterogeneous abundances. During the first 11 days after pulse exposure the emergence of Baetidae revealed

no indication of an increased or premature emergence patter in etofenprox treatments (Figure S4). However, the emergence data from day 53 onwards corroborate the exposure-associated decrease in abundances of Baetidae in etofenprox treatments indicated by the frame sampling data.

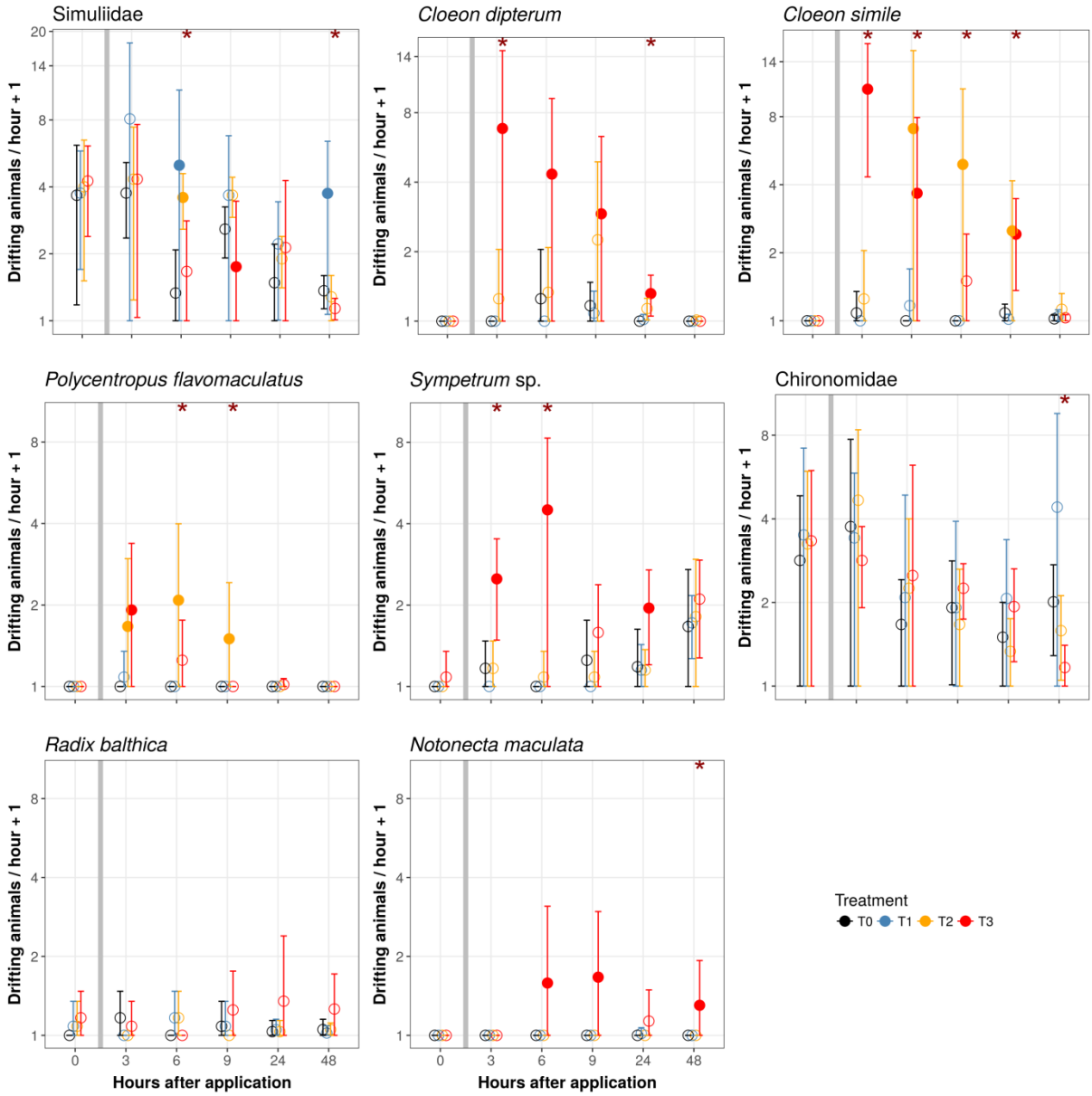

Figure 4: Effects of treatment on drift. Only the eight taxa with a mean drift rate greater than 0.15 animals per hour in the highest treatment are shown. Displayed are means and 95% CI on a logarithmic scale. Negative CIs have been truncated to zero. Stars on top show sampling dates with statistically significant treatment effect (Likelihood-Ration Test), filled circles show statistically different treatments compared to control (Dunnett-contrasts). The vertical grey bar indicates treatment application.

Functional endpoints

In the *in situ* bioassays, mean mortality in control treatments was below 10% for *A. aquaticus* yet increased in concentration-dependent manner. The feeding rates in the 0.05 and

0.5 µg/L treatments were significantly reduced by 44 and 84%, respectively (Table 3). No feeding rate could be calculated for the 5 µg/L treatment as 100% mortality of *A. aquaticus* was observed in this treatment.

Table 3: Mortality (%) and feeding rate of *A. aquaticus* (mg/mg/d± SD) after the experimental period of seven days. A nested design with 10 individual replicates in each of the streams ensuring an independent replication of 4. Asterisks indicate statistically significant differences between the treatment and the control by means of chi square testing and nested ANOVA analysis, respectively.

		Treatment		
	control	0.05 µg/L	0.50 µg/L	5.00 µg/L
Mortality (%)	8 (±10)	15 (±10)	39 (±44)*	100*
Feeding rate (mg/mg/d)	0.25 (±0.04)	0.14 (±0.03)*	0.04 (±0.02)*	-

Discussion

Structural endpoints

Abundance of invertebrates - In the highest treatment, adverse effects on the invertebrate abundance were visible for the species *C. fusca/villosa*, *C. dipterum*, *C. simile* and *P. nymphula*. Rico and Van den Brink (2015) classified the respective families to be more sensitive to pyrethroids than the average of invertebrate families covered by the ECOTOX database of the US Environmental Protection Agency (USEPA). The adverse effects on populations of *P. nymphula* in the two highest etofenprox treatments on day 107 and the indication of a concentration-response relationship throughout the sampling period indicated a high sensitivity of this species to the pyrethroid insecticide. The low sensitivities of the caddisfly *C. fusca/villosa* observed in this study might be partially explained by inhibition of the passive etofenprox uptake via a sealable case as suggested by Rasmussen et al. (2013).

Out of the 11 populations presented in Figure 3, *C. simile* was found to be the most sensitive species within the mesocosm community showing statistically decreased abundances on days 72 and 107 in all treatments. These effects thus occurred at 10-fold lower concentrations than the acute 48-h EC_{50} value for *D. magna*. This is in line with Rasmussen et al. (2013) presenting LC_{50} concentrations of *C. dipterum* being 8 times lower than the 48-h EC_{50} for *D. magna* and several pyrethroids.

Other families such as Gammaridae, Notonectidae, Simuliidae and Asselidae which were also classified as sensitivity to pyrethroid exposure (Rico and Van den Brink 2015) have been detected in low or heterogeneous abundances to allow for a statistical evaluation. Some

families, such as Notonectidae, were present in the streams and showed visual behavioral responses in the highest etofenprox treatment but individuals of this family were hardly captured during the frame sampling – likely due to a pronounced flight behavior. This indicates methodological limitation of the frame sampling for some taxa. In line with the mode-specific sensitivity classification by Rico and Van den Brink (2015), no adverse effects of etofenprox could be observed in this study for the families Chironomidae, Libellulidae and Lymnaeidae.

Some species increased in their abundance and thus might have benefited from the etofenprox exposure, e.g. both snail species *P. acuta* and *R. balthica* in the highest etofenprox treatment (Figure 2). Decreased abundances or reduced fitness of species such as *Cloeon* sp. might have reduced the inter-species competition for food and thus contributed to the snails' increased abundances. Increased abundances were also indicated for the family Aeshnidae on day 23 and 44. These findings indicate that species of Aeshnidae might have benefited indirectly from the etofenprox exposure, for instance, via elevated pray availability. Identifying the underlying mechanisms for their increased abundance should be followed up during laboratory based experiments targeting behavioral variables.

Recovery – At the population level, significantly reduced abundances mainly in the highest treatment were observed even at the end of the experimental period of 107 days for *C. simile*, *C. dipterum* and *P. nymphula* (Figure 2). These effects were also reflected at community level in the 5 µg/L treatment (Figure 3). Although there is the tendency that the differences in the community composition between 0.05 and 0.5 µg/L treatments and the control increased over time, no significant effects were demonstrated at the end of the sampling period. Other mesocosm studies using pyrethroids demonstrated recovery of invertebrates (e.g. Baetidae, Ecnomidae, Caenidae and overall biodiversity) within 62 to 149 days (Caquet et al. 2007). Thus, as external recolonization by merolimnic invertebrates was generally possible within the present study, the time study duration might have been too short to enable recovery. The possibility for stream-internal recolonization from untreated upstream sections was restricted by the experimental design of this study: solely during 3 hours prior to and the 48 hours following etofenprox injection, streams were fed with water from the storage pond and, thus, small (mesh size of the spillway = 1 mm) or juvenile individuals might have entered the streams.

Drift of invertebrates - Invertebrate drift is often divided into active (behavioral) and passive (hydraulic) drift (Naman et al. 2016). In this study, passive invertebrate drift over the whole stream length was rather unlikely due to the low flow velocity (approximately 1 cm/s) and dense vegetation at the sampling locations which retained drifting individuals as observed for instance for Notonectidae. Hence, drift numbers presented in this study were most likely based on active drift behavior.

The significantly increased drift numbers of *C. dipterum* and *C. simile* in the highest treatment was similar to the effects observed in the population analyses on day 2 (Figure 4). These findings were in line with several studies reporting increased invertebrate drift following pyrethroids pulse exposures (Heckmann and Friberg 2005; Lauridsen and Friberg 2005; Beketov and Liess 2008). Nevertheless, only 7% of the difference of *C. simile* abundance in the highest treatment between frame sampling day -5 and 2 (extrapolated from the frame samples to 40 m stream length) could be explained by drifting individuals observed over 24 hours following the exposure. This, and the aspect that no indications for treatment-related increased emergence of merolimnic insects were observed, indicates that effects on the invertebrate abundance are mainly related to lethal effects.

Functional endpoints

In this study, bioassays with individuals of *A. aquaticus* revealed significantly reduced feeding rates of the species in the 0.05 and 0.5 µg/L etofenprox treatments. Thus, the endpoint feeding rate was found to be the most sensitive sublethal endpoint. The significantly reduced feeding rate of 44% in the 0.05 µg/L etofenprox treatment indicates the potential of a brief insecticide exposure to adversely affect stream ecosystem functioning, namely the macroinvertebrate mediated leaf litter decomposition. A differentiation between direct (etofenprox dissolved in the water phase) and indirect (etofenprox adsorbed to leaf discs and subsequently ingested by the individuals) functional endpoints was not possible on the data basis available. Earlier studies, however, indicated that the interplay of both exposure pathways (waterborne and food associated) is likely responsible for the joint effect observed in the present study (Bundschuh et al. 2013). With the present approach, a NOEC could not be determined for the endpoint feeding rate, as significant effects were present even at the lowest concentration. Thus, the present study showed effects at etofenprox concentrations 10-fold lower than the acute 48 h LC_{50} value for *D. magna* (Trebon 30EC). The decreased feeding rate in the lowest treatment, likewise as the effects on structural endpoints, such as effects on *C. simile*, were close to the PEC_{sw} indicating that predicted (PEC_{sw} = 0.024 µg/L) and

measured (up to 0.2 µg/L; Tanabe et al. 2001) field concentrations might adversely affect stream ecology. If the reduction in the *in situ* measured feeding rate of *A. aquaticus* by roughly 45% can be directly related to the ecosystem process of leaf litter breakdown, as for instance suggested for gammarids (Maltby et al. 2002), realistic etofenprox concentrations may influence the energetic basis of heterotrophic food webs. Reduced feeding rates might also cause adverse effects at the population level and might also affect inter-species competition between *A. aquaticus* and other shredders (Whitehurst 1991). As suggested by Rasmussen et al. (2013) and emphasized by the results of this study, functional endpoints are highly recommended in higher-tier approaches especially in lotic systems such as stream mesocosms.

Concluding remarks

The 6-h pulse exposure of the pyrethroid etofenprox revealed significant effects below the *D. magna* 48-h LC_{50} for all investigated endpoints. Considering the LOECs for structural and functional endpoints (i.e., 0.05 µg/L) and assuming an assessment factor of 10, a hypothetical RAC of 0.005 could be derived from our study. This RAC fits well to the official tier I RAC of 0.0044 µg/L derived from single species laboratory data (EFSA 2008). Thus, the RAC of the present study represents an exception of findings of van Wijngaarden et al. (2015), who demonstrated that the majority (> 90%) of tier-1 and tier-2 RACs of insecticides were lower compared to ETO-RACs derived from micro- and mesocosm studies. Thus, the present study demonstrated that a higher-tier effect assessment not necessarily leads to a less sensitive RAC compared to lower-tier RACs. As the present RAC was based on the feeding rate of *A. aquaticus*, this study suggests these kind of individual-based functional endpoints as a supportive concept for higher-tier approaches. Furthermore, multiple exposures of the same or different pesticides and mixtures might result in higher effects and a single application might, thus, underestimate the risk under field conditions (EFSA 2013). Hence, further research is needed to evaluate the impact of realistic and repeated pulse exposures especially for insecticides or mixtures including insecticides.

Acknowledgements

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Supplemental data

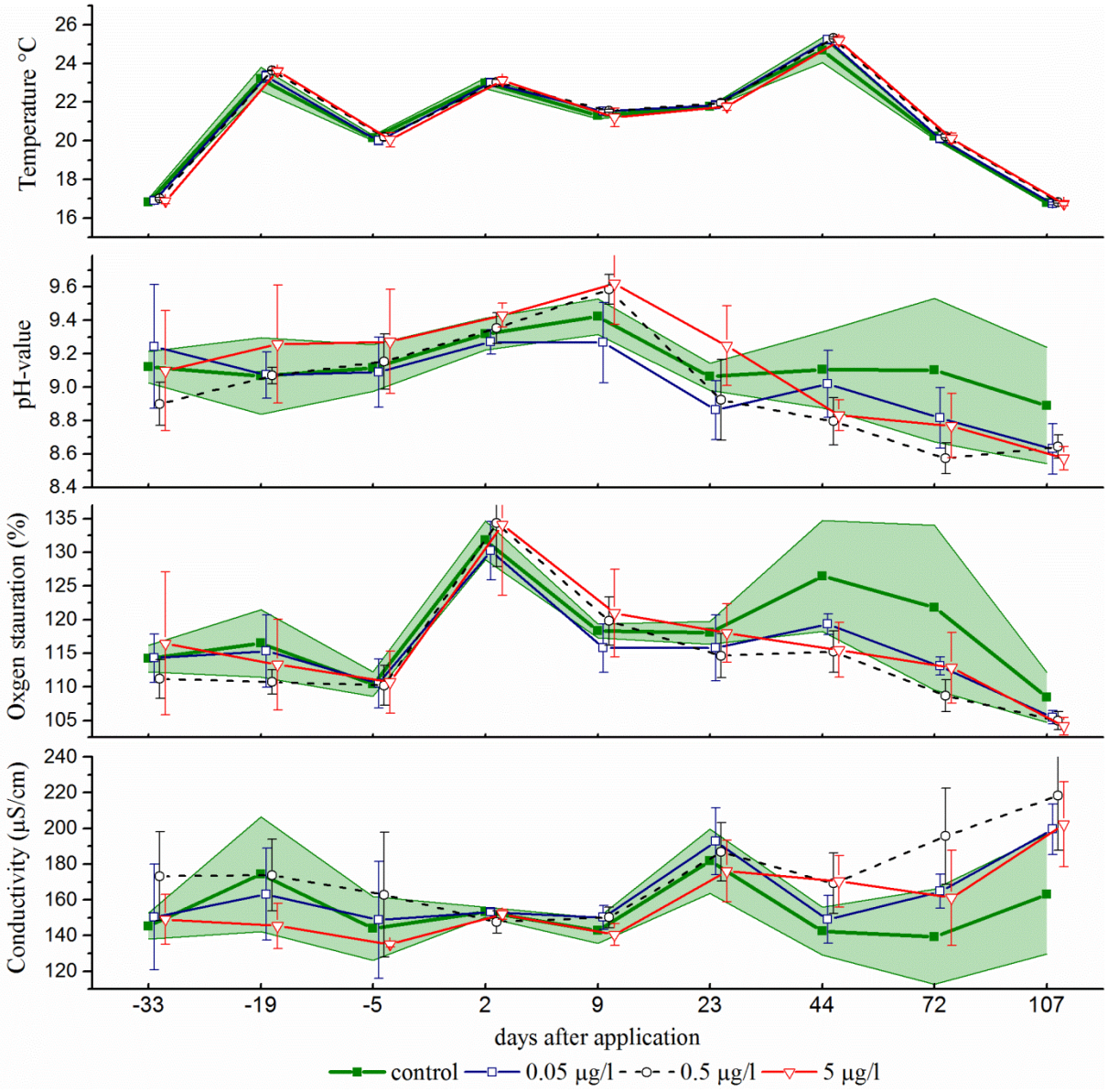

Figure S 1: Mean temperature, pH, oxygen saturation and conductivity ± SD (the green filled area displays the SD of controls). The parameters were measured in control and treatment replicates at 9 a.m. and 4 p.m. on each frame sampling date

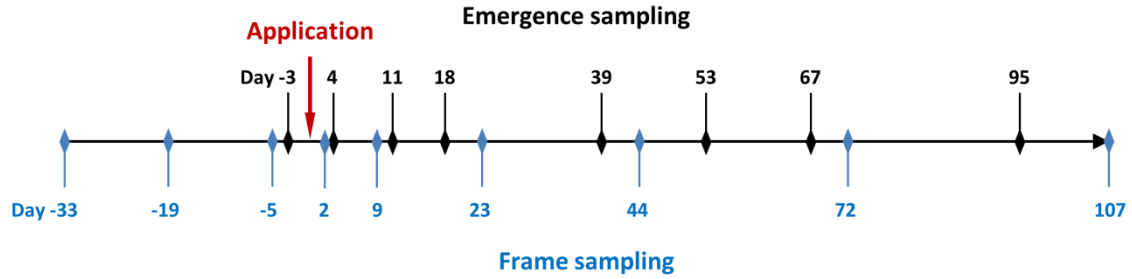

Figure S 2: Schematic time line of emergence (black) and frame (blue) sampling dates. The date of the 6-hour pulse application is indicated with an arrow (red)

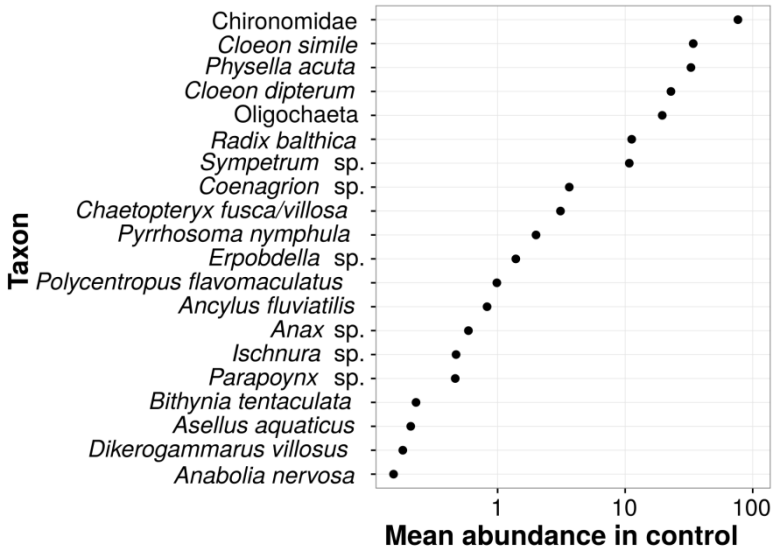

Figure S 3: Species abundance distribution in control treatments per 0.05 m^2. Only the 20 most abundant taxa are shown (total = 57 taxa)

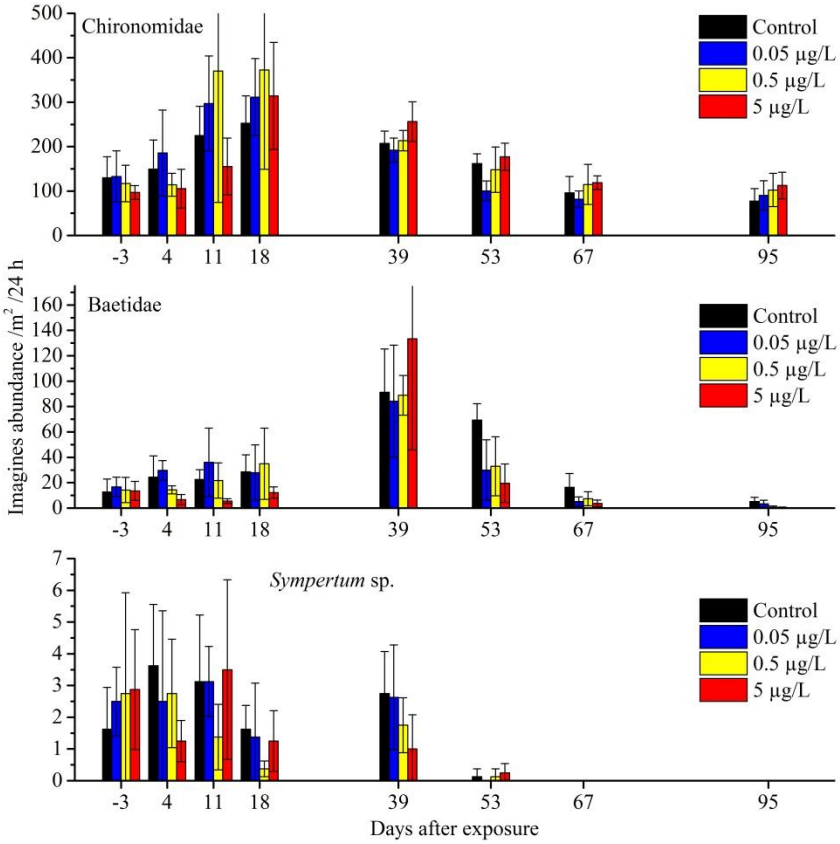

Figure S 4: Mean abundance (± SD) of emerging invertebrate imagines per m^2/24 h.

Table S 1: Mean (n = 2) values of general water quality parameters on June 6 prior to the application (a) and October 2 after the sampling period (b).

Parameter	control		0.05 µg/L		0. 5 µg/L		5 µg/L	
	a	b	a	b	a	b	a	b
Nitrate (mg/l)	0	0	0	0	0	0	0	0
Nitrite (mg/l)	0.01	0	0.01	0	0.015	0	0.01	0
Ammonium (mg/l)	0	0	0	0	0	0	0	0
Phosphate (mg/l)	0.15	0.08	0.18	0.075	0.1	0.08	0.09	0.075
Sulfate (mg/l)	< 25	<2 5	<< 25	<< 25	<< 25	<< 25	<< 25	<< 25
Total hardness (°dH)	4.5	4.5	4.125	4.625	4.375	4.625	4.25	5

Table S2: Settings of the HPLC-MS system for the chemical analysis of etofenprox

HPLC				
Pump	Accela		Surveyor	
mobile phase	Milli-Q water/MeOH		Milli-Q water/MeOH	
	00.00–02.00 min	95/5	00.00–2.00 min	98/2
	02.01–11.00 min	0/100	02.01–6.00 min	98/2
	11.01–13.00 min	95/5	06.01–11.00 min	2/98
			11.01–13.00	98/2
buffer	0.1% CH_2O_2, 4 mM NH_4HCO_2		0.1% CH_2O_2, 4 mM NH_4HCO_2	
flow rate	0.2 mL/min		0.1-1 mL/min	
Autosampler	CTC PAL		CTC PAL	
injection volume	20 µL		1000 µL	
Column	Thermo Hypersil Gold C18		Thermo Hypersil Gold aQ	
type	analytical		preconcentration	
length	50 mm		20 mm	
internal diameter	2.1 mm		2.1 mm	
particle size	1.9 µm		12 µm	
MS				
Scan and ionization				
scan range	100-2000 m/z			
spray voltage	4.0 kV			
capillary temperature	280°C			
Target compound	etofenprox		etofenprox-D5	
m/z	394.2380		399.2691	
ionization mode	ESI+		ESI+	

Table S3: List of invertebrate taxa and the respective orders or (sub) classes recorded via surber sampling. The presence of taxa was categorized in 5 classes: Out of total 9 sampling dates, taxa were present in (-) none, (r) ≤ 1, (+) 2 to 4, (++) 5 to 7, (+++) 8 or 9 of the sampling days.

Order/(sub)class	Taxa	Control				0.05 µg/L				0.5 µg/L				5 µg/L			
		C4	C6	C8	C16	C1	C5	C12	C15	C3	C10	C13	C14	C2	C7	C9	C11
Amphipoda	*Dikerogammarus villosus*[C]	r	r	+	r	-	+	r	+	r	r	r	+	r	r	+	r
	Gammarus fossarum[A]	r	r	r	r	r	r	+	r	r	+	+	r	+	r	+	-
Coleoptera	Dytiscidae (imagines)[B]	r	r	r	r	-	r	-	-	-	-	-	-	r	r	-	r
	Dytiscidae (larvae)[B]	-	-	r	-	r	-	-	-	r	-	-	-	-	-	-	-
Diptera	*Anopheles maculipennis*[C]	-	r	+	-	-	-	-	+	-	-	+	-	-	-	r	-
	Ceratopogonidae [A]	-	r	+	-	-	r	r	-	r	-	+	r	r	-	-	r
	Chaoboridae[A]	r	-	-	-	-	-	-	-	-	-	-	-	-	-	r	-
	Chironomidae	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Simuliidae	-	+	-	-	-	-	-	-	-	-	-	-	+	r	-	-
	Tabanidae[A]	-	-	-	-	-	-	-	-	-	-	r	-	-	-	-	-
	Tipulidae[A]	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Ephemeroptera	*Caenis* sp.[A]	+	r	-	-	-	-	-	-	-	-	-	-	-	-	-	r
	Cloeon dipterum [A]	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Cloeon simile [A]	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Cloeon sp. [A, D]	++	+	++	++	++	++	+	++	++	++	+	+	r	++	+	+
Gastropoda	*Ancylus fluviatilis*[B]	++	+++	-	r	r	+	-	-	++	r	-	r	+	+	+	+
	Bithynia tentaculata[B]	r	+	-	+	-	-	r	-	-	r	-	-	-	-	-	-
	Physella acuta[B]	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Planorbarius corneus[B]	-	r	-	-	-	-	-	-	-	+	-	r	r	+	-	-
	Radix balthica	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++
	Valvata p. piscinalis[B]	-	-	-	-	-	r	-	-	-	-	r	-	r	-	-	r

[A] The taxon was classified as sensitive to organic pesticides according to Liess and Von Der Ohe (2005)
[B] The taxon has a generation time of ≥ 1 year
[C] No data on the specific sensitivity were available in the SPEAR calculator for the respective taxon
[D] The taxon was not included in the count of total taxa richness

Table S4: List of invertebrate taxa and the respective orders or (sub)classes recorded via surber sampling. The presence of taxa was categorized in 5 classes: Out of total 9 sampling dates, taxa were present in (-) none, (r) ≤ 1, (+) 2 to 4, (++) 5 to 7, (+++) 8 or 9 of the sampling days.

Order/(sub)class	Taxa	Control				0.05 µg/L				0.5 µg/L				5 µg/L			
		C4	C6	C8	C16	C1	C5	C12	C15	C3	C10	C13	C14	C2	C7	C9	C11
Hemiptera	*Notonecta maculata*[B]	++	+	r	-	++	r	+	+	++	+	+	r	+	++	+	-
Hirudinea	*Alboglossiphonia* sp.[B]	r	-	+	+	-	-	r	+	r	-	-	+	r	r	r	-
	Erpobdella spp.[B]	++	++	+++	++	++	++	++	++	++	++	+++	++	++	++	+	++
	Glossiphonia complanata[B]	-	+	-	-	-	-	r	-	+	r	-	r	-	r	-	+
	Glossiphonia heteroclita	-	r	r	-	-	-	r	r	-	-	-	-	-	r	-	-
	Glossiphoniidae[D]	-	-	r	-	-	-	-	-	-	-	-	-	-	-	-	-
	Helobdella stagnalis	+	+	-	-	+	-	r	r	-	-	-	r	-	-	-	r
Isopoda	*Asellus aquaticus*	r	++	++	+	+	+	+	++	+	+	++	+	+	+	r	+
Lepidoptera	*Parapoynx* sp.[B, C]	++	++	++	++	+	+	+	r	++	r	+	+	++	+	+	++
Megaloptera	*Sialis* sp.[A, B]	-	-	-	r	-	-	-	-	-	-	-	-	-	-	-	-
Odonata	*Aeshna cyanea*[B]	-	-	-	-	-	-	-	-	-	-	r	-	-	-	-	-
	Aeshna mixta[B]	-	-	-	-	-	-	-	-	-	r	-	-	-	-	-	-
	Anax imperator[B]	+	++	+	+	+	+	+	+	+	+	+	++	+	+	+	+
	Calopteryx splendens[B]	r	r	+	r	r	+	-	r	r	+	+	+	-	+	+	r
	Calopteryx virgo[B]	-	r	-	-	r	-	r	-	-	r	-	-	-	-	-	-
	Coenagrion sp.[A, B]	++	++	++	+++	+	+	++	++	+	++	++	+	+	+	++	+
	Coenagrionidae[A, B,D]	++	++	+	++	++	++	++	++	++	+	++	++	++	++	+	++
	Erythromma sp.[A, B]	+	r	+	-	++	+	+	++	+	++	r	+	++	+	+	-
	Ischnura sp.[A, B]	++	+	+	+	+	+	+	+	+	++	+	++	+	+	r	r
	Lestes sp.[B]	-	r	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[A] The taxon was classified as sensitive to organic pesticides according to Liess and Von Der Ohe (2005)
[B] The taxon has a generation time of ≥ 1 year
[C] No data on the specific sensitivity were available in the SPEAR calculator for the respective taxon
[D] The taxon was not included in the count of total taxa richness

Table S5: List of invertebrate taxa and the respective orders or (sub)classes recorded via surber sampling. The presence of taxa was categorized in 5 classes: Out of total 9 sampling dates, taxa were present in (-) none, (r) ≤ 1, (+) 2 to 4, (++) 5 to 7, (+++) 8 or 9 of the sampling days.

Order/(sub)class	Taxa	Control				0.05 µg/L				0.5 µg/L				5 µg/L			
		C4	C6	C8	C16	C1	C5	C12	C15	C3	C10	C13	C14	C2	C7	C9	C11
Odonata	Libellulidae[B,D]	+	r	+	+	+	+	r	+	+	r	+	+	r	+	r	+
	Platycnemis pennipes[B]	-	-	-	-	+	-	-	r	+	-	-	-	-	-	-	r
	Pyrrhosoma nymphula[A, B]	+	+	+	++	++	++	+	++	++	++	++	+	+	+	r	++
	Sympetrum spp.[B]	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Zygoptera[B,D]	+	+	+	+	+	r	r	r	++	+	+	+	+	r	+	+
Oligochaeta	Oligochaeta	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Plecoptera	Plecoptera[A, B]	-	-	-	-	-	-	-	-	r	-	-	-	-	-	-	-
Trichoptera	*Chaetopteryx fusca/villosa*[A, B]	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Anabolia nervosa[A, B]	+	++	++	+	r	+	+	r	+	++	++	+	+	++	+	++
	Polycentropus flavomaculatus[A, B]	+	++	+++	+++	++	+	++	+++	+	+	++	+	+++	++	++	+
	Hydropsyche siltalai[B]	-	-	-	-	-	-	-	-	-	-	r	-	-	-	-	-
	Silo nigricornis[A]	r	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Athripsodes cinereus[A, B]	r	-	-	-	r	r	-	-	r	-	-	-	-	-	-	-
	Oecetis lacustris[A, B]	-	-	-	-	-	r	-	r	-	-	-	-	-	-	-	-
	Lepidostoma sp.[A, B]	-	-	-	-	-	r	-	-	-	-	r	-	-	-	-	-
	Sericostoma personatum/flavicorne[A, B]	-	r	r	r	-	-	-	-	r	-	-	-	-	-	r	r
	Phryganea grandis[A, B]	-	-	-	-	-	r	-	-	-	-	-	-	-	-	-	-
Tricladia	*Dugesia* sp.[B]	-	r	-	-	-	-	-	r	+	-	-	-	+	+	-	-
Turbellaria	Turbellaria	-	-	+	-	-	-	-	-	-	r	r	-	r	r	-	-
	Polycelis sp.	-	-	-	-	-	-	-	r	-	r	-	r	r	-	-	-

[A] The taxon was classified as sensitive to organic pesticides according to Liess and Von Der Ohe (2005)
[B] The taxon has a generation time of ≥ 1 year
[C] No data on the specific sensitivity were available in the SPEAR calculator for the respective taxon
[D] The taxon was not included in the count of total taxa richness

Table S6: Overview of etofenprox effects on invertebrate abundance on population level. The effect size on log-scale and the direction of the statistical effect (Dunnett-contrast) is given. Bold indicates the LOEC per endpoint and time ($p < 0.05$).

		Pre-treatment (days)			Post-treatment (days)					
Species	Cocncentration	-33	-19	-5	2	9	23	44	72	107
Chaetopteryx fusca/villosa	0.05	0.19 (0.841)	-0.15 (0.910)	-0.11 (0.960)	-0.08 (0.982)	0.54 (0.153)	-0.42 (0.318)	-0.29 (0.606)	-0.34 (0.501)	-0.14 (0.933)
	0.5	0.05 (0.996)	0.33 (0.513)	0.25 (0.723)	0.3 (0.580)	0.59 (0.102)	-0.21 (0.799)	0.05 (0.996)	0.12 (0.949)	-0.38 (0.405)
	5	-0.02 (1.000)	-0.09 (0.976)	0.27 (0.668)	-0.3 (0.601)	0.24 (0.745)	-0.66 (0.056)	**-0.7 (0.039)**	**-0.72 (0.032)**	-0.41 (0.340)
Chironomidae	0.05	-0.28 (0.579)	-0.25 (0.651)	0.16 (0.879)	-0.08 (0.983)	0.28 (0.576)	0.23 (0.697)	-0.04 (0.998)	-0.04 (0.997)	0 (1.000)
	0.5	-0.37 (0.342)	**-0.62 (0.05)**	-0.47 (0.180)	-0.03 (0.999)	0.24 (0.667)	0.49 (0.158)	0.27 (0.604)	0.27 (0.590)	0.08 (0.977)
	5	-0.14 (0.900)	-0.16 (0.861)	-0.56 (0.086)	-0.58 (0.074)	-0.36 (0.37)	0.26 (0.623)	-0.01 (1.000)	-0.17 (0.858)	0.03 (0.998)
Cloeon dipterum	0.05	0.46 (0.500)	-0.06 (0.997)	0.16 (0.957)	-0.27 (0.835)	0.05 (0.999)	-0.09 (0.992)	0.4 (0.601)	-0.62 (0.261)	-0.74 (0.143)
	0.5	0.17 (0.951)	-0.11 (0.983)	-0.09 (0.992)	-0.67 (0.205)	-0.22 (0.899)	0.11 (0.984)	0.21 (0.909)	-0.61 (0.276)	-0.75 (0.141)
	5	0.65 (0.227)	0.09 (0.991)	-0.28 (0.815)	**-1.43 (0.001)**	**-1.05 (0.023)**	-0.62 (0.267)	0.53 (0.386)	**-1.05 (0.022)**	**-1.06 (0.020)**
Cloeon simile	0.05	-0.75 (0.167)	-0.32 (0.766)	0.27 (0.844)	0.06 (0.998)	0.03 (1.000)	-0.08 (0.994)	-0.43 (0.59)	**-1.06 (0.028)**	**-1.06 (0.029)**
	0.5	-0.82 (0.114)	0.13 (0.978)	-0.17 (0.957)	-0.57 (0.363)	-0.66 (0.256)	-0.13 (0.981)	-0.78 (0.146)	**-1.3 (0.005)**	**-1.54 (0.001)**
	5	-0.45 (0.550)	0.51 (0.454)	-0.57 (0.369)	**-1.46 (0.001)**	**-2.12 (< 0.001)**	-0.92 (0.068)	-0.77 (0.151)	**-1.75 (<0.001)**	**-1.84 (<0.001)**
Coenagrion sp.	0.05	-0.11 (0.961)	-0.29 (0.566)	0.07 (0.988)	-0.1 (0.966)	-0.14 (0.920)	-0.4 (0.321)	0.13 (0.926)	**-0.68 (0.035)**	-0.59 (0.076)
	0.5	0.29 (0.566)	-0.26 (0.649)	-0.07 (0.988)	-0.24 (0.703)	-0.14 (0.920)	-0.5 (0.161)	-0.16 (0.881)	0.15 (0.899)	-0.18 (0.837)
	5	-0.14 (0.920)	-0.22 (0.743)	0.07 (0.988)	-0.24 (0.703)	-0.14 (0.920)	-0.46 (0.203)	-0.21 (0.765)	-0.08 (0.985)	-0.26 (0.659)
Erpobdella	0.05	0.04 (0.998)	0 (1.000)	-0.19 (0.878)	0.28 (0.699)	-0.38 (0.472)	-0.08 (0.986)	0.29 (0.676)	0.23 (0.796)	-0.55 (0.185)
	0.5	0.38 (0.467)	-0.21 (0.842)	-0.22 (0.811)	-0.03 (0.999)	-0.31 (0.630)	-0.48 (0.290)	-0.28 (0.687)	-0.01 (1.000)	-0.74 (0.050)
	5	-0.11 (0.969)	-0.18 (0.878)	-0.08 (0.986)	-0.31 (0.628)	-0.33 (0.577)	-0.64 (0.107)	-0.13 (0.947)	-0.07 (0.993)	-0.71 (0.064)
Oligochaeta	0.05	0.52 (0.712)	-0.19 (0.977)	-0.04 (1.000)	0.21 (0.970)	-0.14 (0.991)	0.54 (0.692)	0.41 (0.832)	-0.68 (0.529)	-0.05 (1.000)
	0.5	0.14 (0.990)	-0.47 (0.766)	-0.2 (0.974)	-0.31 (0.913)	-0.56 (0.663)	-0.7 (0.508)	-0.49 (0.745)	-0.9 (0.308)	-0.22 (0.966)
	5	0.68 (0.524)	-0.14 (0.991)	-0.36 (0.877)	0.14 (0.991)	-0.02 (1.000)	-0.29 (0.926)	0.36 (0.878)	-1.15 (0.147)	0.27 (0.943)
Physella acuta	0.05	0.33 (0.863)	-0.38 (0.812)	-0.47 (0.700)	-0.58 (0.556)	-0.93 (0.198)	-0.15 (0.984)	0.04 (1.000)	-0.48 (0.682)	-0.39 (0.799)
	0.5	-0.27 (0.92)	-0.39 (0.799)	-0.11 (0.994)	-0.77 (0.329)	**-1.66 (0.007)**	0.24 (0.942)	-0.29 (0.905)	0.63 (0.491)	-0.3 (0.894)
	5	0.49 (0.673)	-0.35 (0.840)	-0.04 (1.000)	-0.6 (0.534)	-0.37 (0.82)	0.87 (0.244)	0.91 (0.209)	1.21 (0.067)	0 (1.000)
Pyrrhosoma nymphula	0.05	0 (1.000)	0.07 (0.992)	0 (1.000)	0.21 (0.844)	0.07 (0.992)	0.22 (0.822)	-0.03 (0.999)	0.14 (0.940)	-0.62 (0.119)
	0.5	0 (1.000)	0 (1.000)	0.07 (0.992)	0.07 (0.992)	0 (1.000)	0.01 (1.000)	-0.55 (0.186)	-0.36 (0.519)	**-1.01 (0.005)**
	5	0 (1.000)	0 (1.000)	0.07 (0.992)	0.07 (0.992)	-0.1 (0.977)	**-0.85 (0.021)**	**-1.39 (< 0.001)**	**-0.81 (0.031)**	**-1.19 (0.001)**
Radix balthica	0.05	-0.36 (0.891)	0.31 (0.923)	-0.27 (0.948)	0.1 (0.997)	-0.01 (1.000)	-0.64 (0.610)	-0.41 (0.847)	-0.21 (0.974)	-0.25 (0.957)
	0.5	-0.28 (0.945)	-0.66 (0.590)	-0.7 (0.549)	-1 (0.270)	-0.98 (0.288)	-1.04 (0.240)	0.11 (0.996)	-0.03 (1.000)	-0.57 (0.691)
	5	-0.22 (0.971)	0.34 (0.902)	0.09 (0.998)	-0.2 (0.977)	0.11 (0.996)	-0.62 (0.629)	0.37 (0.882)	-0.06 (0.999)	0.9 (0.352)
Sympetrum sp.	0.05	-0.16 (0.911)	-0.23 (0.79)	-0.08 (0.989)	-0.08 (0.99)	-0.18 (0.881)	-0.03 (0.999)	0.09 (0.985)	-0.27 (0.722)	-0.5 (0.249)
	0.5	0.06 (0.995)	-0.15 (0.928)	0.11 (0.973)	-0.23 (0.804)	-0.26 (0.738)	0.2 (0.853)	0.21 (0.832)	-0.05 (0.996)	**-0.74 (0.049)**
	5	0 (1.000)	-0.02 (1.000)	0.15 (0.925)	-0.45 (0.329)	-0.38 (0.466)	-0.28 (0.686)	-0.38 (0.463)	-0.31 (0.634)	-0.62 (0.118)

Table S7: Overview of etofenprox effects on invertebrate drift. The effect size on log-scale and the direction of the statistical effect (Dunnett-contrast) is given. Bold indicates the LOEC per endpoint and time ($p < 0.05$).

		Pre-treatment	Post-treatment				
Species	Concentration	-3-0 h	0-3 h	3-6 h	6-9 h	9-24 h	24-48 h
Chironomidae	0.05	0.03 (1.000)	0.8 (0.753)	-1.17 (0.499)	-1.47 (0.317)	0.56 (0.894)	0.83 (0.734)
	0.5	0.02 (1.000)	1.23 (0.460)	-0.1 (0.999)	-0.93 (0.665)	-0.8 (0.757)	-0.58 (0.885)
	5	0.06 (1.000)	0.68 (0.832)	-0.38 (0.962)	0.43 (0.946)	0.63 (0.856)	-1.87 (0.151)
Cloeon dipterum	0.05	0 (1.000)	0 (1.000)	-0.97 (0.491)	-0.71 (0.714)	0.36 (0.946)	0 (1.000)
	0.5	0 (1.000)	0.97 (0.491)	0.71 (0.714)	1.56 (0.144)	1.9 (0.058)	0.27 (0.974)
	5	0 (1.000)	**5.37 (< 0.001)**	**2.97 (0.001)**	**2.64 (0.005)**	**2.69 (0.004)**	0 (1.000)
Cloeon simile	0.05	0 (1.000)	-0.71 (0.741)	0.87 (0.608)	0 (1.000)	-1 (0.506)	0.26 (0.982)
	0.5	0 (1.000)	0.26 (0.980)	**5.29 (< 0.001)**	**4.05 (< 0.001)**	**2.75 (0.005)**	0.97 (0.533)
	5	0 (1.000)	**5.34 (< 0.001)**	**3.74 (< 0.001)**	1.95 (0.065)	**2.75 (0.005)**	0.27 (0.977)
Notonecta maculata	0.05	0 (1.000)	0 (1.000)	0 (1.000)	0 (1.000)	0.36 (0.861)	0 (1.000)
	0.5	0 (1.000)	0 (1.000)	0 (1.000)	0 (1.000)	0 (1.000)	0 (1.000)
	5	0 (1.000)	0 (1.000)	**1.85 (0.004)**	**2.07 (0.001)**	1.15 (0.114)	**2.13 (0.001)**
Polycentropus	0.05	0 (1.000)	0.71 (0.621)	0 (1.000)	0 (1.000)	0 (1.000)	0 (1.000)
flavomaculatus	0.5	0 (1.000)	**2.07 (0.012)**	**2.97 (< 0.001)**	**2.46 (0.002)**	0 (1.000)	0 (1.000)
	5	0 (1.000)	**2.9 (< 0.001)**	1.58 (0.071)	0 (1.000)	0.36 (0.920)	0 (1.000)
Radix balthica	0.05	0.71 (0.751)	-1.42 (0.248)	1.42 (0.247)	0 (1.000)	0.36 (0.955)	-0.49 (0.898)
	0.5	0.71 (0.751)	-1.42 (0.247)	1.42 (0.247)	-0.71 (0.751)	0 (1.000)	0.19 (0.993)
	5	1.42 (0.247)	-0.71 (0.751)	0 (1.000)	0.87 (0.62)	0.9 (0.597)	0.99 (0.528)
Simuliidae	0.05	0.07 (0.999)	0.74 (0.549)	**3.28 (< 0.001)**	0.27 (0.955)	1.19 (0.184)	**1.84 (0.020)**
	0.5	0.13 (0.994)	0.07 (0.999)	**3.12 (< 0.001)**	0.54 (0.759)	1.01 (0.300)	-0.45 (0.841)
	5	0.3 (0.941)	0.04 (1.000)	1 (0.310)	**-1.72 (0.032)**	0.84 (0.444)	-0.93 (0.367)
Sympetrum sp.	0.05	0 (1.000)	-1.42 (0.167)	0 (1.000)	-1.58 (0.107)	-0.2 (0.987)	0.33 (0.951)
	0.5	0 (1.000)	0 (1.000)	0.71 (0.679)	-0.87 (0.53)	0.1 (0.998)	0.24 (0.979)
	5	0.71 (0.680)	**2.81 (0.001)**	**4.73 (< 0.001)**	1.07 (0.368)	**2.15 (0.018)**	0.73 (0.657)

Appendix IV: **Scientific publication 4**

Using stable isotope analysis in stream mesocosms to study potential effects of environmental chemicals on aquatic-terrestrial subsidies

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Abstract

While recent research has provided evidence that the emergence of merolimnic insects (specis with an aquatic larval stage) provides a considerable energy subsidy to riparian food webs it has also shown that merolimnic insects may serve as a vector for contaminants. Therefore, riparian food webs may be at risk from either an aquatic-terrestrial transfer of contaminants or from the contaminant-driven reductions of emerging merolimnic insects. The objective of the present study was to develop an integrated stream mesocosms test design capable of identifying these inter-ecosystem boundary effects and to provide a comprehensive approach as a basis for ecotoxicological testing. We chose the widely distributed web-building spider *Tetragnatha extensa* as a representative species for riparian predators. Trophic aspects of riparian food webs were investigated by stable isotope analysis of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$). Utilization of stable isotope ratios provided detailed information on the riparian food web structure and the dietary composition of *T. extensa*. Merolimnic invertebrates (mainly *Cloeon* spp. and Chironomidae) were found to contribute up to 71% of *T. extensa*'s diet, demonstrating their importance in riparian food webs in ecotoxicological mesocosm testing. This study provides a conceptual and methodological basis for assessing aquatic insect emergence-related pollutant transfer or effect translation from aquatic to adjacent terrestrial systems.

Introduction

The inter-habitat transfer of material, also referred to as subsidies (Ballinger and Lake 2006), is a key aspect of aquatic-terrestrial food web coupling (Daley et al. 2011). Taking into consideration reciprocal inter-habitat prey fluxes, the energy subsidy of adult merolimnic insects contributes 25 - 100% of riparian consumer's energy (Sanzone et al. 2003; Baxter et al. 2005; Paetzold et al. 2005). Aquatic subsidies are of great importance for consumers who preferentially focus on the emergence of merolimnic insects (Kato et al. 2003, Blanchette et al. 2014, Gergs et al. 2014) and for consumers living in less productive habitats (Sanzone et al. 2003; Paetzold et al. 2005).

Pesticide contamination has been identified in surface waters surrounded by agriculture which regularly receive high pesticide loads (Stehle et al. 2011). In such ecosystems, aquatic energy subsidies have high ecotoxicological relevance as they provide a potential vector for the transfer of contamination from aquatic environments to terrestrial ecosystems (Fairchild and Muir 1992, Walters et al. 2008, Walters et al. 2010, Tsui et al. 2012). Aquatic contamination may lead to trophic transfer of contaminants via the emergence of merolimnic

insects and subsequently to biomagnification in riparian spiders and vertebrates such as bats or birds (Walters et al. 2010). Web-building riparian spiders play a major role within aquatic-terrestrial food web coupling. They show high ingestion rates of adult merolimnic insects (Kato et al. 2003; Akamatsu et al. 2004; Ballinger and Lake 2006) and thus high contaminant residues in the presence of aquatic sediment contamination (Baxter et al. 2005; Walters et al. 2008). Therefore, the horizontal web-building spider *Tetragnatha extensa* was selected as a riparian predator suitable for the purposes of this study. Tetragnathid spiders feed on both merolimnic and terrestrial insects (Gergs et al. 2014) but prefer merolimnic insects (Kato et al. 2003; Walters et al. 2008) and are thus suitable for the evaluation of the dietary composition of inter-habitat predators. The ability to evaluate dietary intake is of use in the case of contaminant-induced alterations of the emergence of merolimnic insects. For the quantitative evaluation of the dietary composition of *T. extensa*, the analysis of stable isotopes $\delta^{13}C$ and $\delta^{15}N$ was used. The high abundance and global occurrence of tetragnathid spiders in riparian ecosystems (Williams et al. 1995; Walters et al. 2008) makes the assessment of the dietary composition in this species widely applicable.

Furthermore, the contamination of aquatic ecosystems may lead to reduced emergence of merolimnic insects (Schulz and Liess 2001; Schmidt et al. 2013) and thus reduced aquatic-terrestrial energy transfer, which may directly affect terrestrial predators directly. However, these potential interactions have been investigated only in field studies, and an experimental approach allowing for the manipulation of aquatic contamination in order to study aquatic-terrestrial energy or effect transfer does not yet exist.

The objective of the present study was to develop an integrated stream mesocosm and terrestrial habitat test design capable of identifying cross-ecosystem boundary effects and to provide a comprehensive approach for future ecotoxicological testing. In order to simulate this, 45 m long vegetated stream mesocosms (Elsaesser et al. 2013; Stang et al. 2014) coupled with caged terrestrial model ecosystems were used. The study approach integrates the implementation of the spider *T. extensa* as a riparian model predator in stream mesocosm studies and the use of stable isotopes to evaluate the aquatic-terrestrial energy transfer as a basis for specific ecotoxicological studies on contaminant-related alterations of combined aquatic-terrestrial system.

Materials and methods

Stream mesocosm design

We conducted an experiment using stable isotope analysis of spiders and their merolimnic and terrestrial insect prey in four replicated combined stream mesocosm-terrestrial model ecosystems in order to provide a setup for future ecotoxicological work on contaminant effects on aquatic-terrestrial subsidies. The study was conducted at the vegetated flow-through stream mesocosm facility, at the University of Koblenz-Landau, Campus Landau (Germany). In total, 4 high density concrete channels were used (length = 45 m; width = 0.4; average water depth = 0.26 m; Figure 1). The stream mesocosms were run in recirculation mode with flow rates of approximately 3 L s^{-1}. During the entire experimental phase (July 1 to August 20, 2012) the complete water volume of the mesocosms was renewed daily with municipal tap water. Further details of the stream mesocosm facility are described elsewhere (Elsaesser et al. 2013; Stang et al. 2014).

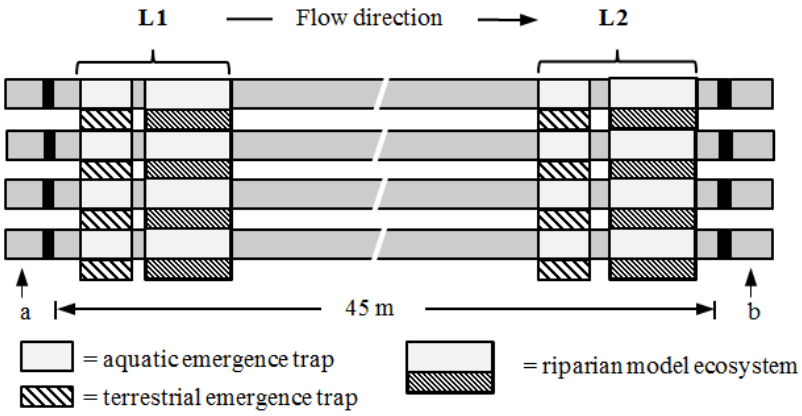

Figure 16: Schematic outline of the stream mesocosms with the two sampling sites L1 and L2, located about 5 and 40 m below the inlet, respectively. The water was pumped from the outlet (b) to the inlet (a). The stream channels are separated from inlet and outlet areas by a concrete overflow (black bars).

To establish an aquatic model ecosystem, the stream mesocosms were equipped with sieved top soil (height = 0.09 m), the macrophyte species cutleaf water parsnip (*Berula erecta* (HUDS.) Coville) and various merolimnic macroinvertes six weeks prior to the start of the experiment. *B. erecta* was collected from the Sauerbach, a small stream located in the Palatinate forest (49°05'N; 7°38'E). *B. erecta* established in the stream mesocosms a macrophyte coverage between 30 and 40% during the experimental phase. Additional organic material for shredders, such as amphipods, i.e. dried leaf material (> 95% *Alnus glutinosa)* which was pre-soaked in tap water for 12 hours was added to each stream mesocosm on a monthly basis. Thereby, the amount of leaf material added was the same for each channel and

was adapted at each addition event in order to maintain the amount of leafs present at the start of the experiment (about 109 ± 11 alder leafs per m^2) as close as possible.

To establish emerging aquatic invertebrates as prey species for riparian spiders, various taxa were added to the stream mesocosms. Mayfly species were collected at the Hainbach (49°14'N; 8°03'E), homogenized in a 80 litter container and subsamples were randomly introduced to the mesocosms at amounts considered as qualitatively equal. Additional merolimnic invertebrate species were added passively to the streams along with the macrophytes and the sediment associated with their roots. The occurrence of additional species in the stream mesocosms can be ascribed to natural colonization from nearby surface waters. Since the four streams were exactly next to each other and their setup was identical, we assume colonization to be equally distributed among the streams. In order to avoid initial drift of invertebrates, the stream mesocosms were separated into 10 m sections using polyester meshes (mesh size = 1 mm) for a period of two weeks. To reduce high solar exposure in the mesocosms, 50% of the stream surface was covered with shading funnels made of white cotton mesh (reduction of solar radiation = 40%). The reduction of radiation was within the natural range between open pasture streams (minimum 10% shading) and streams with patchy riparian shade (up to 70% shading) (Wiley et al. 1990; Quinn et al. 1997).

Abiotic parameters such as temperature, oxygen saturation, specific conductivity and pH were measured in all channels twice a week at 9 a.m. and 4 p.m. (WTW Multi 340i, WTW GmbH, Weilheim, Germany). Ammonium, nitrate, nitrite, phosphate and total hardness was measured weekly in all channels using visocolor Test-Kits (Macherey-Nagel, Düren, Germany).

The aquatic-terrestrial model ecosystem

To mimic a riparian ecosystem, aquatic-terrestrial model ecosystems with the riparian predator *T. extensa* were combined with the stream mesocosms using mesh cages (Figure 1 and 2). The cages were established at two sampling locations, 5 and 40 m (L1 and L2) downstream of the inlet (Figure 1). Each cage consisted of a wooden frame (length = 1.5 m; height = 1 m) and cotton mesh (mesh width = 2 mm; Figure 1; Figure 2). Each of the cages contained a meadow section (terrestrial meadow model ecosystem; area = 0.5 m^2) and an associated water section (aquatic model ecosystem; area = 0.6 m^2). The meadow was removed from a vegetated retention pond (49°9'N; 8°1'E). The location was chosen based on the results of a preliminary survey with ground photoeclectors (area = 0.264 m^2) at several spots around Landau, which quantified total emerged insect abundance and taxa richness (duration

= 5 days). One day prior to the start of the experiment, each spider cage was equipped with three randomly chosen meadow strips (0.5 m^2 in total). Hence, equal distribution of terrestrial insects among the meadow strips was assumed. The strips were placed beside the aquatic stream sections and were watered daily in the evening. Four wooden sticks with horizontal wires at the top were located between the meadow strip and the water in each cage. These constructions enhanced the structural complexity of the inter-habitat system and therefore provided additional possibilities for the web positioning of *T. extensa*.

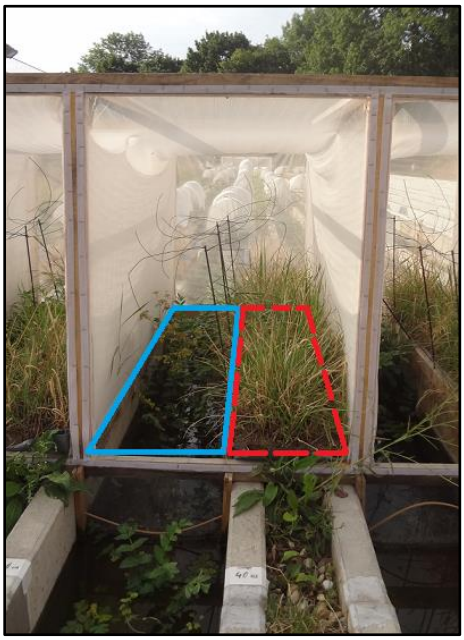

Figure 2: Photograph of one mesh-separated compartment with a schematic outline of the aquatic model ecosystem (solid line) and the terrestrial model ecosystem (dotted line)

Individuals of the *T. extensa* were collected at the Sauerbach on the 1st day of the experimental phase. Four equal-sized adult individuals of *T. extensa* (at least three females per cage) were inserted into each cage compartment within 10 hours after collection (total n = 32). During the experimental phase survival of adult spiders and the presence of clutches of eggs and spiderlings were monitored daily by visual inspection of the plants with a particular emphasis on locations preferred by the spiders.

To prevent unnatural high consumption rates of the spiders, insects were regularly removed from the cages. Insects being present on the interior cage mesh, considered as surplus insects, were removed with an aspirator at 48 – 72 h intervals and subsequently stored at -18°C for stable isotope analysis. This procedure was based on the assumption that under field conditions insects passing the areas with the horizontally-orientated spider nets and not being trapped in the net should not have the chance of being trapped at a later stage just due to

the fact that they lived in a caged environment. The aspirator was equipped with a removable collection chamber which was covered with fine-meshed net (mesh size = 100 µm).

Insect emergence and taxonomic classification

To quantify the emergence of merolimnic and terrestrial insects, representative model ecosystems equal to the meadow and water sections as described above but without spider predation were used (Figure 1). Insect emergence was quantified with emergence traps (area = 0.25 m^2) which were equipped with collecting chambers filled with water and a detergent. Insect emergence was collected continuously at the sampling locations L1 and L2 during the whole experimental phase. Terrestrial control insect emergence was quantified using meadow strips (n = 8) which were placed besides the streams (Figure 1). Emerged insects were removed at each sampling date from the collecting chambers and the inner side of the mesh material of the traps with an aspirator (sampling intervals = 48 – 72 h) and stored at -18°C until further processing.

Samples of emerged insects were identified to the lowest feasible taxonomic level (to at least the family level). Taxonomic determination of insects was done according to Johannsen (1977), Chinery (1984), Haupt and Haupt (1998), Bauernfeind and Humpesch (2001) and Bährmann (2011). Following identification, insects were dried at 60°C for at least 48 h and dry weights were determined. Dry weights of merolimnic insects (Figure 3) were averaged for the n = 4 mesocosms based on values for L1 and L2 combined. Terrestrial dry weights were averaged using the above mentioned n = 8 control meadow strips.

Sample processing and stable isotope analysis

One month after the start of the experimental phase, all remaining spiders (first generation; n = 18) were removed from the cages and subsequently transferred into 1.5 mL safe-lock tubes (Eppendorf, Hamburg, Germany). Samples were stored at -18°C until further processing. Spider bodies were subsampled by dissecting prosoma and opisthosoma in order to address potential differences in isotope assimilation among these two major body parts. Subsamples were dried at 60°C for at least 48 h until weights were constant and then ground using stirring spatulas (120 mm, 30 x 3 mm, Ø 3 mm, PS, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Afterwards, subsamples of 0.5 to 1.0 mg of ground spider tissue was weighed into tin capsules (8x5 mm, ThermoFisher Scientific) and stored at 60°C until isotope analysis.

To demonstrate the magnitude of spiders' isotope signals at the start of the experiment,

additional spiders originating from the Sauerbach (n = 3, coefficient of variation = 0.04 and 0.025 for C^{13} values of prosoma and opisthosoma, respectively) being collected at the same time as the spiders of the experiment were analyzed. To evaluate the isotope signal of spiders solely kept under experimental mesocosm conditions, individuals of the second spider generation (n = 7), that hatched until August 21, 2012, were analyzed.

For evaluation of the spiders' dietary composition, isotope ratios of potential merolimnic and terrestrial prey were analyzed. For the isotope analyses merolimnic insects were sampled on July 30 and terrestrial insects, due to low emergence rates, during the three days before this date. Whole animals were taken for measurements and were processed as described above for *T. extensa*.

The analyses of stable isotope ratios were conducted at the Landau Stable Isotope Facility with the isotope ratio mass spectrometer (Delta V Advantage) which is interfaced to a high-performance elemental analyzer Flash HT (Thermo Finnigan, Bremen, Germany). The isotope values of stable carbon and nitrogen are displayed using the standard δ notation. The δ-value is defined as the difference in per mille (‰) between the stable isotope value of the sample and the international reference standards atmospheric N_2 for nitrogen and Vienna Peedee belemnite for carbon (Paul et al. 2007, Gergs et al. 2014). The typical accuracy for repeated analyses of the international standard (±1 SD) was 0.05 and 0.12% for nitrogen and carbon, respectively (Gergs et al. 2014).

Data analysis

Unless otherwise stated, all statistical analyses were conducted with R (version 2.13.1, R core team 2014). Analysis of dietary composition was conducted with the SIAR package, stable isotope analysis in R (Parnell et al. 2010), using default settings (number of iterations = 500,000; number of initial iterations to discard = 50,000). SIAR, based on the Bayesian inference method, was used to calculate most likely dietary proportions of spiders' ingested or assimilated diets. This included trophic enrichment factors (TEFs) as recommended by McCutchan et al. (2003) for terrestrial animals 0.5 ± 0.19 ‰ and for $\delta^{13}C$ and 2.3 ± 0.24 ‰ for $\delta^{15}N$. After TEF addition, source and consumer data were plotted with standard deviations to identify consumers lying outside the mixing space (Phillips and Koch 2002; Blanchette et al. 2014). A single outlier of *T. extensa* was excluded for SIAR evaluation to prevent unlikely model solutions (Parnell et al. 2010; Smith et al. 2013). Prey species were considered as likely contributors in the case of contributions ≥ 20% to the spiders diets (Blanchette et al. 2014).

Differences in isotope ratios of spiders' prosoma and opisthosoma were investigated using analysis of variance (ANOVA) and were, due to heteroscedasticity, followed by a Games-Howell test (SPSS; Version 21). For SIAR analyses, mean values of different prey species were calculated in case of no statistical significant differences ($p > 0.05$) in isotope ratios (Phillips et al. 2005). Due to unequal sample size and heteroscedasticity, ANOVA procedure was followed by a Games-Howell test (SPSS; Version 21).

As the composition of insects being ingested in the short period prior to isotope analysis potentially affected the isotopic signal of the spiders or selected body parts thereof, the community composition of merolimnic insects was statistically analyzed for differences between L1 and L2 to exclude location-related differences in the prey composition. For this purpose the permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001; McArdle and Anderson 2001) was conducted for the last two sampling dates with detailed taxonomic classification (July 23 and 30). Prior to PERMANOVA, a permutation test of multivariate homogeneity of group dispersions (variances) was conducted.

Results

Between July 1 and July 30, emergence of merolimnic insects was observed for all stream mesocosms (Figure 3). No statistical differences were found for the community structure of adult merolimnic insects between the sites L1 and L2 for July 23 and 30, 2012 (PERMANOVA: Pseudo-F = 1.65; $p > 0.05$; n = 4). For each channel, location-independent mean values of insect isotope ratios were used for further SIAR calculations.

Over the entire experimental phase the cumulated abundance of adult merolimnic insects consisted of 93.9% Chironomidae, 4.4% Baetidae, 1.5% Trichoptera and 0.2% other taxa. The most abundant family, Chironomidae, consisted of 59.5% Tanytarsini, 16.7% Orthocladiinae and 12.8% Tanypodinae (based on the following dates: July 4, 12, 18, 23, 30). Total dry weights for the entire experimental phase consisted of 39.1% Chironomidae, 50.8% Baetidae, 9.7% Trichoptera and 0.3% other taxa. Mean dry weights of merolimnic insect were 42.1 ± 14.9 mg m^{-2} d^{-1} for days with detailed taxonomic determination (July 4, 12, 18, 23, 30) and 56.6 ± 24.6 mg m^{-2} d for the entire experimental phase, respectively.

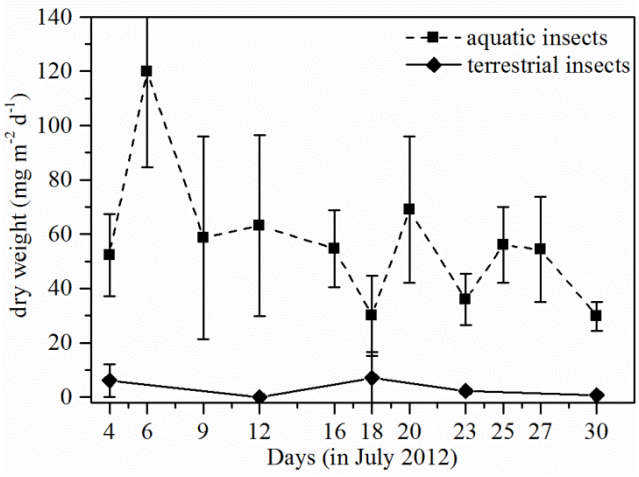

Figure 3: Average dry weights (± SD) of merolimnic and terrestrial insects.

Average terrestrial emergence rate and dry weights were highly variable and not consistent (several dates had little or no emergence). The average dry weight was 1.6 ± 1.7 mg m^{-2} d^{-1} based on the following dates: July 4, 12, 18, 23, 30 (Figure 3). The physico-chemical conditions of the stream mesocosms are displayed in Table 1, confirming that measured parameters were within the limits for small vegetated streams or slow-flowing wetlands.

Table 5: Mean values (± SD) of the physico-chemical water parameters in the stream mesocosms (July 1 – July 30)

Parameter	
pH	8.3 ± 0.2
Oxygen saturation (%)	102.1 ± 6.8
Temperature (°C)	19.9 ± 1.3
Conductivety (µS cm^{-1})	138.6 ± 2.5
Nitrate (mg L^{-1})	3.1 ± 0.14
Nitrite (µg L^{-1})	12.1 ± 0.4
Ammonium (µg L^{-1})	10 ± 1.8
Phosphate (mg L^{-1})	0.14 ± 0.02
Total hardness (°dH)	3.58 ± 0.04

Daily monitoring showed that spider web construction above aquatic, terrestrial or both compartments, prey-capture success, egg deposition and hatching of *T. extensa* occurred in all cages. At the end of the experimental phase (July 31), the survival of *T. extensa* was 56.3%. During the experimental phase, 18.8% of *T. extensa* were recorded as dead and 25.0% could not be found at the end of experimental phase.

Table 6: $\delta^{13}C$ and $\delta^{15}N$ ratios for *T. extensa* (p = prosoma; o = opisthosoma) and merolimnic and terrestrial insect taxa

Taxon	n	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
T. extensa (p)	18	-25.7 ± 1.3	3.9 ± 0.4
T. extensa (o)	18	-22.0 ± 0.8	3.6 ± 0.3
T. extensa (p; spiderlings)	7	-20.9 ± 0.5	4.8 ± 0.5
T. extensa (o; spiderlings)	7	-21.6 ± 1.1	4.0 ± 0.8
T. extensa (p; Sauerbach)	3	-28.0 ± 1.1	4.4 ± 0.7
T. extensa (o; Sauerbach)	3	-27.8 ± 0.7	4.3 ±0.3
Tanypodinae	8	-20.1 ±0.5	3.4 ± 0.3
Tanytarsini	8	-22.6 ± 1.1	2.5 ± 0.4
Orthocladiinae	7	-21.9 ± 1.3	2.8 ± 0.5
Cloeon spp.	8	-20.6 ± 1.4	1.3 ± 0.5
Oecetis lacustris	7	-19.9 ± 0.5	3.4 ± 0.3
Cicadellidae	8	-27.9 ± 1.5	3.9 ± 0.9
Staphylinidae	3	-27.1 ± 0.5	8.2 ± 0.5
Tipulidae	3	-27.2 ± 0.5	1.0 ± 0.3
Empididae	3	-26.2 ± 0.5	8.5 ± 0.9

Isotope measurements of the prey species revealed that natural $\delta^{13}C$ ratios were sufficient for a statistical significant differentiation (Games-Howell: < 0.05) of $\delta^{13}C$ merolimnic and terrestrial species signals (Figure 4, Table 2). Ratios of $\delta^{13}C$ were generally higher (factor = 1.3) for merolimnic insects compared to those of terrestrial insects. Several prey organisms showed the same $\delta^{13}C$ and $\delta^{15}N$ ratios, respectively (no statistically significant differences for each isotope ratio). For prey organisms showing no statistically significant differences for $\delta^{13}C$ and $\delta^{15}N$ isotope ratios, mean values of the respective taxa were calculated as follows: Tanytarsini and Orthocladiinae (p > 0.99), *Oecetis lacustris* and Tanypodinae (p = 1.0), Empididae and Staphylinidae (p > 0.56). Statistically significant differences in at least one of the isotope ratios were found for *Cloeon* spp., Cicadellidae and Tipulidae. These groups were thus treated individually.

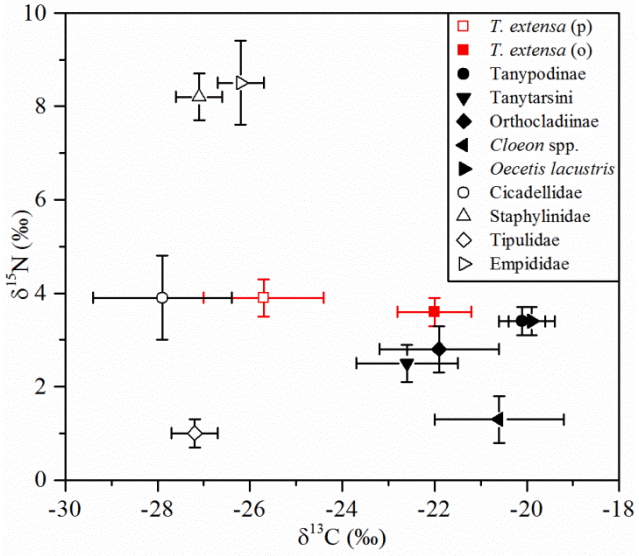

Figure 4: Average stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N \pm SD$) of *T. extensa* (red symbols: prosoma (p; open symbol) and opisthosoma (o; filled symbol)) and merolimnic (filled black symbols) and terrestrial prey (open symbol) species.

Average isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) of *T. extensa* and the merolimnic and terrestrial prey species are shown in Table 2 and displayed in Figure 4. Statistically significant differences of $\delta^{13}C$ isotope ratios ($p = 0.001$) were found for different body parts (prosoma and opisthosoma) of adult spiders sampled from the experiment, whereas no such differences ($p = 0.5$) were found for $\delta^{15}N$ isotope ratios. In order to address these differences, isotope ratios of prosoma and opisthosoma were considered separately (Table 2). No statistically significant differences for $\delta^{13}C$ and $\delta^{15}N$ isotope ratios ($p > 0.86$) were found between the opisthosoma of adult mesocosm spiders and the opisthosoma of spiderlings. Furthermore, no statistically significant differences ($p > 0.21$) were found among $\delta^{13}C$ and $\delta^{15}N$ isotope ratios for the prosoma and the opisthosoma of spiderlings (Figure 5).

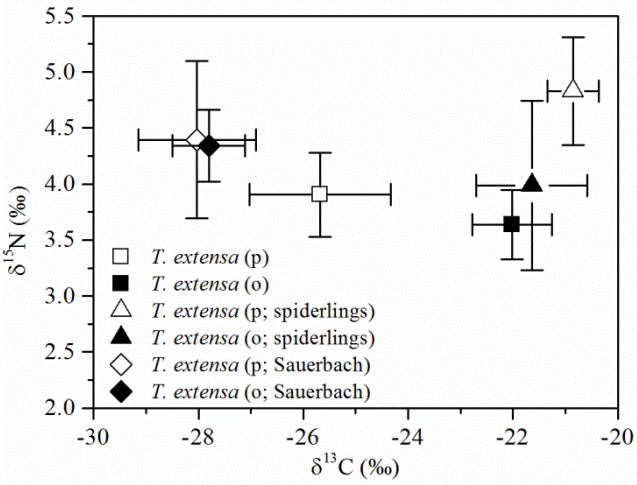

Figure 5: Average stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N \pm SD$) of prosoma (p; open symbols) and opisthosoma (o; filled symbols) of adult and juvenile *T. extensa* from the stream mesocosms and from the Sauerbach.

The prey contribution to the diet of *T. extensa* was considered separately for (A) opisthosoma and (B) prosoma. The results of the SIAR mixing model are displayed as proportion plots with 95 and 50% credibility intervals (Figure 6). *Cloeon* spp. (merolimnic prey species) and Tipulidae (terrestrial prey) were identified as the two major contributors to the diet of *T. extensa*. According to the opisthosoma (A) measurements, the diet of *T. extensa* consisted of 71% and 29% merolimnic and terrestrial prey, respectively. The mean contribution of the major contributors to the diet of *T. extensa* was 62% and 26% of merolimnic *Cloeon* spp. and terrestrial Tipulidae, respectively.

According to the prosoma (B) measurements, total prey contribution to the diet of *T. extensa* was 32% and 68% of merolimnic and terrestrial prey, respectively. The mean

contribution of the major contributors to the diet of *T. extensa* was 16% and 58% of merolimnic *Cloeon* spp. and terrestrial Tipulidae, respectively.

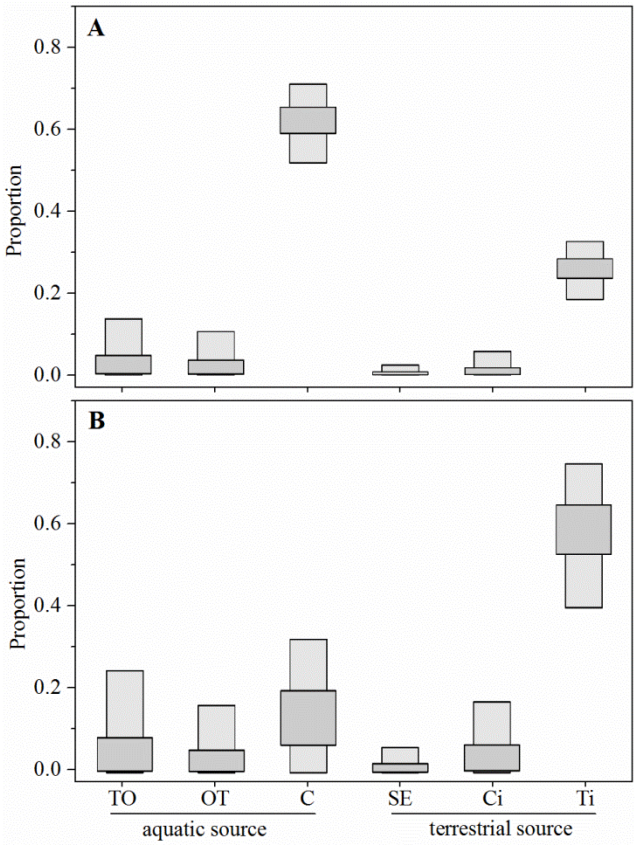

Figure 6: SIAR output with 95 (light grey) and 50% (dark grey) credibility intervals, showing the estimated prey contribution to (**A**) opisthosoma and (**B**) prosoma of *T. extensa*. Abbreviations of merolimnic and terrestrial prey organisms: TO = Tanytarsini and Orthocladiinae, OT = *Oecetis lacustris* and Tanypodinae, C = *Cloeon* spp., SE = Staphylinidae and Empididae, Ci = Cicadellidae and Ti = Tipulidae

Discussion

The inter-ecosystem boundary approach in stream mesocosms

The inter-ecosystem boundary approach provided the vegetated stream mesocosms with an additional level of complexity. The study's ecological relevance with respect to natural ecosystems (aquatic, terrestrial and riparian compartments), reproducibility and applicability in current ecotoxicological testing is evaluated in the following discussion.

The mean dry weights of emerging insects in this study (42.1 ± 14.9 and 56.6 ± 24.6 mg m^{-2} d^{-1}) were within the range of several field studies (Kato et al. 2003; Paetzold and Tockner 2005). Dry weights of merolimnic insects below the range reported here were ~1 - 13 mg m^{-2} d^{-1} (Paetzold et al. 2008), 8.2 - 19.2 mg m^{-2} d^{-1} summarized by Jackson and Fisher (1986), 20.3 mg m^{-2} d^{-1}(Gray 1989), 30.2 ± 7.0 (Paetzold et al. 2005). Higher production of merolimnic insects was reported by Jackson and Fisher (1986) (63.3 – 130 mg m^{-2} d^{-1}). Differences in dry weight levels might be attributed to different time scales, e.g. the inclusion

of periods with low emergence in the case of an annual temporal scale, different weather conditions and different spatial scaling of the stream ecosystems. Generally, the abundance of emerged merolimnic insects varied over time. Regarding secondary aquatic production, comparability of the aquatic stream mesocosms with natural streams was demonstrated. Hence, future ecotoxicological approaches including the emergence of merolimnic insects and fluxes of potentially contaminated prey insects to terrestrial ecosystems might provide a more comprehensive addition to current testing design.

The terrestrial model ecosystem approach showed a mean dry weight rate of 1.6 ± 1.7 mg m^{-2} d^{-1} and therefore demonstrated lower emergence rates and high inherent system variability. The results of this study are at the lower end of results (3.0 ± 0.7 und 58.6 ± 17.5 mg m^{-2} d^{-1}) reported for 9 field study sites conducted within tributaries of the River Rhine (Gergs et al. 2014).

Combining aquatic and terrestrial model ecosystems provided a realistic approximation of naturally occurring riparian ecosystems. The standardized design used here combines the emergence of merolimnic and terrestrial insects and provided structural elements for riparian spiders e.g. overhanging grass and artificial wire constructions. The current inter-ecosystem boundary approach excluded allochthonous input into the streams e.g. leaf litter (Tank et al. 2010) and terrestrial insects (Kawaguchi and Nakano 2001; Kawaguchi et al. 2003; Baxter et al. 2005) and thus focused predominantly on the flux of merolimnic prey organisms to the riparian zone. Except *T. extensa*, riparian predators were excluded.

The inclusion of *T. extensa* was standardized by using only female, adult spiders of the same size. The observed egg deposition may be attributed to pre-experimental mating. The mortality of 6 individuals might have occurred due to spatial competition between the four spiders per cage or from age-related reasons. Insufficient prey supply can be excluded as a cause of mortality as mean emergence of merolimnic insects, on average 6.3 mg dry weight per spider per day, exceeded the proposed minimum daily intake of 0.87 to 2.34 mg d^{-1} reported for *Tetragnatha elongatha* (Gillespie and Caraco 1987) and successful reproduction was observed. In total, 25% of *T. extensa* (8 of 32 individuals) could not be found at the end of experimental phase, which might be referred to as unobserved mortality. As individuals of *T. extensa* were occasionally found directly outside the cages at the end of experimental phase, loss of missing *T. extensa* might be partially attributed to unobserved escape during daily monitoring or during the removal of surplus emergence.

Tetragnathid spiders are globally distributed (Walters et al. 2008; Walters et al. 2010), are relatively easy to handle, and thus appear suitable for standardized testing approaches. Their

general suitability to serve as indicator species for aquatic contamination and the transfer of contaminants to terrestrial ecosystems (Walters et al. 2010; Raikow et al. 2011; Otter et al. 2013) allows for the use of tetragnathid spiders as model riparian predators (Wise 1995). According to the PPR Panel, which is involved in pesticide risk assessment in the EU, alternative micro- or mesocosm approaches are needed to test bioaccumulation and secondary poisoning in edge-of-field ecosystems (European Food Safety Authority 2013). Hence, more investigations are needed to evaluate the suitability and the applicability of tetragnathid spiders for bioaccumulation and secondary poisoning in combined aquatic-terrestrial mesocosm testing. For reasons of simplification and standardization in future studies, the current inter-ecosystem boundary approach might be able to be simplified to cages restricted to the aquatic section which only contain single individuals of tetragnathid spiders.

Dietary composition

Statistically significant higher isotopic $\delta^{13}C$ ratios were shown for merolimnic insects in comparison to terrestrial insects and are thus in agreement with the findings reported by Akamatsu et al. (2004) and Akamatsu and Toda (2011). Inverse findings were shown by Walters et al. (2010) and Sanzone et al. (2003). The clear differentiation between $\delta^{13}C$ ratios of merolimnic and terrestrial insects in the current study is probably due to the fact that the insects from the terrestrial model ecosystem and aquatic section originated from different locations and not from adjacent locations.

Based on natural isotope ratios, the current study approach provided a detailed insight into the dietary composition of riparian spiders. The classification of Chironomids to the subfamily level and the subsequent isotope analyses provided significantly different isotope ratios and thus more detailed information compared to the conservative approach using only two groups of as prey sources (merolimnic and terrestrial insects). Total prey contributions of merolimnic insects (32 – 71%) to the diet of *T. extensa* are within the range of several studies with *Tetragnatha* sp. (Henschel et al. 2001; Kato et al. 2003; Akamatsu et al. 2004) but are lower than those reported for Tetragnathidae by Sanzone et al. (2003) and Iwata (2006). Higher contributions may be due to different habitat conditions e.g. low productive terrestrial ecosystems adjacent to a desert stream (Sanzone et al. 2003) or forest stream conditions (Iwata 2006). Nevertheless, the results of the current study reflect the expected foraging behavior of orb-weaving spiders (Tetragnathidae) to preferentially feed on emerging merolimnic insects within river habitats (Sanzone et al. 2003; Akamatsu and Toda 2011). However, high contributions of merolimnic insects to the dietary composition of spiders might be attributed to the high abundance of merolimnic prey organisms. However,

abundance does not entirely explain dietary composition as *Cloeon* spp. was less abundant than Chironomidae but contributed more to the diet of *T. extensa*. This may be due to the higher dry weight of single individuals. For the terrestrial compartment the largest contribution to spider diets was also from species with the highest dry weight (Tipulidae).

Although total prey contributions were within the range of several field studies, the differences between the prosoma and the opisthosoma of adult spiders are striking. To evaluate the overall plausibility of the results for the prosoma and the opisthosoma, isotope ratios of adult spiders from the mesocosms were compared to those from the Sauerbach and to those of spiderlings from the mesocosms. Considering $\delta^{13}C$ ratios no significant differences were demonstrated between the prosoma and the opisthosoma for spiders from the Sauerbach and spiderlings from the mesocosms (Figure 5). Contrary to these findings, isotope ratios of adult spiders from the mesocosms showed significant differences between the prosoma and the opisthosoma. Furthermore, the $\delta^{13}C$ isotope ratios of the prosoma for mesocosm spiders lay between those from the Sauerbach and spiderlings (Figure 5). Therefore, it may be concluded that mesocosm spider tissue of the prosoma is still biased by the isotope signal of the Sauerbach. This indicates that the experimental phase was too short for a complete assimilation of the community-related isotope signal of the stream mesocosms in the prosoma body tissue. As no statistical differences occurred between the opisthosoma of spiderlings and the opisthosoma of the adult mesocosm spiders it can be assumed that the utilization of the opisthosoma values is more appropriate and less prone to effects of short experimental time periods. Plausibility of opisthosoma isotope values is supported by the fact that the low dry weights of terrestrial emergence make prey contributions of terrestrial insects of 68% to the spiders' diet as found for the prosoma samples very unlikely. In the current study no gut clearance was allowed and therefore signal ratios of the opisthosoma samples might have been biased by isotope ratios of the last ingested prey. The decision against gut clearance was made based on the findings showing alterations in $\delta^{15}N$ (Hobson et al. 1993; McCutchan et al. 2003; Haubert et al. 2005) and $\delta^{13}C$ ratios (Haubert et al. 2005). Furthermore, cutting of the gastrointestinal tract might lead to the loss of hemolymph (Ponsard and Arditi 2000).

To address problems of potentially biased isotope signals, future experiments should be developed in several ways: (a) the experimental phase may be prolonged so that isotope assimilation is also possible in the prosoma of spiders, (b) the younger developmental stages of *T. extensa* may be used and therefore might provide more time for isotope assimilation and

(c) the spiderlings from the second generation may be used, whose isotope signal is solely characterized by the mesocosm community.

Further application of stable isotope mediated evaluation of the prey fluxes of merolimnic insects and aquatic-terrestrial food web coupling is possible in the case of exposure scenarios with bioaccumulating substances in stream mesocosms. Detailed information on the environmental fate of contaminants in food webs might be obtained by additional analytical quantification of contaminants in merolimnic prey species and spiders (Walters et al. 2010). This could be combined with the analysis of stable isotopes to identify the exposure routes of spiders (Walters et al. 2010) and thus quantify contributions of different merolimnic prey organisms to the total chemical body burden of the spiders.

Conclusion

Overall, this study demonstrated the application of a merolimnic invertebrate community in simulating lateral prey flux between aquatic and terrestrial sections of a riparian model ecosystem. The current approach demonstrated for the first time the potential of integrating $\delta^{13}C$ and $\delta^{15}N$ stable isotope analysis in coupled aquatic-terrestrial mesocosm testing. Utilization of stable isotope ratios provided detailed information of the riparian food web structure and thus might be applied in further projects to identify and describe the exposure pathways of contaminants in stream mesocosm food webs. For instance, applications of environmental chemicals to stream mesocosm may be used to link alterations in lateral prey fluxes of merolimnic insects with spatial changes of aquatic contamination e.g. due to macrophyte-related retention of contaminants or dispersion processes within the stream mesocosms (Stang et al. 2014). Furthermore, this study evaluated the applicability of riparian spiders as a model predator for bioaccumulation assessment in stream mesocosm testing. Assuming further development concerning the terrestrial model ecosystem and the related terrestrial emergence, this study demonstrated the potential of the cross-ecosystem boundary approach in improving our understanding of food web coupling in ecological or ecotoxicological stream mesocosm studies.

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***Appendix V*: Curriculum vitae**

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Berufliche Erfahrungen

01/2016 bis heute	**Wissenschaftlicher Mitarbeiter** Universität Koblenz-Landau
04/2016 – 09/2016	**Organisator der Sommerakademie** "International Summer Academy on Spatial Ecotoxicology and Ecotoxicological Risk Assessment – Using an Open Community Approach" an der Universität Koblenz-Landau
2012 - 2015	**Organisation und Leitung** von zwei Seminaren über Invertebraten von Fließgewässern an der Universität Koblenz-Landau
2013	**Projektleiter** "Response and recovery of the macrophytes *Elodea canadensis* and *Myriophyllum spicatum* following a pulse exposure to the herbicide iofensulfuron-sodium in outdoor stream mesocosms" in Kooperation mit Bayer CropScience
2013 - 2014	**Mitarbeiter des Projekts** "Validating models to predict the influence of aquatic macrophyte species *Elodea nuttallii* and *Myriophyllum spicatum* on the elimination of Plant Protection Products from aqueous matrices (uptake, elimination and distribution)" in Kooperation mit Bayer CropScience

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11/2010 - 12/2010	**Praktikum bei BASF SE** Abteilung: Global Product Safety & Registration Terrestrische Ökotoxikologie
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Präsentationen	**Wieczorek, MV**, Kötter, D, Gergs, R and Schulz, R. Using stable isotope analysis in vegetated flow-through stream mesocosms to study aquatic-terrestrial subsidies. SETAC 24th Annual Meeting 2016 (Basel)
	Wieczorek, MV, Bakanov, N, Stang, C, Bilancia, D, Kötter, D, Lagadic, L, Bruns E and Ralf Schulz. The Landau stream mesocosm facility. SETAC Europe 2016 (Nantes)