# Risk assessment of plant protection products in stream mesocosms with special consideration of aquatic biofilm communities and macrozoobenthos

by

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Dissertation thesis

for fulfilment of the requirements for a

Doctor of Natural Sciences (Dr. rer. nat.)

Faculty 08: Biology and Chemistry

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# List of Abbreviations

| | |
|---|---|
| ANOVA | Analysis of Variance |
| CPOM | Coarse particulate organic matter |
| CV | Coefficient of variation |
| DF | Delayed fluorescence |
| DMI | Demethylation inhibitor |
| DOM | Dissolved organic matter |
| $DT_{50}$ | Dissipation time 50 % |
| $EC_x$ | Effect concentration x |
| EEA | Extracellular enzyme activity |
| EFSA | European Food Safety Authority |
| EPT taxa | Ephemeroptera, Plecoptera, Trichoptera taxa |
| ERA | Ecological risk assessment |
| ERO | Ecological recovery option |
| $ESI^+$ | Electrospray ionisation positive mode |
| ESS | Ecosystem services |
| ETO | Ecological threshold option |
| EU | European Union |
| FFG | Functional feeding group |
| FOCUS | Forum for the coordination of pesticide fate models and their use |
| FPOM | Fine particulate organic matter |
| GD | Guidance Document |
| HLNUG | Hessisches Landesamt für Naturschutz, Umwelt und Geologie (Hessian Agency for Nature Conservation, Environment and Geology) |
| HPLC | High performance liquid chromatography |
| $LC_{50}$ | 50 % lethal concentration |
| LC-MS/MS | Liquid chromatography with mass spectrometry |
| L-DOPA | 3,4-Dihydroxy-L-phenylalanine |
| LOEC | Lowest observed effect concentration |
| LOQ | Limit of quantification |
| MDD | Minimum detectable difference |
| MRM | Multi reaction monitoring |
| MUF | Methyl-umbelliferone |
| NOEC | No observed effect concentration |
| OECD | Organisation for economic co-operation and development |

| | |
|---|---|
| PBS | Phosphate-buffer saline |
| PCR-DGGE | Polymerase chain reaction-denaturing gradient gel electrophoresis |
| PEC | Predicted environmental concentration |
| PECsw;max | Maximum predicted environmental concentration in surface water |
| PECsw;twa | Predicted environmental concentration in longer-term time-weighted average concentration in surface water |
| PET | Polyethylene |
| PPDB | Pesticide properties database |
| PPP | Plant protection product |
| PRC | Principal response curve |
| RAC | Regulatory acceptable concentration |
| RDA | Redundancy analysis |
| RFU | Relative fluorescence unit |
| SD | Standard Deviation |
| SLWB | Small lotic water bodies |
| SPE | Solid phase extraction |
| $SPEAR_{pesticide}$ | Species at risk against pesticides |
| SPG | Specific protection goal |
| SSD | Species sensitivity distribution |
| UPLC | Ultra performance liquid chromatography |
| WWF | World Wide Fund for Nature |

# Zusammenfassung

Süßgewässer stellen für uns Menschen essentielle Ökosystemleistungen bereit. Insbesondere der Einsatz von Pflanzenschutzmitteln auf landwirtschaftlichen Flächen kann enorme Einflüsse auf das aquatische Nahrungsnetz haben. Innerhalb der prospektiven Risikobewertung von Pflanzenschutzmitteln können Higher-Tier-Studien in Form von aquatischen Mesokosmosstudien im Halbfreiland Erkenntnisse im ökosystemaren Kontext liefern. Hierbei ist besonders wichtig, dass in der zu testenden Lebensgemeinschaft besonders empfindliche Arten (*vulnerable species*) vertreten sind, z.B. Makroinvertebraten mit einer langen Generationszeit. Der Anteil dieser Arten ist in Fließgewässern häufig höher als in Stillgewässern. Des Weiteren spielen Mikroorganismen eine wichtige Rolle in Fließgewässern, da sie z.B. Laub zersetzen und die so enthaltenen Nährstoffe anderen Organismen verfügbar machen. Innerhalb der heute üblichen ökotoxikologischen Risikobewertung von Pflanzenschutzmitteln werden allerdings mit Ausnahme von Algen keine direkten bzw. indirekten Effekte auf die Mikroorganismengemeinschaft in aquatischen Labor- bzw. Halbfreilandstudien berücksichtigt.

In der vorliegenden Arbeit wurden Ansätze entwickelt, mit denen das Risiko von Pflanzenschutzmitteln auf repräsentative Fließgewässerbiozönosen bewertet werden kann. Hierbei wurden neu entwickelte Fließgewässermesokosmen verwendet. Das Augenmerk lag dabei auf Makroinvertebraten und Mikroorganismen. In der ersten Studie wurde das Pflanzenschutzmittel Carbaryl mit insektizider Wirkung als Modellpestizid verwendet. Die zweite Studie ging die Herausforderung an, das Risiko eines Fungizids (Modellfungizid Tebuconazol) auf die aquatische Biozönose, vor allem Mikroorganismen, zu untersuchen und gleichzeitig neu entwickelte Methoden für die Risikobewertung in die Mesokosmosprüfungen einzuführen.

Innerhalb der beiden Studien wurden die neu entwickelten Fließgewässermesokosmen realitätsnah entsprechend einem kleinen Fließgewässer in der Nähe einer landwirtschaftlichen Fläche bestückt und es konnten pestizidbedingte Effekte bei Makroinvertebraten und Mikroorganismen beobachtet werden. Diese Fließgewässermesokosmen besitzen eine hohe ökologische Aussagekraft und können innerhalb des höherstufigen Testverfahrens zur aquatischen Risikobewertung von Pflanzenschutzmitteln verwendet werden.

# Abstract

Freshwaters provide essential ecosystem services. Particularly, the use of plant protection products in agricultural areas and other chemicals used in industry might influence the aquatic food web. Within the prospective risk assessment of plant protection products, higher-tier studies with semi-field aquatic mesocosms can provide further information on the risk of a pesticide on ecosystem level. Hereby, it is important that vulnerable species are represented within the tested aquatic biocenosis, e. g. macroinvertebrates with long generation times or minor fecundity. The number of vulnerable species is often higher in lotic waterbodies compared to lentic ones. Furthermore, microorganisms play an important role in running waters, because they e.g. decompose leaf litter and in doing so they make nutrients available for other aquatic organisms. However, within the current ecotoxicological risk assessment of plant protection products no direct and indirect effect on microorganism community apart from algae will be assessed in laboratory and semi-field studies.

In the present work approaches were developed to assess the risk of plant protection products on representative lotic waterbody biocenosis. Here, newly constructed stream mesocosms were used for conducting two higher-tier studies. Close attention was focused on macroinvertebrates and microorganisms. In the first study the plant protection products carbaryl with insecticidal mode of action was used as a model pesticide. Focus of the second study was the risk assessment of fungicides on the aquatic biocenosis and simultaneously develop new suitable methods for the risk assessment of fungicides within higher-tier studies on aquatic microorganisms. Tebuconazole was used as model fungicide.

For both case studies the newly constructed stream mesocosms were realistically assembled representing a small lotic waterbody within an agricultural area and pesticide related effects on macroinvertebrates and microorganisms were observed. The newly constructed stream mesocosms possess a high ecological force of expression and can be used within aquatic risk assessment of plant protection products and other chemicals for higher-tier testing.

# 1 Introduction

## 1.1 Importance of Freshwater ecosystems

According to the International Convention on Biological Diversity an ecosystem is defined as "*a dynamic complex of plant, animal and micro-organism communities and their non-living environment interacting as a functional unit*"(Secretariat of the Convention on Biological Diversity, 2005). Aquatic freshwater ecosystems like lakes, reservoirs, rivers, and wetlands are widely spread over the continents and of high importance for provisioning of *ecosystem services* (ESS). Although less than 1 % of the global land surface area is covered by inland waters, they represent about 6 % of global biodiversity and up to 35 % of vertebrate diversity (Balian et al., 2008; Rinke et al., 2019). Next to a high proportion of global diversity, freshwater ecosystems provide habitat for a great quantity of endemic species (Dudgeon et al., 2006). According to the Millennium Ecosystem Assessment ,water, energy and nutrients are the centrepieces for the delivery of ESS to humankind (Millennium Ecosystem Assessment, 2005). While *ecosystem services* are defined as "*the benefits people obtain from ecosystems*"(Millennium Ecosystem Assessment (MEA), 2005), they are distributed into four major categories (Fig. 1). *Provisioning Services* of inland waters include for example the production of food and fibres or the direct or indirect use of water (Rinke et al., 2019). *Regulating Services* of inland waters are e.g. biological self-purification, the regulation of local hydrology or air quality, while *Supporting Services* from inland waters contain indirect benefits and long-term stability like soil formation, nutrient cycling, and carbon sequestration. Another important EES category is *Cultural Services*, which covers non-material values like recreation, tourism or educational, ethical, and aesthetic values. Often, freshwater only counts as "provisioning" service because it serves as drinking water, for domestic use, for agricultural and industrial use, irrigation, power generation, transportation and several more (Millennium Ecosystem Assessment, 2005).

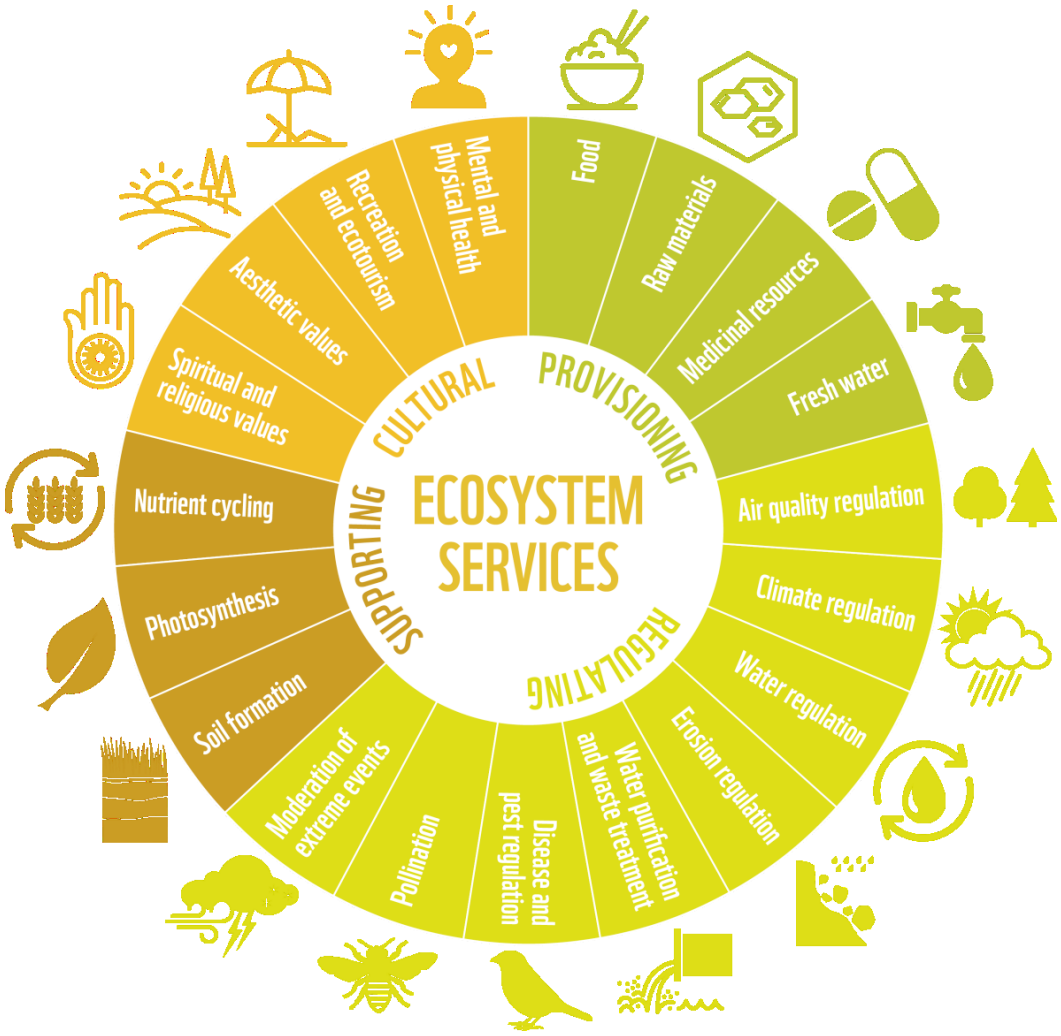


**Fig. 1: Distribution of e*cosystem services* in four categories: *Provisioning, regulating, supporting* and *cultural ecosystem services* (source WWF, 2016)**.

### 1.1.1 Lotic water bodies

Fresh surface waters are divided into rivers (lotic surface waters), lakes/ponds (lentic surface waters), reservoirs and wetlands. In Germany, 2.4 % of the surface area is covered by water, while a high proportion is covered by lotic waters (Jekel et al., 2013). One of the main characteristics of lotic water bodies is the permanent horizontal (unidirectional) water movement, which affects the morphology of streams, sedimentation patterns, water chemistry and biology of organisms inhabiting them (Wetzel, 2001). Lotic surface waters can be characterized according to their longitudinal zonation in comparison to the horizontal zonation in lakes and ponds. Rivers can be subdivided into three principal zones (upstream-downstream zonation): The area near the source of the river ("*crenon*"), the steep and torrential upper course ("*rithron*") and the flat, slow-flowing lower course ("*potamon*" Hawkes, 1975; Illies & Botosaneanu, 1963). The *crenon* zone is identified by low temperatures, reduced oxygen

content and slight flow velocities. In comparison the *rhitron* zone consists of a coarse substrate (rocks, rubble, pepples, and gravel), the monthly mean temperature does not exceed 20 °C, the dissolved oxygen concentration is equal to saturation and the flow is mostly fast and turbulent (Angelier, 2019; Lamberti et al., 2007). In contrast to the *rhitron* zone, the *potamon* zone is identified by a slow-flowing lower course, where the monthly mean temperatures may exceed 20 °C and the oxygen level and flow velocity are lower. Small lotic water bodies (SLWB), which are often located in agricultural landscapes and are frequently characterised by the *rhitron* zone, represent a large proportion of the surface water resources and the species composition can be very heterogenous (Biggs et al., 2014; Lorenz et al., 2017). Furthermore, the contribution of SLWB on aquatic biodiversity, which has only recently been recognised, is greater than expected from their size. For example, 91.3 % of the 544.967 km of German streams are < 3 m in width and belong to first-, second- and third-order streams (Lorenz et al., 2017). SLWB are often defined into first-, second- and third-order streams. Therefore, the SLWB are currently playing an important role for humankind, but the influence of the global population is often destructive to e.g. freshwater biodiversity or the provisioning of *ecosystem services*.

## 1.2 Aquatic food web and energy consumption

### Leaf litter decomposition and detritivores

Next to energy from sunlight, the energy from allochthonous organic material is one of the most important energy resources in small lotic water bodies (Vannote et al., 1980; Wallace et al., 1999; Wetzel, 2001; Wurzbacher et al., 2011). Allochthonous organic material originates from terrestrial ecosystems and includes leaves, stems, flowers, seeds and logs, which fall into the lotic water bodies (Benfield, 1997; Pozo et al., 1997). Organic matter (OM) from allochthonous and autochthonous (dead macrophytes, animal faeces etc., Hanlon, 1982) sources is called detritus and can be divided into three size classes: 1) coarse particulate OM (CPOM, > 1 mm), 2) fine particulate OM (FPOM, 0.5 μm – 1 mm) and 3) dissolved OM (DOM, < 0.5 μm; J. D. Allan & Castillo, 2007). For the decomposition of CPOM (e.g. litterfall) three main interacting mechanisms are essential: leaching, microbial conditioning and fragmentation (through shredding, consumption and production of faeces by invertebrates or through physical fragmentation due to water abrasion (Fig. 2; Abelho, 2001; Gessner et al., 1999; Petersen & Cummins, 1974; Webster & Benfield, 1986). During the leaching process of fresh fallen leaves into the water soluble compounds like phenolics, carbohydrates and amino

acids will be removed, which results in a rapid mass loss in the first seven days (Abelho, 2001; Bärlocher, 2005). In the simultaneously starting conditioning process, heterotrophic microorganisms like various bacteria and fungi colonise the litterfall and promote the breakdown process of the litter. In general, by breaking of complex leaf molecules into simpler ones through extracellular enzymes, mechanical alteration of leaves mainly due to fungal hyphae growing, and microbial nutrient incorporation, the chemical composition of leaf litter and other plant tissue will be modified (e.g. increased protein and lipid content, Bärlocher & Kendrick, 1975; Gessner et al., 1999). For example aquatic fungi, particularly the polyphyletic fungi group known as "aquatic hyphomycetes" (Wurzbacher et al., 2011), are one of the first microorganisms to colonise leaf litter and are able to effectively degrade the recalcitrant lignin by using extracellular enzymes like lignin-peroxidase, manganese-peroxidase or laccase (phenol oxidase; Beek, 2001; Gulis, Suberkropp, & Rosemond, 2008; Likens, 2010; Romaní, Fischer, Mille-Lindblom, & Tranvik, 2006). Leaf palatability and nutrition content for detritivores macroinvertebrate leaf shredders (aquatic insects and crustaceans such as *Tipula* sp. (Insecta, Diptera), *Gammarus* sp. (Crustacea; Amphipoda), *Asellus* sp. (Crustacea, Isopoda) etc.) is increased by microbial colonisation (Bärlocher, 1985; Cummins & Klug, 1979). Hereby, aquatic insects and crustaceans are the most common consumers of CPOM and detritivores significantly accelerate the decomposition process of leaves (Allan & Castillo, 2007). In addition to shredder organisms other macroinvertebrate functional feeding groups based on morpho-behavioural characteristics of food acquisition and food supply like collectors, scrapers, piercers and predators also play an important role within the aquatic food web and the energy processing

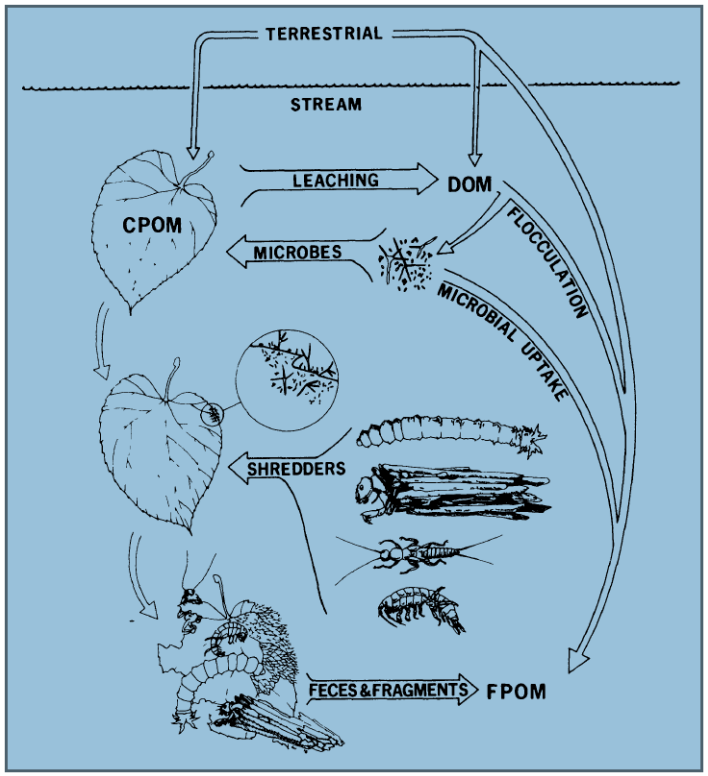


**Fig. 2: The shredder : CPOM : fungal-bacterial system and the importance in energy processing from leaf litter according to Cummins & Klug (1979).** CPOM: coarse particulate organic matter (OM); FPOM: fine particulate OM; DOM: dissolved OM.

in flowing waters. They consume among other things FPOM or ultrafine POM (Cummins & Klug, 1979).

### Primary producers (algae and higher plants)

Primary producers are autotrophic organisms, which conduct photosynthesis by using energy from sunlight. Algae, higher plants like *Potamogeton* sp., *Lemna* sp., other flowering aquatic plants and some autotrophic bacteria are important primary producers in lotic water bodies (Allan & Castillo, 2007; Lamberti et al., 2007). Particularly algal biomass is not only an important food source for herbivores, since it can also serve as a source of detritus or be collected by filtering heterotrophic organisms (Steinman et al., 2007). Especially benthic algae play a key role for primary production in small streams and ditches, because they are morphologically adapted to shear stress (Breuer et al., 2017; Lamberti, 1996; Nienhuis, 1993). They are also known as *periphyton* or biofilm, because they occur on many surfaces within lotic water bodies (Allan & Castillo, 2007). Benthic algae can be categorised according to their size (macro- and microalgae) and/or their growth on different substrates like stones (*epilithon*), sediment (*epipelon*) or sand (*epipsammon*). Free-floating algae (suspended algae) mainly originating from attached benthic algae also seem to play an important role in the food web of small lotic water bodies (Breuer et al., 2017). Aquatic invertebrates and many fish species (primary consumers) feed on *periphyton*, which is called grazing (Lamberti, 1996).

### Macroinvertrebrates

Macroinvertebrates are ubiquitous in streams and rivers and they display a high level of diversity (Hauer & Resh, 2007). This huge group of aquatic organisms consists of the phylum Arthropoda (insects, mites, scuds, and crayfish), Mollusca (snails, limpets, mussels, and clams), Annelida (segmented worms, leeches), Nematoda (roundworms) and Tubellaria (flatworms). The group can be classified for aquatic invertebrate trophic relations, according to functional feeding groups (FFG), which are based on the functional feeding mechanisms and the nutritional food resource like CPOM, FPOM, periphyton and prey (Tab. 1; Cummins, 1973; K. W. Cummins & Klug, 1979; Merritt & Cummins, 1996, 2007). Shredders feed on CPOM, while collectors feed on FPOM. In contrast, scrapers consume periphyton and predators feed on prey. Macroinvertebrates often possess special mouth parts and/or organs,

e.g. mandibles, for reaching and ingesting the preferred food sources (Merritt & Cummins, 2007).

**Tab. 1: Classification of macroinvertebrates based on functional feeding groups** modified from Merritt & Cummins, 1996). FPOM: fine particulate organic matter, CPOM: coarse particulate organic matter.

| Functional feeding group | Dominant food | Feeding mechanisms | General particle size range of food [mm] |
|---|---|---|---|
| *Shredders* | Living vascular hydrophyte plant tissue | Herbivores (chewers and miners of live macrophytes) | > 1 |
| | Decomposing vascular plant tissue and wood-CPOM | Detritivores (chewers, wood borers, gougers) | > 1 |
| *Collectors* | Decomposing FPOM | Detritivores (filterers/suspension feeders, gatherers/deposit feeders) | < 1 |
| *Scrapers* | Periphyton-attached algae and associated material | Herbivores (grazing scrapers of mineral and organic surfaces) | < 1 |
| *Piercers-Herbivores* | | Herbivores (suck contents of algal cells) | < 1 |
| *Predators* | Living animal tissue | Carnivores (attack prey, pierce tissues and cells, suck fluids or ingest whole animals (or parts)) | > 1 |

Macroinvertebrates represent a large part of the stream community and are the link between processing organic matter resources and fishes. Hereby, macroinvertebrates are considered to be one of the most sensitive groups against organic enrichment and eutrophication induced by urbanisation and agricultural activities (Brabec et al., 2004; Rosenberg & Resh, 1993). Because of macroinvertebrate presence in most habitats and their generally limited mobility and easy collectability due to established sampling techniques, they are often used for biological monitoring of freshwater ecosystems (Hellawell, 1986; Hussain & Pandit, 2012). Functional feeding groups of potentially sensitive species of Ephemeroptera, Plecoptera and Trichoptera (EPT) are widely used for the assessment of organic pollution and eutrophication biotic indices (Kolkewitz & Marsson, 1902; Rawer-Jost et al., 2000; Rolauffs et al., 2004). In this regard they are also utilised to monitor and reach the goals of the European Union Water Framework Directive (among others a "good" ecological and chemical quality status of surface waters) by tracing changes in the environment (Richter et al., 2013; Verdonschot & Nijboer, 2004).

#### Fishes and other vertebrates

In lotic food webs fishes play the most important role as vertebrates. However, amphibians, reptiles, birds, and mammals are also represented in the aquatic food web (Allan & Castillo, 2007). Out of more than 20 000 fish species, 42.5 % of them are living in freshwater (Angelier, 2019). Stream fishes are located at the top of the food webs and can be separated according to their trophic guilds: piscivore (consume fish and/or invertebrates), benthic invertebrate feeder, surface and water column feeder, generalized invertebrate feeder, planktivore (consume phytoplankton and zooplankton), herbivore-detritivore (feed on periphyton and detritus), omnivore (consume wide range of animal, plants, detritus) and parasites (Allan & Castillo, 2007; Horwitz, 1978). Because fishes are most often at the top of the aquatic food cascade, environmental pollution through eutrophication, organic compounds and other chemical substances have high impacts on the diversity and on the amount of fishes (e.g. Hamilton et al., 2016; Lewis, 1991; Pereira Maduenho & Martinez, 2008).

## 1.3 Impact of plant protection products on the environment and their risk assessment

Approximately 45 % of the annual food production is lost due to pest infestation (Abhilash & Singh, 2009). Therefore, plant protection products (= pesticides) like insecticides, herbicides, fungicides, acaricides or rodenticides are widely used throughout the world to enhance crop production and to protect plants from harmful organisms or diseases. In 2019, more than 2 million tonnes of pesticides were applied worldwide (Sharma et al., 2019). Furthermore, it is estimated that the global pesticide usage will increase up to 3.5 million tonnes by the year 2020 (Zhang, 2018). In Germany, 285 active ingredients in 872 different formulations were permitted for the use as pesticides in the year 2018 (BVL, 2019). Overall 104 634 t were sold in-country in 2018, whereby the vast amount was characterized as insecticides and acaricides (36.1 %), herbicides (32.4 %) and fungicides (26.0 %; BVL, 2019). Pesticides are an important stressor for freshwater ecosystems (Beketov et al., 2013; Sánchez-Bayo & Wyckhuys, 2019). They enter streams and ditches near agricultural areas through spray drift, drainage or surface runoff and can have severe effects on non-target aquatic organisms taxonomically related to target pest organisms (Berenzen et al., 2005; Brock et al., 2010). Direct and indirect effects of pesticides on freshwater communities can lead to changes in biocenosis, decrease of

biodiversity, or important *ecosystem services* cannot be maintained (e.g. Nabi, Youssouf & Manzoor, 2019; Nienstedt et al., 2012; Schäfer et al., 2012).

The prospective environmental risk assessment (ERA) for pesticides in edge-of-field surface waters (streams, ditches, ponds) in the European Union (EU) is based on a tiered approach (Brock, 2013; EFSA, 2013). The main objective of the prospective risk assessment is the protection of the environment and humans before a substance is released in the environment and might have harmful effects (see also Regulation 1107/2009/EC for plant protection products, EC, 2009). The Tier-1 and -2 effect assessment is based on acute and chronic laboratory single-species tests, while Tier-3 deals with experiments on population and community level in semi-field micro- and/or mesocosm experiments. Tier-4 deals with experimental field studies and landscape models (Fig. 3). By using a tiered approach it can be started with a simple effect assessment making use of data obtained from more complex and time-consuming experiments (Boesten et al., 2007; Rico & Van den Brink, 2015). Based on experimental results and by using different safety factors for acute and chronic effect assessment the regulatory acceptable concentration (RAC) for a pesticide is determined. Assuming a pesticide is applied to a certain crop under good agricultural praxis the predicted environmental concentration (PEC) for edge-of-field surface waters will be estimated by using FOCUS (Forum for the Coordination of Pesticide Fate Models and their Use) surface water scenarios and models (FOCUS 2001, 2007). In case the calculated PEC value for a pesticide is lower than the RAC, the exerted risk on surface waters by a pesticide is legally acceptable. By contrast, if the RAC is higher than the PEC, several opportunities might decrease the RAC value or increase the PEC value to enable the registration of a pesticide. Higher tier studies like semi-field outdoor studies with aquatic mesocosms assess not only the acute and chronic effect assessment on a single species, but also on population and community level under highly realistic conditions. Thus, the assessment factor for RAC derivation can be decreased to values of 2 to 3 by conducting a Tier-3 study (EFSA, 2013). In comparison the assessment factor for acute single-species tests for RAC derivation is 100, while it is 10 for chronic single-species studies. Furthermore, the predicted environmental concentration of a pesticide can be refined e.g. by reduced exposition of the crop plants or increased distance of pesticide application to surface waters.

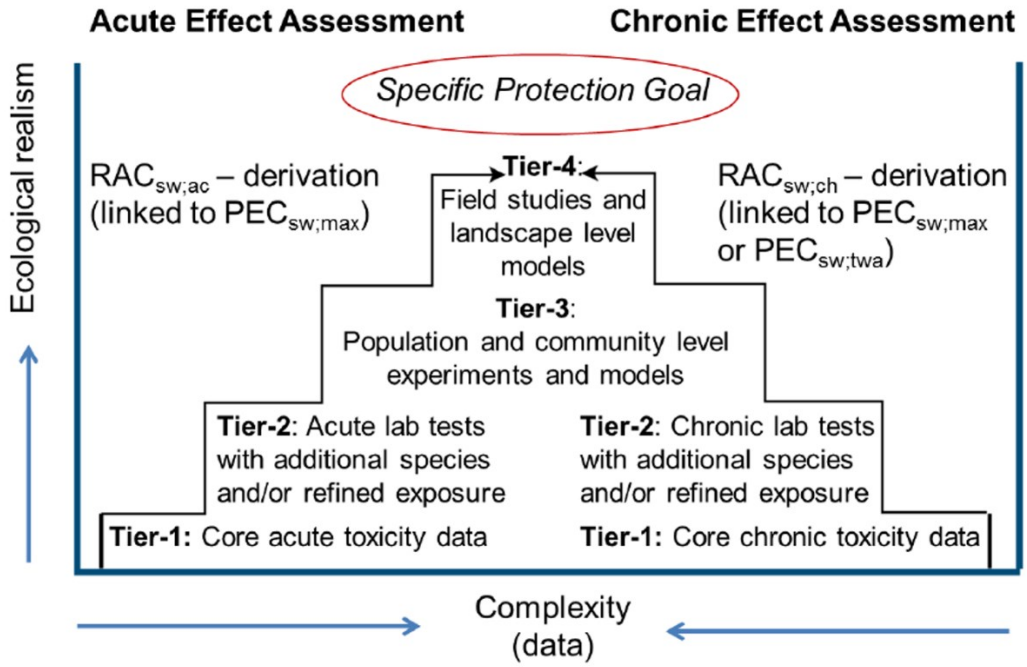


**Fig. 3: Schematic overview of acute (left part) and chronic (right part) effect assessment of pesticides within the tiered approach (Tier 1- 4).**
$RAC_{sw:ac/sw:ch}$: Regulatory acceptable concentration derived by means of acute (ac) or chronic (ch) toxicity data effect assessment scheme.
$PEC_{sw:max/sw:twa}$: Maximal (max)/ longer-term time-weighted average (twa) predicted environmental concentration; Figure modified from EFSA (2013).

In accordance to the Millenium Ecosystem Assessment the EFSA defined Specific Protection Goals (SPG) for organisms in surface waters (Fig. 3; EFSA, 2013; Nienstedt et al., 2012). The SPG of algae, macrophytes and invertebrates is the population (biomass, abundance), while for aquatic vertebrates the SPG is survival on the individual level (mortality and suffering due to acute toxicity needs to be avoided). According to the EFSA guidance document microorganisms are characterised as a functional group whose SPG is the maintenance of the functional processes they are part of (e.g. litter decomposition).

### Higher tier studies with aquatic model ecosystems

If a risk of a pesticide is observed for aquatic organisms in standard laboratory tests, higher tier studies with aquatic mesocosms may be useful to gain information on the risk of the pesticide on aquatic population and on community level. Aquatic mesocosms (artificial ponds/ditches and streams or marine/estuarine facilities) are enclosed and self-sufficient and can be seen as a link between observations in laboratory and the natural habitat (Nordberg et al., 2009). Although long-term studies in outdoor aquatic mesocosms can be costly and the

variability between replicates is increased compared to laboratory conditions, the assessment of fate and effects of pesticides and other toxicants is markedly more realistic (Fig. 4; Caquet, 2013). Under highly realistic conditions the fate of a toxicant can be assessed (e.g. adsorption onto sediment, photodegradation of solar light, bioturbation). Furthermore, direct and indirect effects of a toxicant on the aquatic community can be assessed. Direct effects are, e.g., a decrease of taxa abundance because a toxicant is lethal or impairs the physiology of the individuals. Indirect effects are, e.g., increase/decrease of taxa benefitting from declines of other taxa whose abundance was decreased due to direct effects of a toxicant (Fleeger et al., 2003). Moreover, natural abiotic factors like temperature, light, pH, oxygen level etc. will also be assessed in aquatic mesocosm studies and can influence the fate and effects of a toxicant and the response of certain taxa (EFSA, 2013; Kennedy, LaPoint, Balci, Stanley, & Johnson, 2003). Although higher tier studies with mesocosms provide essential data to evaluate possible effects of chemicals at species, population and community level under highly realistic conditions, the use of these model ecosystems in Europe and worldwide is rare compared to laboratory toxicity test facilities and only a few facilities have the ability to test pesticides and other chemicals in lotic and lentic mesocosms (Berger & Nejstgaard, 2020: http://mesocosm.org/ (Retrieved: 01 Ocotober 2020)).

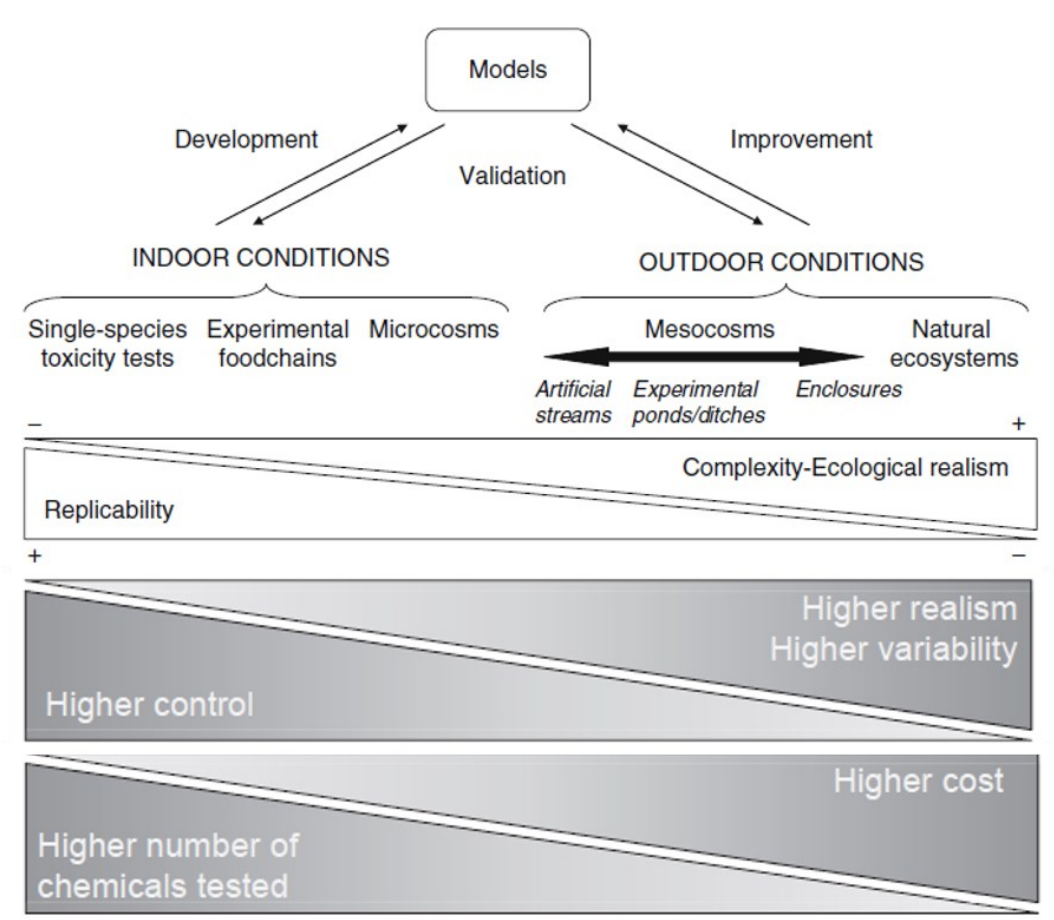


**Fig. 4: The main experimental approaches used in aquatic toxicology** (modified from Amiard-Triquet, 2015; Caquet, Lagadic, & Sheffield, 2000; Caquet et al., 1996).

### Aquatic model ecosystems in ecological risk assessment of pesticides

The use of aquatic mesocosms is explicitly mentioned for RAC derivation in Tier-3 in the EFSA aquatic guidance document (EFSA, 2013). One essential benefit of aquatic mesocosm studies is that they last longer (~2 – 3 months) compared to laboratory studies. Thus, recoveries of organisms through exposition to pesticides might be observed during the study. Hereby, the focus is on treatment-related responses of primary producers and invertebrates. Vertebrates like fishes and amphibians are normally not tested within these mesocosm studies. For the derivation of the RAC from mesocosm studies two different options can be chosen: ETO (Ecological Threshold Option) and ERO (Ecological Recovery Option). While the ETO only accepts negligible population effects, the ERO accepts population-level effects if ecological recovery takes place within an acceptable time period (~ 8 weeks). The option for the ERO-RAC derivation is only possible for data from Tier-3 mesocosm studies, while the ETO-RAC derivation is also possible for Tier-1 and -2 studies (EFSA, 2013). Within the ecological risk assessment of pesticides, static mesocosm studies simulating a lentic waterbody are conducted

most often (Brock et al., 2010; EFSA, 2019, 2013, 2009; Wieczorek, Bakanov, Stang, et al., 2016). For the ERO-RAC derivation, the studies must allow the assessment of effects and the recovery of sensitive/vulnerable taxa (EFSA, 2016). Taxa with, e.g., long generation time or low dispersal ability or macrophytes with a relatively slow growth rate are considered to be "vulnerable" (Berger et al., 2018; Kattwinkel et al., 2012). For example, Arthropoda taxa belonging to Ephemeroptera, Plecoptera, Trichoptera or to crustaceans are often considered to be sensitive / vulnerable to pesticides, especially insecticides (EFSA, 2019, 2016; Gergs et al., 2016; Rico & Van den Brink, 2015). While species sensitivity distributions (SSD) from Arthropoda taxa from lentic and lotic waterbodies seem to be similar (Maltby et al., 2005), the amount of taxa vulnerable to pesticides is higher in lotic waterbodies than in lentic waterbodies (Biggs et al., 2007). For example stoneflies (Plecoptera) exist almost entirely in running waters and more than half of the species possess an univoltine lifecycle (one brood per season, Sánchez-Bayo & Wyckhuys, 2019). They are highly sensitive to organic pollution (Biggs et al., 2007; Sánchez-Bayo & Wyckhuys, 2019). Within static mesocosms the exerted risk of pesticides for stoneflies cannot be assessed. Furthermore, small lotic waterbodies are most often in close proximity to agricultural areas and pollution by pesticides is quite high (Lorenz et al., 2017; Schulz & Liess, 1999; Tada & Shiraishi, 1994). Therefore, it should be essential to assess the effects of pesticides on a representative aquatic community present in small lotic waterbodies.

### Special requirements for the effect assessment of pesticides with specific mode of action

When substances with insecticidal mode of action are tested in acute Tier-1 studies, the EFSA GD requires to assess an additional acute toxicity test with an additional arthropod taxon (*Chrionomus* sp. or *Americamysis bahia*) next to *Daphnia* sp. (EFSA, 2013), because aquatic Arthropoda are usually most sensitive (Maltby et al., 2005). In the case of testing an insecticide in a mesocosm study, the focus of the study should be on populations of zooplankton and macroinvertebrates. In the case of herbicides, algae and/or macrophytes seem to be most sensitive (Van Den Brink et al., 2006). Thus, for Tier-1 risk assessment next to the standard toxicity tests with algae, *Dapnia* sp. and fish (e.g. *Oncorhynchus mykiss*) an additional non-green algae species and a macrophyte species (e.g. *Lemna* sp.) are tested. Primary producers like algae (phytoplankton, periphyton) and aquatic macrophytes should be focused on in a mesocosm study with a pesticide of herbicidal mode of action.

With regard to pesticides with fungicidal mode of action no additional standard tests in Tier-1 are required according to the EFSA guidance document (EFSA, 2013). It is recommended to test fungicides within a range of taxonomic groups (primary producers, macrophytes, invertebrates, vertebrates) for higher tier studies, because sensitivity of organisms against a fungicide is dependent on the mode of action (e.g. biocidal fungicide). However, within the prospective risk assessment of fungicides, with the exception of algae, no aquatic fungi or other microorganisms are included for evaluating adverse effects. Despite the fact that although non-target aquatic fungal communities seem to be quite sensitive to fungicides, e.g. ergosterol-inhibiting fungicides such as triazoles (Dijksterhuis et al., 2011; Ittner et al., 2018; Lin et al., 2012; Zubrod et al., 2015). Laboratory and semi-field studies showed that fungicides alter microorganism related processes like food processing, reduce energy reserves, effect survival of leaf-shredding macroinvertebrates or change the fungal community or ergosterol content of aquatic fungi even at low concentrations (Bundschuh et al., 2011; Dimitrov et al., 2014; Feckler et al., 2016; Rasmussen et al., 2012; Zubrod et al., 2014, 2015). Currently, several working groups are participating in developing potential laboratory studies for lower tier effect assessment with aquatic fungi and other microorganisms (Lategan et al., 2016; Lategan & Hose, 2014; Nagai, 2018). Although several semi-field mesocosm studies with fungicides exist, most often only one functional endpoint (leaf litter decomposition) associated with microbes was assessed (Maltby et al., 2009). However, to assess the risk of fungicides on microorganisms it is essential to gain further information on the effect of structural endpoints (e.g. bacterial / fungal biomass, microorganism community structure (Dimitrov et al., 2014)) and effects on higher trophic level (e.g. feeding behaviours, survival of shredders (Bundschuh et al., 2011)). Further research into the potential effects on fungi and other microorganisms is needed to cover the risk for pesticides (especially fungicides) on aquatic microorganisms adequately within the risk assessment scheme (EFSA, 2013; Ittner et al., 2018; Rico et al., 2019; Zubrod et al., 2019).

## 1.4 Aim of the thesis

The aim of this thesis was the testing of newly constructed stream mesocosms regarding the usability for higher-tier assessment studies of plant protection products and other chemicals. A broad spectrum of the biocenosis of small lotic water bodies like primary producers (e.g. algae, macrophytes), decomposers (e.g. microorganisms) and consumers (e.g. macroinvertebrates (Wogram, 2010)) should be present in stream mesocosms. The main focus of this work was on the potential risk of pesticides for macroinvertebrates and microorganisms. Both organism groups play a major role for the provisioning of *ecosystem services* in surface-waters (Nienstedt et al., 2012). The thesis was divided into two parts. The first part consists of a case study conducted in stream mesocosms with the insecticide carbaryl. Because macroinvertebrates, particularly Arthropoda, react quite sensitive to insecticides (Beketov et al., 2013), the main focus was the establishment of a macroinvertebrate community with a representative amount of potential vulnerable taxa common in lotic water bodies near agricultural areas. The assessment of effects on the macroinvertebrate community and on population level (abundance), drift of invertebrates and insect emergence was assessed. Furthermore, indirect effects on primary producers like suspended algae and periphyton were also assessed. Parts of the results from the case study (referred to as "case study I") are published in the following paper (Beuter et al., 2019):

Beuter, L. K., Dören, L., Hommen, U., Kotthoff, M., Schäfers, C., & Ebke, K. P. (2019). Testing effects of pesticides on macroinvertebrate communities in outdoor stream mesocosms using carbaryl as example test item. *Environmental Sciences Europe*, *31*(1), 1–17. https://doi.org/10.1186/s12302-019-0185-1

Regarding case study I the following questions were targeted:

1. Can populations of potentially sensitive and vulnerable species, which are characteristic for streams in agricultural landscapes, be established in the test systems?
2. How many and which species can be expected to be sufficiently abundant for effect evaluation?
3. Is there a need to modify the test systems and/or the sampling technique?
4. Considering the limited number of test systems for this pilot study, does the study provide data which are consistent with the existing data on carbaryl?

The second part of this thesis focuses on the risk assessment of a fungicide to aquatic organisms. Currently there is a big gap in the evaluation of the trophic level of destruents. Current risk assessment is based more or less on indirect effects to primary producers and consumer levels. Microorganisms form a complex and diverse group and play an important role in lotic waterbodies for example in leaf litter decomposition or by enhancing the leaf palatability for shredder organisms (Abelho, 2001). In current pesticide risk assessments no specific risk assessment scheme is developed for microorganisms with the exception of algae (EFSA, 2013). To receive more information about potential effects of fungicides on microorganisms under highly realistic exposure and assessment conditions, a second study with the triazole fungicide tebuconazole was conducted. In this study the effect of a fungicide on microorganisms was assessed by testing and developing new and innovative methods, which assess the risk on a whole bandwidth of different endpoints (functional entity: leaf decomposition, extracellular enzyme activity; structural entity: fungal biomass, bacterial biomass; effects on higher trophic levels: lipid value of a shredder organism).

Case study II aimed to answer the following questions:

1. Are selected methods available to detect ecological endpoints sensitive against fungicides?
2. Is the test system useful for the focus on the risk assessment of fungicides on bacterio- and fungi-coenosis?
3. Does the principal of the dose-response relation work in this case?
4. Does the modified higher tier study provide data which are consistent with the existing data from lower tier studies and other higher tier test systems (e.g. Dimitrov et al., 2014) for tebuconazole?

# 2 Material and methods

## 2.1 Study site and test system

Two stream mesocosm studies with several sampling techniques were conducted. Newly constructed stream mesocosms (years of construction 2013 – 2014 ) at the test site of the Mesocosm GmbH in Homberg/Ohm (Hesse, Germany, 50°45'9.9"N, 9°1'51.2"E) were used (Janz, 2016). Five artificial ponds are located in close proximity to the stream mesocosms and are in use for higher-tier studies in lentic mesocosms. The test site is surrounded by hedges and meadows protecting the experimental site from contamination by pesticides.

The stream mesocosms were built of stainless steel and possess a flow length of 10 metres in a recirculating system (Fig. 5). By using paddle wheels, two guide plates, a flow conditioner, and a middle wall for each stream mesocosm, reproducible streaming profiles were generated. The paddle wheels were driven by the same single electric motor to ensure the same average flow velocity of 0.10 – 0.11 m/s (measurement flow velocity: Flow-Mate Model 2000, Marsh-Mc Birney, INC). The mesocosm streams were filled with filtered pond water (mesh size: 63 µm) and tap water (ratio 2 : 1) up to a water level of ~30 cm (Tab. 2). The used pond water was taken from a non-polluted lake on site. Macrophytes like *Glyceria maxima*, *Myriophyllum spicatum* and *Ceratophyllum demersum* from surrounding ponds were applied. Other substrates were basalt stones originating from a quarry in Nieder-Ofleiden (Hessen, Germany) and leaves from trees (*Salix* sp., *Alnus glutinosa*) in close proximity. Most of the chosen parameters in the two case studies were similar (Tab. 2). A few differences arose, e.g., in case study II an additional habitat by supplying a sediment mixture was provided. The sediment was used from a nearby pond and mixed with sand (ratio 1 : 1). Sediment covered the bottom with a flow length of one metre (1 m x 0.50 m). The stream mesocosms were colonised with macroinvertebrates from streams (for details see chapter 2.2). Because case study I was performed during summer, a tent was installed as sun protection for four times to avoid too high water temperature (see. Fig. 14 a). The tent was installed from day 7 until day 2 before carbaryl application, day -1 until day 8, day 13 to 18 and day 20 to 26 after application.

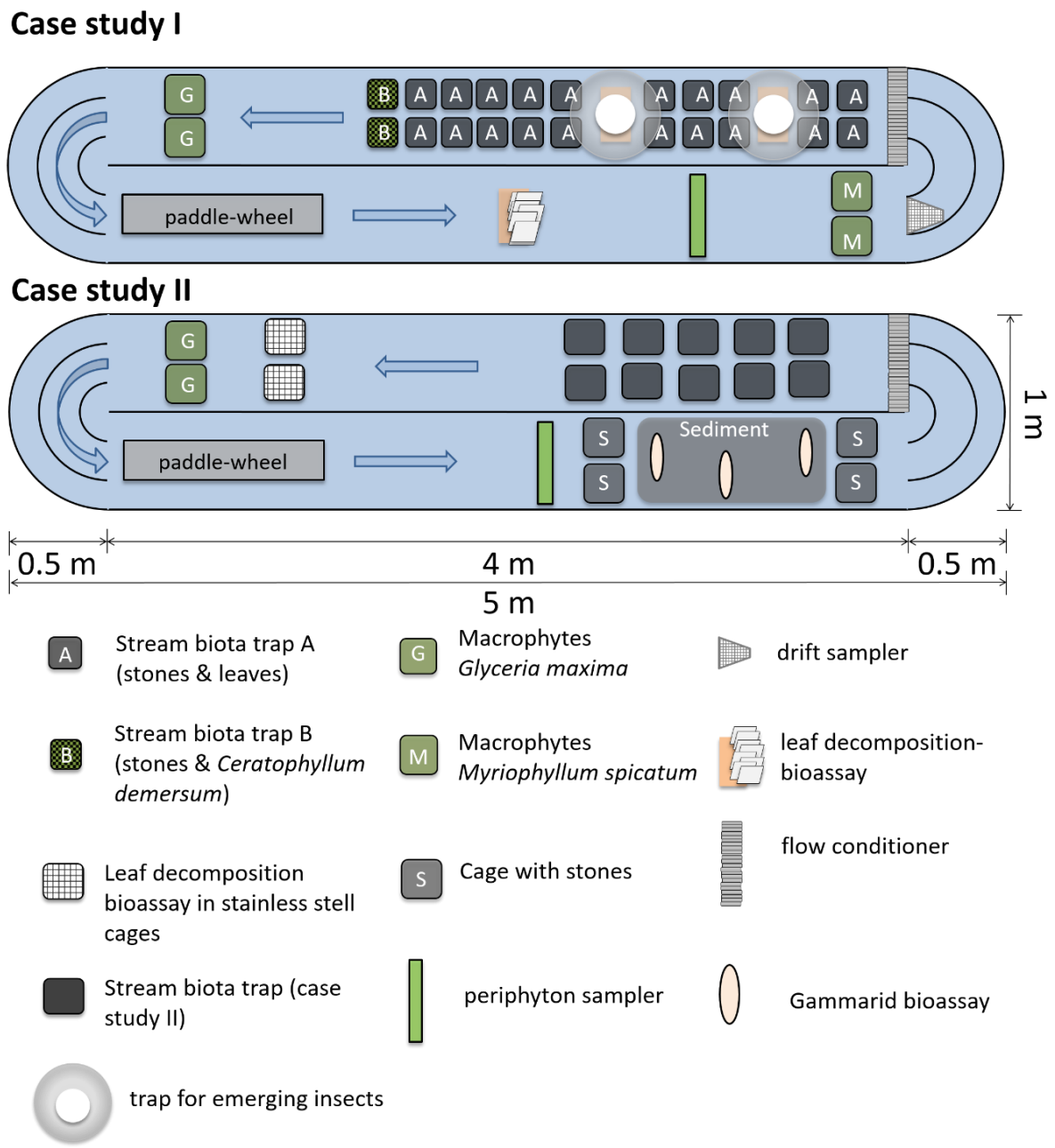


**Fig. 5: Experimental setup of one stream mesocosm with different sampling endpoints in case study I and II.**

**Tab. 2: Components of the stream mesocosms in case study I and II.**

| Components | Case study I | Case study II |
|---|---|---|
| **Number of stream mesocosms [n]** | 8 | 12 |
| **Composition of water [%]** | 66.6 filtered pond water<br>33.3 tap water | 66.6. filtered pond water<br>33.3 tap water |
| **Water volume [L]** | ~1300 | ~1300 |
| **Water level before start of application [cm]** | ~30 | ~30 |
| **Flow velocity [m/s]** | ~0.11 | ~0.10 |
| **Sediment** | No extra sediment | 100 cm x 50 x 15 cm (volume ca. 75 L), sediment – sand ratio (1:1) |
| **Macrophytes** | 2 baskets (6 L) *Glyceria maxima*<br>2 baskets (3 L) *Myriophyllum spicatum*<br>2 baskets (3 L) *Ceratophyllum demersum* | 2 baskets (6 L) *Glyceria maxima*<br>2 baskets (3 L) *Myriophyllum spicatum* |
| **Other substrate** | Basalt stones, willow (*Salix* sp.) leaves | Basalt stones, alder (*A. glutinosa*) and willow (*Salix* sp.) leaves |

## 2.2 Colonisation with organisms

Macroinvertebrates were taken from two nearby streams with drainage areas within an agricultural landscape (see also Wogram, 2010). The stream Pferdsbach (50°41'50.7"N, 8°59'58.6"E, DEHE_2582.2) is an upland stream with a length of 7 km and a small drainage area of 12 km² (HLNUG, 2018). The chosen location at the Pferdsbach in Büßfeld, where the organisms were entrapped, was surrounded by trees and bushes and consequently shady. On this site the stream was approximately 1.2 metres wide and 0.4 m deep. The other stream Gleenbach (50°46'32.0"N, 9°03'20.0"E; DEHE 25826) is 23 km long and the drainage area amounts to 163 km². Organisms were entrapped in Lehrbach where the stream was ca. 4 m wide, 0.5 – 0.8 m deep and partly shaded by trees. Both streams are classified in different running water types and among others the river soles differ. While the Pferdsbach had a sandy-loamy river sole, the Gleenbach was characterised by gravel and stones. The different types of streams were chosen to get a large spectrum of organisms, especially potentially sensitive and vulnerable taxa. Baskets made out of synthetic material in case study I (size basket: 15 cm x 15 cm x 9 cm, size of holes on top: 1 cm x 1 cm) and stainless steel in case study II (size basket: 15 cm x 15cm x 12.5 cm, size of holes: 1 cm x 1 cm) were filled with basalt stones (size: 2 – 8 cm) and leaf material (case study I: freshly picked *Salix* sp., case study II: dried *Alnus glutinosa*, Fig. 6) and were used for entrapment. Baskets were placed in the two water bodies for a period of four weeks to reach colonisation by target organisms. Afterwards

all baskets were placed in tubs filled with the respective stream water and transported to the study site. For case study I ten baskets of each stream were randomly transferred into each stream mesocosm, while for case study II six baskets of each stream were transferred. Furthermore, additional organisms were actively transferred by hand to the stream mesocosms. For case study I approximately 30 stoneflies were transferred to each stream mesocosm by collecting them under stones in the Gleenbach. For case study II individuals of the shredder *Gammarus pulex* were actively captured by hand in a small forest stream near Kirtorf-Wahlen (50°48'9.8"N, 9°6'37.2"E) and transferred to the stream mesocosms. Algae, zooplankton organisms and microorganisms were transferred with the water and the applied substrate (see also chapter 2.9) from the streams Pferdsbach and Gleenbach to the test site.

**Fig. 6: Baskets for attracting macroinvertebrates (filled with basalt stones and leaf material**). Left: basket for case study I, right: basket for case study II.

## 2.3 Timing of case studies

Because the focus of case study I was on the establishment of a suitable habitat for particularly potential sensitive and vulnerable taxa of macroinvertebrates, the study was performed from July until October 2015. In case study II the focus was on assessing the risk of a fungicide on the aquatic food web, particularly on microorganisms. This study was performed from September to November 2017, because litter fall inputs to streams in Europe are highest in this season (Abelho, 2001). The type of samples and the timing of sampling for case study I and II are summarized in Tab. 3 and Tab. 4.

**Tab. 3: Timetable for sampling points and measurements in case study I in the year 2015.** Intro: Introduction of macroinvertebrate sampler, emergence traps; temp: temperature; * introduction of plates for periphyton sampling was 43 days before carbaryl application; x: number of sampling time points per week.

| Month | July | | | | August | | | | September | | | October |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Week after application | -4 | -3 | -2 | -1 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Macrozoo-benthos | intro | x | | x | x | x | x | x | x | x | x | x |
| Invertebrate drift | | | | | xxx | | | | | | | |
| Insect emergence | | intro | x | x | x | x | x | x | x | x | x | x |
| Suspended algae | x | | x | x | x | x | x | x | x | x | x | x |
| Periphyton* | | x | | x | x | x | x | x | x | x | x | x |
| $O_2$, pH, temp, conductivity | x | x | x | xx | xx | x | x | x | x | x | x | x |
| $NO_3^-$, $NH_4^+$, $PO_4^{2-}$, water hardness | | | x | | | | | | x | | | x |
| Carbaryl in water | | | | | xxxx xx | x | | | | | | |

**Tab. 4: Timetable for sampling points and measurements in case study II in the year 2017.** Intro: Introduction of macroinvertebrate sampler, cages with gammarids, fine and coarse mesh bags; decompos.: decomposition, temp: temperature; EEA: extracellular enzyme activity; bact: bacterial; * introduction of plates for periphyton sampling was 25 days before tebuconazole application; x: number of sampling time points per week.

| Month | September | | | | October | | | | November | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Week after application | -2 | -1 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Macrozoo-benthos | intro | x | x | | x | | x | | x | | |
| Gammarid biosassay | | intro | | | x | | x | | | x | |
| Macrobial litter decompos. | | intro | x | | x | | x | | | x | |
| Microbial litter decompos. | | intro | x | x | x | | x | | x | | x |
| EEA | | | x | x | x | | x | | x | | |
| Fungal biomass | | | x | x | x | | x | | x | | |
| Bact. biomass | | | x | x | x | | x | | x | | |
| Suspended algae | | x | x | x | x | x | x | x | x | x | x |
| Periphyton* | | x | x | x | x | x | x | x | x | x | x |
| $O_2$, pH, temp, conductivity | | x | x | x | x | x | x | x | x | x | x |
| $NO_3^-$, $NH_4^+$, $PO_4^{2-}$, water hardness | | x | | | | | x | | | x | |
| Tebuconazole in water | | | xx | x | x | x | | x | | | x |

## 2.4 Study design

In case study I five carbaryl concentrations (1.2, 3.6, 12, 36, 120 active ingredients (a.i.) µg carbaryl/L) were tested with one replicate per treatment level (Fig. 7). This concentration range was chosen because laboratory studies have shown that especially insects from fast flowing habitats seem to have a high sensitivity against the exposure of carbaryl (96h-$LC_{50}$: 1.7 – 17 µg/L, seven species of stoneflies and heptageniid mayflies (Schäfers, 2012)). Three stream mesocosms were used as untreated controls. The allocation of the stream mesocosms was randomized. Carbaryl was applied once, on 6th August 2015 (Day 0).

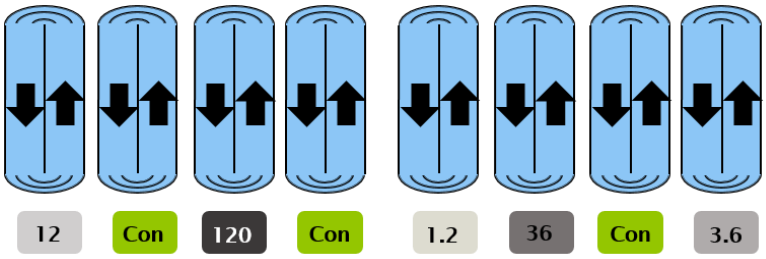


**Fig. 7: Study design and allocation of stream mesocosms for carbaryl application (case study I).** Con: control, numbers in boxes: carbaryl concentration (µg a.i./L).

For case study II the fungicide tebuconazole was used for assessing the potential risk on the aquatic food web, especially the risk on microorganisms. Because of further construction of additional stream mesocosms in spring 2017, twelve stream mesocosms were used for case study II. Four different tebuconazole concentrations (119, 238, 476 and 952 µg a.i. tebuconazole/L) with two replicates each were applied. Another four stream mesocosms served as untreated controls (Fig. 8). Tebuconazole was applied to the stream mesocosms on 25th September 2017 (Day 0).

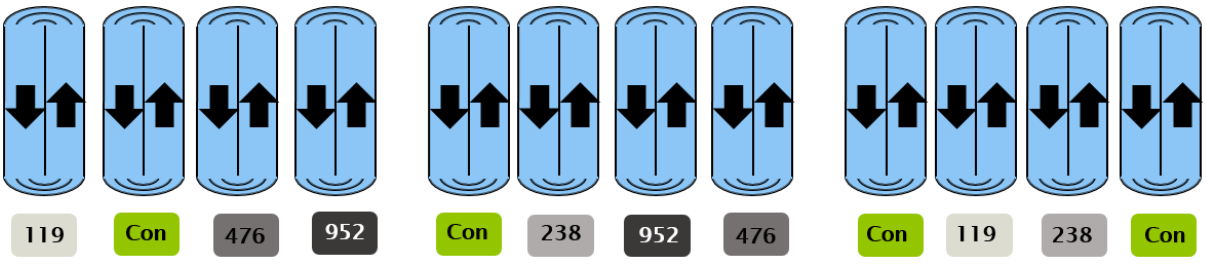


**Fig. 8: Study design and allocation of stream mesocosms for tebuconazole application (case study II).** Con: control, numbers in boxes: tebuconazole concentration (µg a.i/L).

## 2.5 Application of test substances

### 2.5.1 Test item and application (case study I)

One day before application the stock solution of carbaryl (CAS No. 63-25-2, Sigma-Aldrich, purity: 99.9 %) was prepared. Because of low solubility of carbaryl (9.1 mg/L; PPDB (Pesticide properties database), 2018), the stock solution (240 mg a.i. in 10 L deionized water) was mixed with 5 mL acetone as a solvent and 10 µL acetic acid (98 – 100 %) for hydrolytic stability. The solution was stirred overnight on a magnetic stirrer. Application solutions (volume 2000 mL) for the different treatment levels were prepared by using separating funnels (Tab. 5). In case more than 1000 mL of the stock solution were needed for the application solution, the application of this treatment was subdivided into as many application solutions as needed. The solutions were uniformly distributed through the whole water column by moving the glass tip of the opened funnel in a circular pattern. After application, each treatment funnel was rinsed three times with water (volume: 1000 mL), which was added to the respective stream mesocosm. To ensure all stream mesocosms received the same amount of acetone for preventing a potential effect due to the use of a solvent and water for rinsing the funnel, additional water and acetone were applied to the respective stream mesocosms (Tab. 5). The final amount of acetone and water was determined by the amount in the highest carbaryl concentration of 120 µg/L (in total 17 L water and 3220 µL acetone). To exclude any effect of the application procedure on the mesocosms, the control stream mesocosms received a mock treatment containing tap water instead of the application solution and acetone in the same way as described before. A potential acetone effect in the control stream mesocosms can be excluded since only 3220 µL acetone per ~1300 L water was added. According to OECD Guideline 23 and several other OECD guidelines for laboratory tests with aquatic organisms, the used solvent should not exceed 100 µL solvent per liter test medium (OECD 2012, 2019a, 2019b).

**Tab. 5: Scheme for preparation of the carbaryl application solutions for stream mesocosms (F1 – F8).** * amount of acetone was adapted to the amount of the highest carbaryl concentration.

| Carbaryl application solution for stream mesocosm | Volume of carbaryl stock solution [mL] | Volume of tap water [mL] | Volume of acetone [µL] * | Volume of water for rinsing [L] | Preparations of application solution [mL] |
|---|---|---|---|---|---|
| **Control 1 (F 2)** | - | - | 3220 | 17 | - |
| **Control 2 (F 5)** | - | - | 3220 | 17 | - |
| **Control 3 (F 8)** | - | - | 3220 | 17 | - |
| **1.2 µg/L (F 6)** | 61.28 | 1938.72 | 3180 | 15 | 1 x 2000 |
| **3.6 µg/L (F 1)** | 199.95 | 1800.05 | 3120 | 15 | 1 x 2000 |
| **12 µg/L (F 3)** | 698.75 | 1301.25 | 2870 | 15 | 1 x 2000 |
| **36 µg/L (F 7)** | 1935.00 | 2065.00 | 2250 | 13 | 2 x 2000 |
| **120 µg/L (F 4)** | 6450.00 | 7550.00 | - | 3 | 7 x 2000 |

## 2.5.2 Test item and application (case study II)

Tebuconazole was applied as the commercially available formulation Folicur (250 g tebuconazole/L, additive: N,N-dimethyldecanamide; Bayer CropScience, Germany). For preparation of the stock solution, Folicur (volume: 25 mL) was diluted in deionised water (volume: 4975 mL) on the day of application. Because of two replicates for each treatment level, two application solutions per nominal concentration were prepared. The application solutions were prepared in a separating funnel with a final volume of 2000 mL application solution (Tab. 6). After application of an individual stream mesocosm the funnel was rinsed three times with tap water (volume: 1 L), which was also added to the respective stream mesocosm. For each treatment level one separating funnel was used. To exclude any effects of the application procedure, control stream mesocosms were treated with tap water in the same way as the treated stream mesocosms.

**Tab. 6: Scheme for preparation of the tebuconazole application solutions for stream mesocosms (F1 – F12).**

| Tebuconazole application solution for stream mesocosm | Volume of tebuconazole stock solution [mL] | Volume of water [mL] | Volume of water for rinsing [mL] |
|---|---|---|---|
| **Control 1 (F 2)** | - | - | 5000 |
| **Control 2 (F 5)** | - | - | 5000 |
| **Control 3 (F 9)** | - | - | 5000 |
| **Control 4 (F 12)** | | | 5000 |
| **119 µg/L (F1)** | 124.82 | 1875.18 | 3 x 1000 |
| **119 µg/L (F10)** | 132.52 | 1867.48 | 3 x 1000 |
| **238 µg/L (F6)** | 246.06 | 1753.94 | 3 x 1000 |
| **238 µg/L (F11)** | 250.03 | 1749.97 | 3 x 1000 |
| **476 µg/L (F3)** | 516.20 | 1483.80 | 3 x 1000 |
| **476 µg/L (F8)** | 542.64 | 1457.36 | 3 x 1000 |
| **952 µg/L (F4)** | 979.20 | 1020.80 | 3 x 1000 |
| **952 µg/L (F7)** | 967.68 | 1032.32 | 3 x 1000 |

## 2.6 Residue analysis of test substances

### 2.6.1 Sampling water for exposure analysis

Water from stream mesocosms was analysed to confirm the intended dosing of the stream mesocosms and investigate the fate of the test substances (case study I: carbaryl, case study II: tebuconazole). By use of a stainless-steel pipe (diameter: 4 cm) depth-integrated water samples at three defined locations per stream mesocosm were collected and pooled in a stainless steel pot. By using these pipes the whole water column between ground and water surface was collected.

For analysing carbaryl in water, two subsamples of 10 mL were taken from the pooled sample per stream mesocosm. 10 µL formic acid (98 – 100 %) were added to every subsample to stabilise the sample. Depth-integrated water samples from untreated controls were pooled for analysis on every sampling occasion. The subsamples were stored frozen (≤ -18 °C) until analysis. Water samples for carbaryl analysis were taken in each stream mesocosm 1, 3, 24, 48, 120 and 144 hours after application. In addition, two subsamples of the carbaryl stock solution, which was used for producing the intended carbaryl concentrations were kept frozen (< -18 °C).

For residue analysis of tebuconazole in water, three subsamples of 40 mL were taken in the same way as for carbaryl. Water samples were taken 4 hours after application (day 0) and 1, 7, 14, 21, 37 and 59 days after application. The water samples were kept frozen until analysis (< -18 °C).

### 2.6.2 Carbaryl analysis (case study I)

Residue analysis of carbaryl in water was performed by the working group of Matthias Kotthoff from the Fraunhofer Institute for Molecular Biology and Applied Ecology (Schmallenberg, Germany). After thawing, water samples were centrifuged (4000 rpm; 10 minutes). Subsequently, the samples were diluted with methanol (high performance liquid chromatography (HPLC)-grade, 1 : 1 (v/v)) and directly measured using ultra-performance liquid-chromatography tandem mass spectrometry (UPLC (model: Acquity, Waters)-mass spectrometry (MS) / mass spectrometry (Xevo TQ-S, Waters)) with the following conditions: chromatographic column: BEH C18 (100 x 2.1 mm, 1.7 µm, Waters); injection volume: 20 µL, flow rate: 300 µL/L; mobile phase A: 89.9 % water, 10 % methanol and

0.1 % formic acid; mobile phase B: 99.9 % methanol and 0.1 % formic acid; gradient programme: 50 % / 50 % of A and B initially, then ramping three minutes to 100 % mobile phase B and ramping back to 50 % / 50 % A and B; retention time carbaryl: 1min 45 s. Carbaryl was detected in multi reaction monitoring (MRM) with electrospray ionisation positive mode ($ESI^+$) using argon as collision gas. Captured mass transitions were m / z 202 → 145 as quantifier and m / z 202 → 127 as qualifier. While the cone voltage was fixed at 20 V for either transition, the collision energy was 10 eV for the quantifier and 25 eV for the qualifier. The absolute detection level of carbaryl was at 0.15 µg/L. The dissipation half-life time ($DT_{50}$) for carbaryl was calculated using the Computer Assisted Kinetic Evaluation application programme (CAKE, Version 3.2, Tessella Technology & Consulting) assuming first order kinetics.

### 2.6.3 Tebuconazole analysis (case study II)

Residue analysis of tebuconazole in water was performed by the working group of Walter Böhmer from the Fraunhofer Institute for Molecular Biology and Applied Ecology (Schmallenberg, Germany). Quantitative determinations of tebuconazole in aqueous test media were performed by direct liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) after addition of the internal standard (tebuconazole- (*tert*-butyl-$d_9$)) and acetonitrile (volume ratio water : acetonitrile, 5 : 1). The measurements were performed on a Waters Quattro Micro LC-MS/MS system in the positive ionisation mode. The mass transition used for the analyte tebuconazole was m / z 308.05 → 69.98 as quantifier and m / z 317.05 → 69.98 as qualifier for the internal standard, while the collision energy was 22 eV for both compounds. Liquid chromatography was done in a Phenomenex Gemini® C18 HPLC column, the mobile phase used consisted of solvent mixtures of water, methanol and ammonium acetate solution. The absolute detection level of quantification was at 5.0 µg/L, while the accuracy was 98.7 % (mean recovery of three fortification level). The dissipation half-life time for tebuconazole was also calculated using the Computer Assisted Kinetic Evaluation application programme (CAKE, Version 3.2, Tessella Technology & Consulting) assuming first order kinetics.

## 2.7 Sampling of ecological endpoints in the case studies

### 2.7.1 Physicochemical water parameters

Water temperature, dissolved oxygen, pH and conductivity were measured *in situ* using WTW measuring probes (pH: SenTix 940 IDS; conductivity: TetraCon 925; content of oxygen: FDO 925; WTW Multi 3430, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim). Temperature was measured with the integrated sensor of the pH probe. Measurements were done 0.2 m beneath the water surface and approximately at the same time of the day and at the same position in the stream mesocosms. Concentrations of ammonium, nitrate, phosphate as well as water hardness were measured in subsamples of the depth-integrated water samples as described above (chapter 2.7.1.) Samples were sieved (mesh size 60 – 70 µm) before they were analysed photometrically using WTW cube tests (ammonium: WTW test 14739; nitrate: WTW test 14556; phosphate: WTW test P6/25; water hardness: WTW test 00961; WTW-Photometer PhotoLab Spektral).

### 2.7.2 Suspended algae

For chlorophyll a analysis a depth-integrated water sample (volume: approximately 3 L) from at least six different locations was taken (see also chapter 2.7.1). A sieved subsample (mesh size: 2 mm) of 350 mL was obtained and stored in a lightproof PET bottle until analysis by using a Delayed Fluorescence (DF) spectrometer. The chlorophyll a content and the respective fractions of green algae (Chlorophyceae, Euglenophyceae, Conjugatophyceae), diatoms (Bacillariophyceae, Chrysophyceae, Dionphyta, Xyanthophyceae), blue-green algae (Cyanophyceae) and cryptophyte algae (Cryptophyceae) were expressed in µg/L.

### 2.7.3 Periphyton

Periphyton was collected on glass slides (total surface area per slide: 1.855 $dm^2$) that served as artificial substrate. By fixing the glass slides in a retainer they were placed on the bottom of the flume. On every sampling occasion periphyton was scraped with a razor blade from one glass slide and mixed with tap water (volume: 350 mL). Periphyton samples were also stored in lightproof PET bottles until chlorophyll a analysis through a DF spectrometer. The chlorophyll a content of the suspension (µg/L) was referred to the settlement density of the substrate (µg/L x 0.35 L/0.01855 $m^2$) and is expressed as µg/$m^2$.

### 2.7.4 Chlorophyll a analysis

By using a delayed fluorescence spectrometer the chlorophyll a content and the respective fractions of green algae (Chlorophyceae, Euglenophyceae, Conjugatophyceae), diatoms (Bacillariophyceae, Chrysophyceae, Dionphyta, Xyanthophyceae), blue-green algae (Cyanoprokaryota) and cryptophytes were analysed according to Gerhardt & Bodemer (1998, 2000). Before measurement, the samples were brought to a temperature of 21 °C ± 1 °C by using a water bath and adapted to the dark for 15 minutes. By using a DF-excitation spectrometer ("DFPOP, apparatus "Steffi 4", University of Regensburg, Experimental and Applied Physics) the samples were exposed to monochromatic light emit photons ($\lambda$ = 680 – 720 nm) in the excitation cuvette. By excitation of photosynthetic pigments of the living cells, the emitted light quantum of the primary producers can be measured through delayed fluorescence. Different algae classes (green algae, diatoms, Cyanoprokaryota and Cryptophyta) possess different pigment composition and therefore use different wavelength ranges for photosynthesis. The DF-kinetic photometer was calibrated by parallel determination of chlorophyll a of a young growing culture of green algae (extractive spectrophotometric method; DIN 38.412, 1985). The integral of the DF is proportional to the photosynthetically active chlorophyll concentration (Gerhardt & Bodemer, 1998).

### 2.7.5 Macroinvertebrates

For investigating acute effects of the applied pesticides on the abundance of macroinvertebrates, the baskets used for colonisation with organisms from two nearby streams were also used for macroinvertebrate sampling (Fig. 6). The invertebrates were rinsed with stream water from the substrate inside the baskets (basalt stones and willow leaves in case study I, alder leaves in case study II; see also chapter 2.2) into white vessels. Organisms were identified alive as far as possible per eye or stereomicroscope (Stereomikroskop Wiloskop, Hund (Wetzlar)) with transmitted light illumination (FLQ 150) and counted. Afterwards the organisms were placed back into each stream mesocosm.

For case study I the macroinvertebrates of three baskets per sampling occasion per stream mesocosm were counted and identified. The content of two baskets previously incubated for four weeks in the Pferdsbach or Gleenbach (one basket each) was analysed. Furthermore, the content of the third basket filled with stones and *Ceratophyllum demersum (*hornwort*)* was also

analysed on every sampling occasion. After counting, the third basket filled with the macrophyte *C. demersum* was placed back completely. From the remaining two baskets only the content (without the basket) was replaced to their respective stream mesocosm. The numbers of organisms per taxon found in the three baskets of a stream mesocosm were added.

For case study II the macroinvertebrates of two baskets per sampling occasion per stream mesocosm were counted and identified. Like in case study I one basket each that was previously incubated in the Pferdsbach or the Gleenbach was analysed. In addition to rinsing organisms from stones and other substrates into white vessels, one coarse mesh bag per basket was rinsed and the organisms found there were also counted (see chapter 2.9.3 for description of coarse mesh bags). After counting and identification, the baskets were re-transferred to their respective stream mesocosm. Likewise, the numbers of organisms per taxon found in the two baskets of a stream mesocosm were added up and for every sampling occasion another two baskets were used to investigate macroinvertebrate abundances. Seven days before tebuconazole application gammarids were counted in each stream mesocosm. Low abundances of gammarids were determined and additional gammarids (150 – 300) were introduced three days before application into each stream mesocosm.

## 2.8 Sampling of further ecological endpoints in case study I

### 2.8.1 Drift of invertebrates

The drift behaviour of invertebrates was analysed by using a drift net (synthetic material, width: 20 cm, height: 50 cm, mesh size: 1 mm, Fig. 9). The drift net was fixated between two guide plates circa 3 m behind the paddle wheel. Approximately one third of the whole water cross-section was covered by the net. The net was introduced immediately before application and emptied 4 hours, 8 hours and 24 hours after application. Collected organisms were fixated in 70 % ethanol. They were counted and identified to the lowest possible taxonomic level by using a stereomicroscope (see chapter 2.7.5).

**Fig. 9: Drift net.**

### 2.8.2 Insect emergence

**Fig. 10: Emergence trap.**

Two emergence traps were installed to assess the emergence of insects (Fig. 10). Each emergence trap consisted of a stainless-steel ring (height: 15 cm, diameter: 40 cm, area: 1257 $cm^2$) that ended circa 5 – 10 cm above the water surface. The ring was covered with a rectangular fine mesh (synthetic material, mesh size: 250 µm) and was withdrawn with an eclector head box (ecoTech Umwelt-Meßsysteme). The eclector head box was filled with tap water (ca. 10 mL) and detergent (ca. 1 mL). The traps were set straight above the macroinvertebrate baskets. Two wood sticks per trap protruded out of the water directly underneath the mesh to facilitate emergence of insects outside the water. On each sampling occasion the content of the eclector head boxes were emptied through a stainless-steel sieve (mesh size: 63 µm) and the organisms were fixated in 70 % ethanol. Insects were counted and identified with a stereomicroscope to the lowest possible taxonomic level. The numbers of both traps per stream mesocosms were pooled.

## 2.9 Sampling of further ecological endpoints in case study II

### 2.9.1 Preparation of exposed leaves

Leaf-litter decomposition was not yet evaluated in the working group before. The method was modified in size and material as follows: Leaf-litter decomposition experiments were performed with *Alnus glutinosa* L. leaves collected in October 2015 and October-November 2016 from trees in the area surrounding the experimental site in Homberg (Ohm). Leaves were air-dried for at least 21 days and stored in the dark in cardboards. Additionally, alder leaves were collected in August 2017 and dried at 60 °C for at least 48 h. They were used for coarse/fine mesh bags and served as food source for gammarids in the *in situ* bioassay.

### 2.9.2 Fine mesh bags


**Fig. 11: Fine mesh bag.**

Fine mesh bags were used for analysing the following endpoints: microbial litter decomposition, extracellular enzyme activity of five different enzymes, ergosterol content and number of bacterial cells. All aforementioned parameters were newly established at the test facility and adapted to the test systems. Each fine mesh bag (material: nylon; pore size: 460 µm diameter) contained 3 g (± 0.01 g) of air-dried alder leaves for analysing microbial litter decomposition (Fig. 11). The main leaf vein was previously removed. Additionally, 50 leaf discs (diameter: 1 cm) per bag were placed separately next to the 3 g sample. They were used for analysing EEA (15 leaf discs), ergosterol content (15 leaf discs), number of bacterial cells (15 leaf discs) and the remaining dry weight of 15 leaf discs to assess the used leaf mass to the aforementioned endpoints. Leaf discs were prepared with a cork borer. To produce microbial inoculum for the leaf material, leaves were exposed for 20 days in the Gleenbach (50°46'32.0"N 9°03'20.0"E) near Kirtorf-Lehrbach. Four days before tebuconazole application, 12 fine mesh bags were randomly selected and placed in each stream mesocosm, respectively. Seven bags were used to quantify mass loss during the conditioning period. Additionally, the leaf discs were used to measure EEA, ergosterol content and bacterial biomass under post-conditioning time. On every sampling occasion on leaf two leaf bags per concentration level (one per replicate) were used for the aforementioned assessment.

### 2.9.3 Coarse mesh bags


**Fig. 12: Coarse mesh bag.**

Coarse mesh bags were used for determination of macrobial litter decomposition and were also used for the *in situ* gammarid bioassay. Each coarse bag was made out of stainless steel and had a pore size of 0.5 x 0.5 mm with more than 24 blanked out holes (diameter: 0.5 cm) with the result that macroinvertebrates have access to the leaf litter (Fig. 12). For assessing the macrobial litter decomposition, 8 g

(± 0.01 g) of alder leaves (without main leaf vein) were used. These bags were conditioned in a separate experimental stream mesocosm for three days. Three days before application eight coarse mesh bags were placed in macroinvertebrate substrate sampler for each stream mesocosm (one coarse mesh bag per substrate sampler). Five bags were used to quantify mass loss during the conditioning period. For the *in situ* bioassay the same coarse mesh bags were filled with 5 g (± 0.01 g) of alder leaves. They were conditioned for five days in a separate experimental stream mesocosm. Six days before application three bags were transferred to three *in situ* gammarid cages (one bag per cage) to each stream mesocosm (see chapter 2.9.4).

### 2.9.4 *In situ* bioassay with *Gammarus pulex*

In a round stainless-steel cage (diameter: 22 cm, height: 11 cm) surrounded with a stainless-steel net (pore size: < 0.3 mm), 30 individuals of the species *Gammarus pulex* (size: 9 – 11 mm) were placed inside (Fig. 13). Coarse mesh bags with 5 g (dry weight) alder leaves served as food source (see chapter 2.9.3). Three cages per stream mesocosm were placed in the sediment on the ground of the mesocosm, so that water can flow through. On three sampling days gammarids of one cage per stream mesocosm were counted and prepared for lipid analysis. Furthermore, the consumed leaf mass of gammarids were analysed by drying the remaining leaves at 60°C (~48 h) and weighing.

**Fig. 13: Cages for *in situ* bioassay.**

### 2.9.5 Microbial leaf litter decomposition and decomposition by macroinvertebrates

Leaf litter decomposition in fine mesh bags for assessing the microbial decomposition (= microbial leaf litter decomposition) and in coarse mesh bags for assessing the microbial decomposition plus invertebrate consumption (= macrobial leaf litter decomposition) were calculated on six (microbial) respectively four (macrobial) sampling days after application. The plant material was cleaned with tap water, oven-dried (~48 h, 60 °C) and weighed (Shimadzu type AUW220D, Shimadzu Corporation, Kyoto, Japan). Leaf litter decomposition was determined as the difference between leaf mass remaining after the conditioning period

(Day -4 for fine mesh bags, Day -3 for coarse mesh bags) and leaf mass remaining on the sampling days. On every sampling day two fine and coarse leaf bags were used for calculating dry mass per stream mesocosm.

### 2.9.6 Extracellular enzyme activity (EEA)

The activity of five different extracellular enzymes (ß-glucosidase, ß-xylosidase, phosphatase, phenoloxidase and peroxidase, Tab. 7) was measured. Aquatic microorganisms secrete these enzymes for breaking down plant compounds (Battin et al., 2016). The enzyme activity of ß-glucosidase, ß-xylosidase and phosphatase was assessed by using the fluorogenic coumarin-derivate-linked substrate 4-Methylumbelliferyl (MUF). The MUF-linked substrates will be degraded by the enzymes and the fluorescent MUF molecule will be released (Hoppe, 1983).

**Tab. 7 Used artificial substrates for extracellular enzymes and their natural substrates during leaf litter decomposition** (Chamier, 1985; Sinsabaugh et al., 1991)**.**

| Extracellular enzyme | Artificial substrate for EEA | Natural substrate |
|---|---|---|
| ß-glucosidase | MUF-ß-D-glucoside | cellobiose |
| ß-xylosidase | MUF-ß-D-xyloside | hemicellulose |
| phosphatase | MUF-phosphate | polyphosphates |
| Phenol oxidase | L-DOPA | lignin |
| peroxidase | L-DOPA + $H_2O_2$ | lignin |

The activity of the oxidative enzymes phenol oxidase and peroxidase (both involved in lignin degradation) was measured spectrophotometrically by using the artificial substrate 3,4-Dihydroxy-L-phenylalanine (L-DOPA). While L-DOPA is oxidized by phenol oxidase or peroxidase, a red pigment is formed (3-dihydroindole-5,6-quinone-2 carboxylate (DIQC), (Hendel, 1999; Mason, 1948)), whose absorption maximum is at 460 nm. The absorption was determined photometrically.

On each sampling day 7 – 15 leaf discs from litter bag samples were used to analyse enzyme activities. Leaf discs were placed in 60 – 120 mL filtered (filter type 595, Schleicher & Schüll) and autoclaved stream mesocosm water. The leaf-water suspension was homogenized by using a disperser for 15 seconds (UltraTurrax, 16 000 rotations per minute). From every stream mesocosm two replicates per enzyme were analysed. The enzyme activities of ß-glucosidase, ß-xylosidase and phosphatase were measured after a modified version of Pohlon et al. (2010). 3.6 mL of the leaf-water homogenate was pipetted in 15 mL Falcon vials and 0.4 mL of the

three respective MUF-substrate stock solutions (2 mmol/L) were added to reach a final substrate concentration of 0.2 mmol/L per sample. The used substrates were MUF-ß-D-glucoside (CAS No. 18997-57-4; manufacturer Alfa Aesar), MUF-D-xyloside (CAS No. 736-33-4; SIGMA-ALDRICH) and MUF-phosphate (CAS No. 3368-04-5; Alfa Aesar). The samples were vortexed and incubated for 60 minutes at room temperature. By adding 0.4 mL glycine buffer (50 mmol/L, pH 10.4) the enzyme activity was stopped. Apart from stopping the enzyme reaction glycine buffer also enhances the fluorescence activity of MUF (Mead et al., 1955). Subsequently the samples were centrifuged for 7 minutes at 4000 rpm (3345 g, Megafuge 1.0, Kendro Laboratory Products). Supernatant was transferred to quartz cuvettes (Suprasil 10 mm, 3.5 mL, Hellma Analytics) and the extracellular enzyme activity was quantified by fluorescence measurement (excitation at 365 nm, emission at 450 nm, Fluorimeter, Kontron SFM25, Kontron Embedded Computers, Munich Germany). Controls for stream water activity without leaf litter were prepared by using 3.6 mL filtered and autoclaved stream mesocosm water with 0.4 mL glycine buffer. On every sampling occasion fresh MUF-standards with a final concentration of 1, 10 and 50 µmol/L (2 replicates each) were prepared.

The fluorescence activity of the fluorimeter is expressed as relative fluorescence units (RFU). By using calibration lines for MUF standard solution concentrations (0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 50 µmol/L) the converted amount of substrate from RFU was calculated. Therefore 3.6 mL of the final MUF standard solution concentration was mixed with 0.4 mL glycine buffer and measured with the same wavelength settings mentioned above. The extracellular enzyme activity was expressed as µmol MUF per unit of time and dry weight of leaf.

Enzyme activities of phenol oxidase and peroxidase were measured according to the method from Hendel (1999) and R. L. Sinsabaugh & Linkins (1990). Stock solutions (5 mmol/L) of L-DOPA (CAS No. 59-92-7; Alfa Aesar) were freshly prepared in acetate buffer (50 mmol/L, pH 5) on every measurement day because it tends to auto oxidation (Hendel, 1999; Hendel et al., 2005). Phenol oxidase activity was determined by mixing 2 mL of the leaf-water homogenate with 2 mL L-DOPA solution (final substrate concentration: 2.5 mmol/L) in a 15 mL Falcon tube. For peroxidase measurement 0.2 mL $H_2O_2$ (0.3 %, v/v) were added additionally. For negative controls the same amount of leaf-water homogenate was mixed with 2 mL acetate buffer or in the case of peroxidase with an additional 0.2 mL $H_2O_2$. All samples were vortexed and incubated for at least 60 minutes at room temperature. After

incubation the samples were centrifuged for 7 minutes at 4000 rpm (3345 g,). The supernatant was transferred to single-use cuvettes (polystyrol, 4.5 mL). Two replicates per stream mesocosm were prepared and the absorption measured at 460 nm (WTW PhotoLab Spektral). The activity of the enzymes was calculated with the following formula according to Hendel et al. (2005):

Phenol oxidase: $A_{phenoloxidase} = Abs_{460}\, k^{-1}\, h^{-1}$

Peroxidase: $A_{peroxidase} = Abs_{460}\, k^{-1}\, h^{-1} - A_{phenoloxidase}$

A: enzyme activity [nmol $h^{-1}$]

Abs460: absorbance at 460 nm

k: extinction coefficient, which is 1.66 mM for DOPA

Extracellular enzyme activity of phenol oxidase and peroxidase were expressed as μmol per unit of time and dry weight of leaf.

### 2.9.7 Ergosterol analysis

Ergosterol was quantified as a proxy for leaf-associated fungal biomass according to Gessner (2005). On every sampling day 15 leaf discs were frozen at -80 °C until further processing. Additional 15 leaf discs were dried at 60 °C and weighed to determine the sample dry weight (Shimadzu type AUW220D, Shimadzu Corporation, Kyoto, Japan). For every sampling day one replicate per stream mesocosm was analysed. Approximately one week after the last sampling day, leaf discs were freeze dried for 48 hours (Beta 1 – 8 LSCplus, Martin Christ GmbH) and afterwards kept frozen (-20 °C) until ergosterol analysis. Ergosterol analysis was done in cooperation with the working group of Dr. Jochen Zubrod and Prof. Dr. Ralf Schulz at the University of Koblenz-Landau and was performed by myself at the university of Kolbenz-Landau. On the first processing day leaf discs were pestled with a mortar for 10 seconds. For each sample 50 mg of the pestled leaf material was mixed with 10 mL KOH/methanol (8 g KOH in 1 L methanol) in screw cap vials and stored in the refrigerator overnight. For lipid extraction and saponification, the samples were heated in a water bath (80 ± 1°C) for 30 min on the next day. To ensure through mixing, the screw cap vials were shaken every 5 – 10 minutes by hand. After heating, the extracts were cooled down for approximately 20 minutes at room temperature. In the next step the solid phase extraction (SPE) cartridges (Sep-Pak Vac RC tC18 500 mg sorbent, Waters) were conditioned with

methanol (7.5 mL) by using vacuum for initiating the flow. Afterwards the cartridges were rinsed three times with cartridge-conditioning solution (120 mL methanol + 600 mL KOH/methanol + 120 mL 0.65 M HCL (pH 2.3), ratio 1:5:1). During all conditioning steps the sorbent bed was always covered with fluid. After the conditioning phase, the SPE cartridges were loaded with the prepared sample extract. The sample extracts were placed on the cartridges with a Pasteur pipette. Screw cap vials in which the samples were prepared were rinsed four times with methanol (2.5 mL, respectively) and the fluid was added to the cartridges. In addition, 0.65 M HCL (2 mL, pH 2.3) were placed on the cartridges and all solutions were well mixed. Subsequently the solutions were soaked with a flow rate of 1 mL $min^{-1}$ through the cartridges. After complete loading of sample onto cartridges, the sorbent was rinsed with wash solution (2.5 mL of 0.4 M KOH in methanol : $H_2O$ (ratio 6:4; v/v)). Following this, the sorbent was dried for 60 minutes under vacuum conditions. After pre-weighing the HPLC-vials, they were placed under the SPE cartridges. The elution of ergosterol was done by adding isopropanol (4 x 400 µL) to the SPE cartridges at a flow rate of 1 mL $min^{-1}$ and the eluent was captured in the HPLC-vials. These vials again were weighed, and the exact elution volume was calculated (1 g isopropanol = 1.27 mL at room temperature). Ergosterol eluent was quantified by high-performance liquid chromatography (1200 Series, Agilent Technologies) with the following conditions: mobile phase: 100 % methanol, flow rate: 1.4 mL/L, column temperature: 33 °C, detection wavelength: 282 nm, injection volume: 10 µL, retention time ergosterol: 8 minutes.

Calibration line for converting the UV-absorption to ergosterol concentrations was generated with ergosterol standards (0, 1, 2, 5, 10, 15, 20 and 30 µg/mL). Ergosterol standards were generated by dilution of the ergosterol stock solution (20 mg in 100 mL isopropanol). Ergosterol was converted to fungal biomass using a conversion factor of 5.5 µg ergosterol/mg fungal dry mass (Gessner & Chauvet, 1993), whereby the ergosterol content was expressed as µg ergosterol per mg leaf dry weight.

### 2.9.8 Bacterial cell numbers

Bacterial cell numbers are also an endpoint with less experience in use for aquatic risk assessment but need to be addressed to get further information to the bacteriocenosis and their role in ecotoxicological context. Bacterial cell numbers were determined by epifluorescence

microscopy according to Buesing (2005). On every sampling day 15 leaf discs were rinsed with tap water, directly fixated (10 mL 2 % formalin, 0.1 % sodium pyrophosphate) and stored at 4 °C. A second set of 15 leaf discs were dried to determine the sample dry weight. For every sampling day one replicate per stream mesocosm was analysed. Likewise to ergosterol measurement, the determination of bacterial cell numbers was done in cooperation with the working group of Dr. Jochen Zubrod and Prof. Dr. Ralf Schulz at the University of Koblenz-Landau. On the day of sample preparation leaf discs were sonicated on ice for 1 minute to detach bacteria from the leaf discs (output: 80 Q, amplitude: 76 µm, Sonopuls HD 2070 equipped with a TT 13 tip, both Bandelin, Berlin, Germany). For every sample the ultrasonic probe was cleaned with ethanol to prevent cross contamination. On the filtration manifold a cellulose filter (0.45 µm pore size, 25 mm diameter, Merck) was mounted and humidified with filtered (0.2 µm pore size) and autoclaved Nanopure water. On top of the cellulose filter an aluminium oxide filter (0.2 µm pore size, 25 mm diameter, Anodisc, Whatman) was placed. Subsequently, the filtration funnel was connected with the filtration manifold and 1 mL Nanopure water was added. Each sample was vortexed for 10 seconds. After 10 seconds, 40 µL (from 2 mm below the surface) of the homogenate was added to the Nanopure water in the filtration funnel. Additional 1 mL Nanopure water was placed on top to ensure mixing of the sample suspension prior to filtration. A small vacuum was used to suck the sample through the filter tower. By removing the aluminium oxide filter from the filtration manifold and placing it on tissue paper, the filter was carefully dried. After drying, the filter was placed on top of 100 µL SYBRGreen II (diluted 400-fold, Molecular Probes) in a petri dish and incubated for 15 minutes in the dark. After the incubation time the filter was dried again on cleansing tissue. At the end, the filter was placed on ethanol-cleaned object slides prepared with 30 µL anti-fading solution (50 % glycerol, 50 % phosphate-buffered saline (PBS; 120 mM NaCl, 10 mM $NaH_2PO_4$, pH 7.5), 0.1 % *p*-phenylenediamine). A cover glass was added to the object slides. To check for possible contamination during the working process, blanks (Nanopure water without sample) were assessed for every new batch of filters and treated like the samples.

For counting the cell numbers digital photographs were taken using an epifluorescence microscope (Axio Scope. A1 and AxioCam MRm, Carl Zeiss MicroImaging). For each sample the mean number of bacterial cells from 20 microscopic fields (1000x magnification) were counted and analysed with image analysis software (Axio-Vision Rel. 4.8, Carl Zeiss

MicroImaging). Bacterial cell numbers were expressed as number of bacterial cells per mg dry weight of the leaf discs.

### 2.9.9 Lipid analysis of *Gammarus pulex*

The lipid content is a suitable proxy for the evaluation of the physiological fitness of animals (Koop et al., 2008). This parameter is not usually used in aquatic risk assessment. In the context of the focus of the recent study it is necessary to investigate the effects of fungicides on indirect effects of higher trophic level like the measurement of lipid value of a typical shredder organism to evaluate the physiological fitness. On three sampling days (Day 9, 28, 56) the remaining gammarids from the *in situ* bioassay were shock frozen in liquid nitrogen for several seconds and stored in individual glass tubes at -80 °C until further use. For determination of the lipid content the method described in Van Handel (1985) with a few adaptations was used and the working procedures were also conducted at the university of Koblenz-Landau by myself. For every sampling day three gammarids per stream mesocosm were pooled and freeze-dried (Systec, D-65) for approximately 24 h and weighed. To make sure that only lipids from gammarids were extracted, the glass vessels were washed with peroxymonosulfuric acid and 1 : 1 chloroform:methanol solution (v/v) before use. The gammarids were placed in chloroform:methanol solution (0.5 mL, ratio 1:1) for 72 hours. Afterwards gammarids were grounded with a glass mortar and transferred to glass culture tubes. The mortar and the pestle were washed two times with chloroform:methanol solution (ca. 2 mL) into the glass culture tubes. The samples were centrifuged (short spin mode) and the supernatant was tipped over in new glass culture tubes. By using a water bath (95 °C) the solvent was evaporated. Subsequently, 0.2 mL of sulfuric acid (97 %) were added and heated again for 10 minutes at 95 °C. After the samples were cooled down to room temperature, vanillin reagent (5 mL, dissolve 600 mg vanillin standard in 100 mL hot purified water and add 400 mL 85 % phosphoric acid) was added. The samples were vortexed and incubated for 5 minutes. 80 µL of each sample was transferred to a 96-well microwell plate (TC Micro Well 96F SI W/Lies Nunclon D, Nunc) and the absorbance was measured at 490 nm.

By using a standard curve with commercially available soybean oil (Sojola Soja-Öl, Vandemoortele) a calibration line was generated (0 – 430 µg/mL). Lipid standards were generated by dilution of the soybean oil stock solution (56 mg soybean oil in chloroform

brought to a volume of 50 mL). The lipid content was normalised to gammarid dry weight (µg/ mg gammarid) to exclude potential growth effects.

## 2.10 Data evaluation and statistics

The data were statistically analysed by uni- and multivariate statistics. Because the study design of case study I and II differed by the number of replicates per treatment, varying analysis methods were performed. In general, univariate statistics were used to test differences between the treated stream mesocosms and the controls. Multivariate statistical analysis was used to investigate effects of stressors on taxa with low abundances or other dependent variables, which were scarce. For analysing and visualising effects on the community structure (e.g. macroinvertebrate community), Principal Response Curves for case study I (PRC) were calculated.

### 2.10.1 Case study I

### 2.10.2 Dominance

By calculating dominance, the relative frequency of a taxon can be described in relation to other taxa of the community:

$$Dominance = \frac{Number\ of\ individuals\ of\ taxon}{Total\ number\ of\ all\ individuals} * 100\ \%$$

### 2.10.3 $SPEAR_{pesticide}$-Index

The SPEcies At Risk (SPEAR) indicator system was developed for the detection of effects of potential stressors on the species community in aquatic compartments (Liess & Von Der Ohe, 2005; Schäfer et al., 2013). Based on ecological traits like physiological sensitivity of the organisms to organic toxicants, generation time, migration ability and presence of aquatic stages during time of maximum pesticide use period, taxa were classified as species at risk (SPEAR) and species not at risk (not SPEAR). These ecological traits highly influence the recovery time of a population after e.g. pesticide treatment. The relative sensitivity of a species to organic toxicants (physiological sensitivity) was calculated with respect to the sensitivity of *Daphnia magna* in 48 hours laboratory tests by using the following equation (Von der Ohe & Liess, 2004):

$$S = \log(\frac{LC50_{Daphnia\ magna}}{LC50_{Taxon}})$$

Where S is the relative sensitivity, $LC50_{Daphnia\ magna}$ = experimental $LC_{50}$ for *Daphnia magna* and $LC50_{Taxon}$ = experimental $LC_{50}$ for a taxon. The greater the value than zero, the more sensitive the taxon is than *D. magna* consequently. The $SPEAR_{pesticide}$ index is calculated with the following equation:

$$\mathrm{SPEAR_{pesticide}} = \frac{\sum_{i=1}^{n} \log(x_i + 1) y_i}{\sum_{i=1}^{n} \log(x_i + 1)} \times 100$$

$x_i$ = Abundance of the taxon *i*
n = total number of taxa in the sample
$y_i$ = is 1 if taxon *i* is classified as sensitive, else 0

### 2.10.4 Non-linear regression, $EC_x$ calculation

Due to limited number of test systems available, a non-linear regression design for calculation of 50 % Effect concentration ($EC_{50}$) of exposed organisms, as recommended in Azimonti et al., 2015 and Liber et al., 1992 was performed. $EC_{50}$ values were computed by calculating effects as reduction of abundance in a treatment compared to controls:

$$\%\ inhibition = \frac{Abundance\ of\ treatment\ at\ time\ t}{Mean\ abundance\ in\ controls\ at\ time\ t} * 100$$

A three-parameter logistic regression model was fitted to the abundance data by using the following equation (Hose & Van Den Brink, 2004; René P.A. Van Wijngaarden et al., 1996):

$$y` = \frac{c}{1 + e^{-slope[\ln(x) - ln(EC50)]}}$$

with y` = fitted response, c = expected number in the control mesocosms, x = dose [µg/L], slope = slope parameter, $EC_{50}$ = defined at which expected numbers have decreased by 50 %. Regression models were fitted using ToxRat (Version 3.2, ToxRat® Solutions GmbH). $EC_{50}$ values were only taken as reliable if the regression was significant ($p \leq 0.05$).

For the endpoints macroinvertebrates and insect emergence only taxa whose average abundance in the controls was higher than five, on at least two sampling occasions, were analysed using non-linear regression model.

### 2.10.5 Calculation of minimum detectable differences (MDD)

To assess the suitability of the test systems for a mesocosm study meeting the requirements laid down by EFSA PPR (EFSA, 2013), the minimum detectable differences (MDDs) for macroinvertebrate taxa were estimated based on the data shortly before application (day -2). The MDD is defined as the difference between the means of a treatment and the control that must exist to detect a statistically significant effect (Brock et al., 2014). For calculation of MDDs abundance data were log-transformed by using the following equation:

$$y' = \ln(a * y + 1)$$

Hereby *y* is the counted abundance, while *a* is the scaling factor. As a scaling factor the value 2 was chosen to ensure that the lowest abundance is two ($a * \min(y) = 2$; Cuppen et al., 2000). Data transformation was done to weigh down high abundance values and approximate a normal distribution of the data (Cuppen et al., 2000; Van den Brink et al., 1995).

MDDs were calculated with the following equation (Brock et al., 2014):

$$\mathrm{MDD} = (\bar{x}_C - \bar{x}_T)^* = t_{1-\alpha,df,k} \times s \sqrt{\frac{1}{n_C} + \frac{1}{n_T}}$$

$(\bar{x}_C - \bar{x}_T)^*$ = Difference between control and treatment mean
$t_{1-\alpha,df,k}$ = Quantile of t-distribution dependent on significance level ($\alpha$), freedom of degrees (df) and number of treatment groups (k)
s = Residual standard error of the ANOVA (Analysis of variance)
$n_K$; $n_B$ = Number of replicates in control and treatment group

MDDs were estimated by using a one-sided Williams test ($\alpha = 0.05$) assuming a typical test design with five controls and five test concentrations with three replicates each. Subsequently, the MDDs related to the log-transformed data ($MDD_{ln}$) were back-transformed to the original abundance scale and were expressed as % $MDD_{Abu}$:

$$\mathrm{MDD_{Abu}} = (e^{\bar{x}_C} - e^{\bar{x}_C - \mathrm{MDD_{ln}}})/a$$

$$\%\,\mathrm{MDD_{Abu}} = 100 \times \frac{\mathrm{MDD_{Abu}}}{(e^{\bar{x}_C} - 1)/a}$$

% $MDD_{Abu}$ = $MDD_{Abu}$ in percent of the back-transformed mean of the controls

According to the EFSA Aquatic Guidance Document for the risk assessment of pesticides MDDs (% $MDD_{Abu}$) can be classified into five different classes (EFSA, 2013; Tab. 8):

**Tab. 8: Minimum detectable difference classes after the EFSA Aquatic Guidance Document** (EFSA, 2013).

| MDD-class | MDD | Explanation |
|---|---|---|
| 0 | > 100 % | No effects can be determined statistically |
| I | 90 – 100 % | Only large effects can be determined statistically |
| II | 70 – 90 % | Large to medium effects can be determined statistically |
| III | 50 – 70 % | Medium effects can be determined statistically |
| IV | < 50 % | Small effects can be determined statistically |

### 2.10.6 Principal Response Curves

To analyse the effects of carbaryl on the macroinvertebrate and (emerged) insect community structure, Principal Response Curves were calculated (Van den Brink & Ter Braak, 1999, 1998). The PRC method is based on the ordination technique constrained redundancy analysis (RDA) and was specially designed for the analysis of data from mesocosm experiments. The use of PRCs for analysing mesocosm data is recommended in well-established guidance documents in Europe (EFSA, 2013; OECD, 2006). The benefit of this multivariate method is that even taxa which are rare and have low abundances are included in the analysis on the community level. With the PRC method, effects of a stressor (e.g. pesticide) on the natural community in comparison to untreated control can be plotted two-dimensionally during the sampling time, which makes it easier to understand in comparison to a classical ordination diagram. PRC results in a diagram showing the sampling weeks on the *x*-axis and the canonical coefficient ($c_{dt}$) relative to the control (value: 0) on the *y*-axis. The more the $c_{dt}$-values of treatments differ from the control line at point 0, the more the community is influenced by the treatment. Furthermore, the PRC gives information about the sensitivity of specific taxa against the treatment by using species weights ($b_k$). The species weights can be interpreted as the weight of each single taxon following the pattern in the PRC. The higher the weight, the more pronounced the actual response pattern of the species corresponds to the PRCs. In contrast high negative weights are obtained with an opposite pattern as the PRCs (Ter Braak & Smilauer, 2002).

To test if the PRC diagram as a whole displays a significant amount of the total variance, Monte Carlo permutation tests (499 permutations) were performed. Hereby, the F-type criterion is used ($p \leq 0.05$). The null hypothesis implies that the abundance data are not related to the treatment levels (Van Den Brink & Ter Braak, 1998, 1999). Furthermore, redundancy analysis on every sampling day were conducted to determine if the treatments showed significant

differences in community structure ($p \leq 0.05$) on each sampling day. The PRC analysis was performed using the CANOCO software package (Version 4.5, Ter Braak & Smilauer, 2002).

The abundance data of macroinvertebrate sampling and of emerging insects were log-transformed similar to the calculation of MDDs (chapter 2.10.5).

## 2.10.7 Case study II

### 2.10.7.1 Univariate analysis

Data on ergosterol content and bacterial biomass were analysed using analysis of variance (ANOVA) on log-transformed data. Tests showing significant differences were followed by Dunnett´s post hoc test to compare treatments with controls. All data were checked for normal distribution (Shapiro-Wílks Test, $\alpha = 0.01$) and homogeneity of variances (Levene´s Test, $\alpha = 0.01$). Fungicide treatments were compared to the controls by performing analysis of variances (ANOVAs) followed by Dunnett´s tests (if the assumptions for parametric testing were met) or Kuskal-Wallis tests followed by Wilcoxon rank-sum tests (for nonparametric testing). Unless specified otherwise, data are reported as mean value ± SD (standard deviation).

$EC_{50}$ calculation for several endpoints by using non-linear regression model were calculated similar to case study I as described in chapter 2.10.4. For macroinvertebrate abundance only taxa whose average abundance in the controls was higher than five on at least two sampling occasions were analysed using non-linear regression model.

# 3 Results

## 3.1 Case study I – insecticide application

### 3.1.1 Physicochemical water parameters

Physical parameters temperature (15.1 °C ± 2.6 (mean + SD)), pH (8.61 ± 0.23 (mean + SD)), dissolved oxygen (10.34 mg/L ± 0.96 (mean+SD)) and conductivity (362.74 µS/cm ± 67.06 (mean+SD)) were similar in all treated and untreated stream mesocosms during the study. Variabilities over time were particularly observed for temperature (Min-Max: 8.2 – 19.4 °C) and conductivity (Min-Max: 217 – 489 µS/cm), which decreased until the end of the study (Fig. 14).

The nutritional parameters phosphate (1.19 mg/L ± 0.59 (mean+SD)), ammonium (0.07 mg/L ± 0.07 (mean+SD)) and nitrate (0.48 mg/L ± 0.20 (mean+SD)) were also similar in all stream mesocosms. Likewise, water hardness did not show apparent trend due to different treatment levels (9.42 mg/L ± 1.80 (mean+SD)).

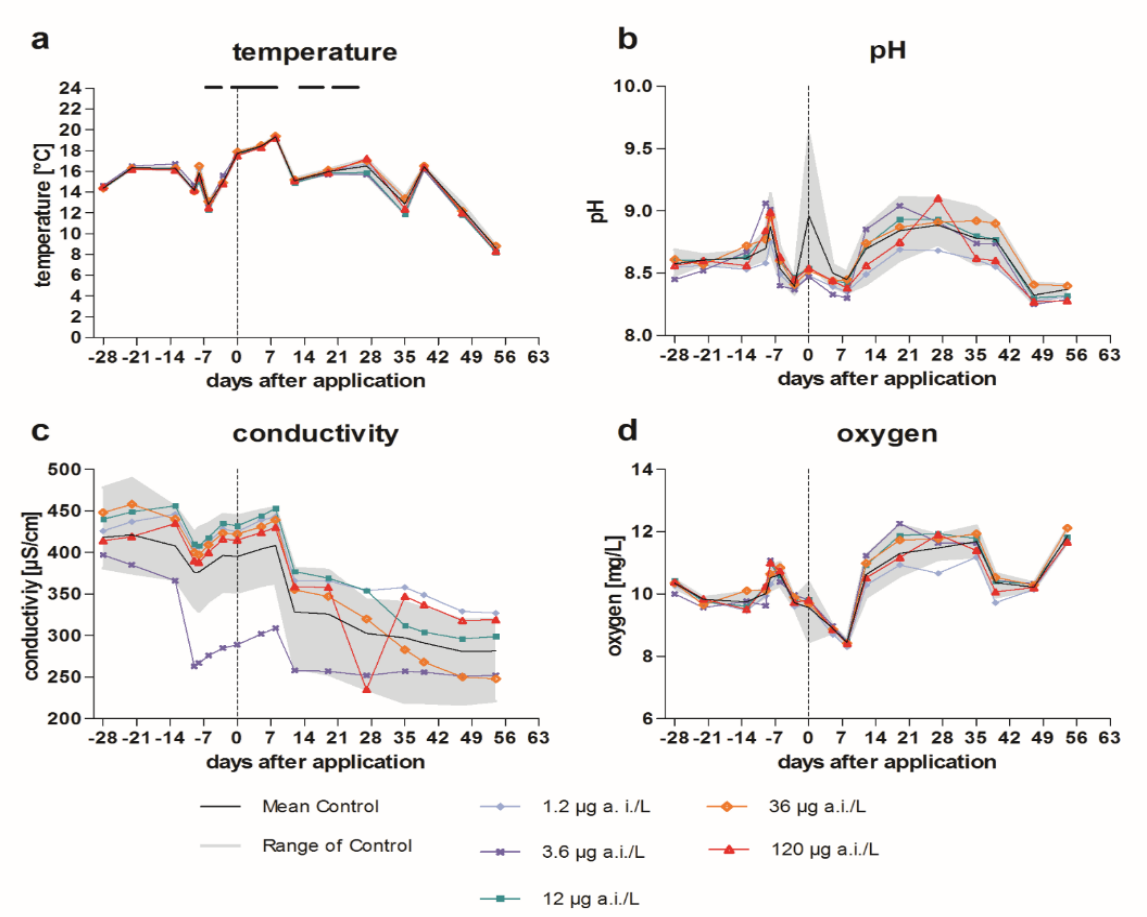


**Fig. 14: Physical parameters a) temperature, b) pH, c) conductivity and d) oxygen content from control and carbaryl-treated stream mesocosms over time.** Dashed line: time point of application; horizontal lines in a) mark the presence of a tent for sun protection, range of control: min-/ max-values of controls per time point.

### 3.1.2 Concentration of carbaryl in water

The measured concentrations of carbaryl one hour after application were within the range of nominal concentrations. 80 – 96 % of the nominal carbaryl concentration were found after one hour in the water samples of all treated stream mesocosms proving the intended dosing of the stream mesocosm system. Carbaryl decreased exponentially in the water with a mean $DT_{50}$ of 38.4 h (range 34.3 – 48.5 h, Fig. 15, A. 1). Carbaryl concentrations in all pooled control samples were below the limit of quantification (LOQ), which was set to be 0.15 µg a.i./L.

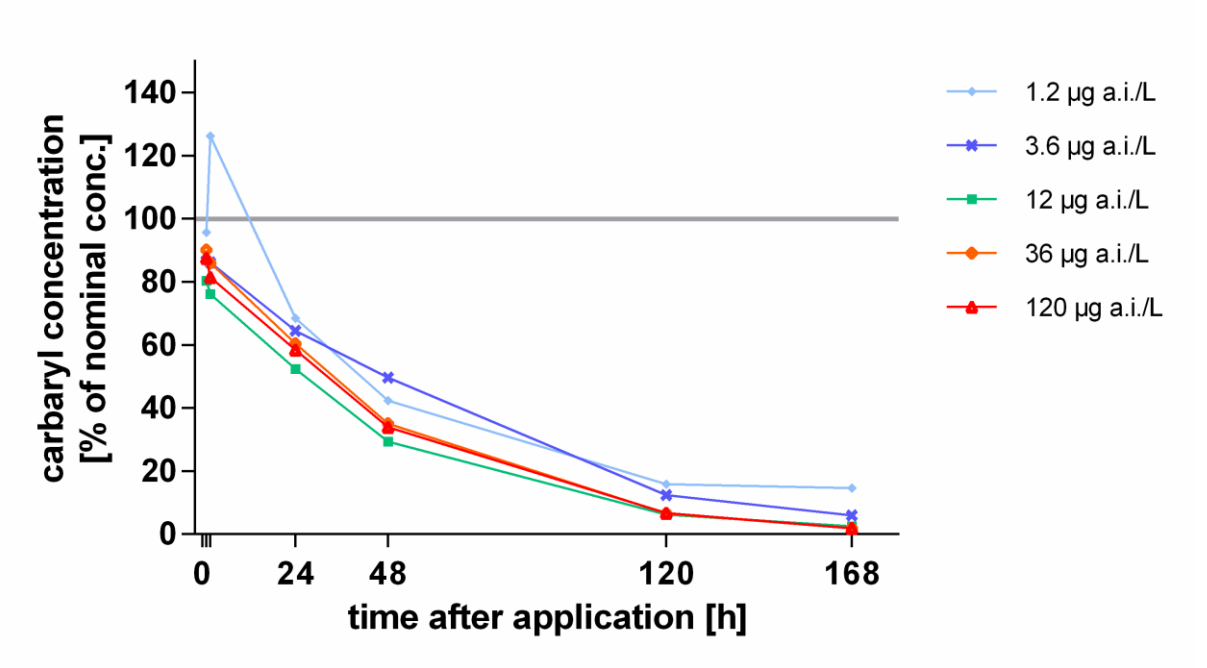


**Fig. 15: Dissipation of carbaryl in the stream mesocosms.**

### 3.1.3 Macroinvertebrates

#### 3.1.3.1 Macroinvertebrate abundance and taxa richness

During macroinvertebrate sampling 51 taxa from 17 different orders were identified overall (A. 3). The mean abundance of macroinvertebrates of all eight stream mesocosms directly before carbaryl application (pre-sampling day -2) was 228 with a coefficient of variation (CV) of 22.8 %. Arthropoda were the most dominant phylum (29 taxa, 80 % of total individuals), which were sampled on pre-application day -21 and -2 in all stream mesocosms and in the control stream mesocosms after application. The most dominant orders were Isopoda (particularly *Asellus aquaticus* (32.6 %)), Amphipoda (particularly *Gammarus* sp. (27.7 %)), Hirudinea (13.5 %), Ephemeroptera (7.0 %) and Trichoptera (5.5 %), which were found with more than 5 % of all individuals each in the samples. 15 taxa out of 51 taxa and 12.9 % of all

individuals belonged to the group of EPT, while mainly mayfly (8 taxa) and caddisfly (6 taxa) larvae were present in comparison to stonefly larvae (1 taxa, (A. 3)). According to the SPEAR database 58 % of all recorded taxa (29 taxa) were characterised with an univoltine life cycle and 8 % (4 taxa) with a semivoltine life cycle and, thus, potentially vulnerable due to long generation times. 11 taxa were categorised as Species at Risk against pesticides. Besides *A. aquaticus* and *Gammarus* sp. the leech *Helobdella stagnalis* (9.5 %) and the dipteran family Chironomidae (4.5 %) were the most dominant taxa, while the organisms of the caddisfly family Limnephilidae, the mayfly species *Ephemerella ignita* and *Ephemera danica*, the leech *Erpobdella octoculata* and the flatworm *Dugesia* sp. were represented with more than 2 % per taxa of total abundance.

A total of 11 taxa were represented with more than five organisms on average in the control stream mesocosms on two sampling occasions (Tab. 9). These taxa were analysed via logistic regression and the MDDs were estimated. For seven of the taxa, who fulfilled the aforementioned criterion, MDDs below 70 % on sampling day -2 were estimated, suggesting that for these taxa effects would be detectable in a replicated test design with 20 test units. Five taxa whose MDDs were estimated below 70 % belonged to the phylum Arthropoda. For the other four taxa estimated MDDs on sampling day -2 were above 70 % suggesting that effects could not be determined in a replicated test design. Abundance data of individual taxa combined to a higher subclass or order showed medium to smaller effects by estimated MDDs for Diptera (MDD class IV), Ephemeroptera (MDD class IV), Trichoptera (MDD class IV) and Hirudinea (MDD class IV. At the subclass oligochaeta (MDD class II) large to medium effects could be determined in a replicated test design.

**Tab. 9: Taxa and combined subclasses/orders considered to be sufficiently abundant to assess the effects of the test item on at least two sampling days (mean control abundance > 5).**

| Taxa | Order | Sampling days with mean in control > 5 | Mean abundance based on sampling day -2 (CV %) | MDD class (%) estimated based on sampling day -2[a, b] | Categorised as species at risk against pesticides according to Liess et al. (2018) | Voltinism according to Liess & Von Der Ohe, 2005; Liess et al., (2018) |
|---|---|---|---|---|---|---|
| **Arthropoda** | | | | | | |
| *Asellus aquaticus* | Isopoda | All sampling days | 70 (35) | IV (28) | No SPEAR | Multivoltine |
| Chironomidae Gen. sp. | Diptera | -21, -2, 6 | 9 (44) | IV (48) | No SPEAR | Multivoltine |
| *Gammarus* sp. | Amphipoda | All sampling days | 69 (35) | IV (36) | No SPEAR | Uni-/bivoltine |
| *Ephemera danica* | Ephemeroptera | 36, 43, 49, 57 | 0 (0) | -[c)] | No SPEAR | Semivoltine |
| *Ephemeralla ignita* | Ephemeroptera | -21, -2 | 9 (56) | III (67) | No SPEAR | Univoltine |
| Limnephilidae Gen. sp. | Trichoptera | -21, -2, 6, 13, 20 | 12 (40) | III (55) | SPEAR | Univoltine |
| *Polycentropus flavomaculatus* | Trichoptera | 27,36,43,49 | 0.2 (213) | 0 (202) | SPEAR | Univoltine |
| **Non-Arthropoda** | | | | | | |
| *Dugesia* sp. | Tricladida | 36, 43, 49, 57 | 4 (55) | IV (48) | No SPEAR | Univoltine |
| *Eiseniella tetraedra* | Crassicllitellata | 20, 43, 49 | 0.2 (185) | 0 (209) | No SPEAR | Multivoltine |
| *Erpobdella octoculata* | Arhynchobdellida | 43, 49, 57 | 1 (86) | 0 (108) | No SPEAR | Univoltine |
| *Helobdella stagnalis* | Hirudinida | -2, 6, 13, 20, 27, 36, 43, 49, 49, 57 | 21 (35) | IV (43) | No SPEAR | Multivoltine |
| **Combined to higher subclass/order** | | | | | | |
| Diptera | - | -21, -2, 6 | 10 (43) | IV (45) | No SPEAR | Multivoltine |
| Ephemeroptera | - | -21, -2, 6, 6, 13, 36, 43, 49, 57 | 16 (44) | IV (47) | SPEAR | Uni-/bivoltine |
| Trichoptera | - | -21, -2, 6, 13, 20, 27, 36, 43, 49 | 12 (36) | IV (49) | SPEAR | Univoltine |
| Oligochaeta | - | 6, 13, 20, 27 | 4 (80) | II (71) | Not classified | Multivoltine |
| Hirudinea | - | All sampling days | 25 (37) | IV (46) | No SPEAR | Uni/bivoltine |

[a] Re-transformed arithmetic mean of log-transformed data

[b] 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 %)

[c] no *E. danica* was found in the stream mesocosms shortly before application

### 3.1.3.2 Effects of carbaryl on macroinvertebrate community

During the study, 10 – 21 taxa were found per sampling day and the number of taxa showed no treatment-related trend (Fig. 16 a). The total macroinvertebrate abundance ranged from 70 to 392 individuals per sample (Fig. 16 b). Treatment-related effects were observed in the first two weeks after carbaryl application. In the highest carbaryl concentration of 120 µg/L, the total number of individuals decreased from 198 on sampling day -2 to 91 on day 6 and 90 on day 13, while the mean abundance of individuals in the control treatment was stable at around 200. On subsequent sampling days the total abundance seems to be lower in the highest carbaryl concentration in comparison to the controls. However, no clear dose-effect relationship was observed on any sampling day, and no reliable effect concentration could be determined via the regression model.

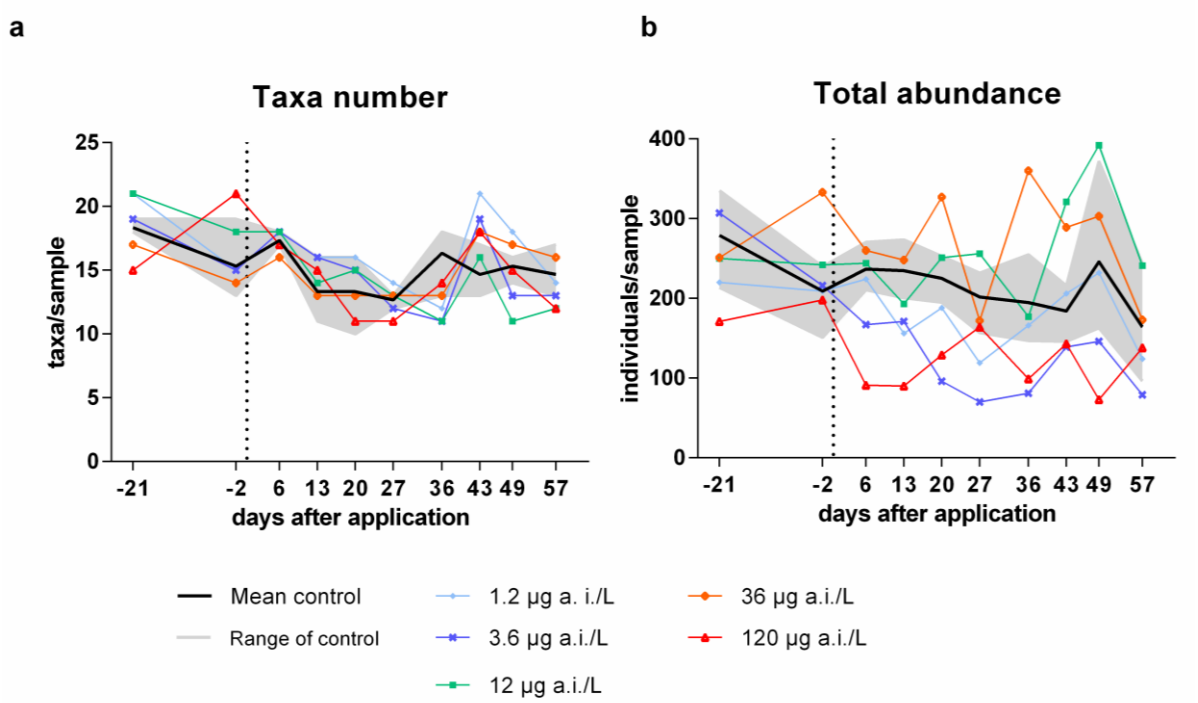


**Fig. 16: Dynamics of a) taxa richness and b) total abundance of macroinvertebrates in the samples.** Dashed line: time point of application, range of control: min-/ max-values of controls per time point.

With respect to the macroinvertebrate community the principal response curve indicates long-term effects for the highest carbaryl concentration of 120 µg/L and a short-term effect for 36 µg/L (Fig. 17 a). The first canonical PRC axis is statistically significant (permutation test, $p = 0.0240$) and 24.9 % of the variance by treatment is captured by the PRC, while the permutation test for all canonical axes did not show any statistical significance ($p = 0.496$, 41.8 % of total variance explained by treatment, while 41.5 % of total variance explained by

time). Redundancy analysis and permutation tests per sampling date revealed a significant treatment effect on days 13 ($p = 0.040$), 36 ($p = 0.016$) and 57 ($p = 0.002$; Fig. 17 a). Taxa, which have a high correspondence to PRCs and therefore show high sensitivity against carbaryl application can be expressed by using species weights (Fig. 17 b). The amphipod *Gammarus* sp., the mayfly *Ephemera danica* and the alderfly *Sialis lutaria* show high species weights and indicate that these taxa are affected through carbaryl application. Other taxa, like Naididae, the caddisfly family Limnephilidae, Plecoptera n. d. and *A. aquaticus* showed smaller positive species weights, while the snails *Radix* sp., *Radix ovata* and *Planorbis planorbis* and the flatworm *Dugesia* sp. indicate an inverse response to the PRCs, i.e. an increase of abundance in the highest carbaryl treatment.

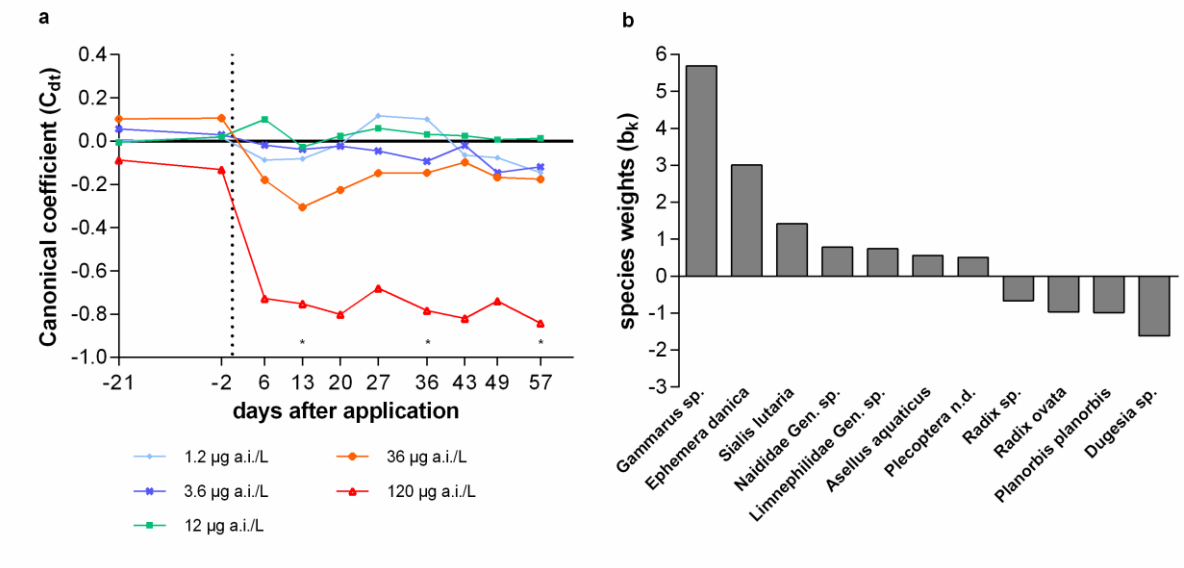


**Fig. 17: Principal response curves ($p = 0.024$, Monte Carlo permutation test) of the macroinvertebrate community (a) and species weights (b) are only shown of > +0.5 or < -0.5.** Asterisks indicate significant ($p < 0.05$) treatment effects according to RDA and followed by permutation test. Dashed line: time point of application.

The pesticide-specific bioindicator system (SPEAR), which is based on biological traits of stream invertebrates, also indicates that the invertebrate community is affected in the two highest carbaryl concentrations in comparison to control treatment (Tab. 10). The SPEAR index was classified into the five classes of ecological status ("high", "good", "moderate", "poor", "bad") according to the EU Water Framework Directive. While the ecological status on the pre-sampling days -21 and -2 was "good" or "moderate" in all stream mesocosms, the SPEAR index decreased in the stream mesocosms after application of the two highest carbaryl concentrations and the ecological status was predominantly "moderate" with high tendencies

to "poor" or "bad". In the control treatments the SPEAR index also decreased and the ecological status changed from "good" on pre-sampling dates to "moderate" on the sampling days after carbaryl application. However, this decrease was less severe in comparison to the two highest carbaryl treatments.

**Tab. 10: Values of $SPEAR_{pesticide}$ indices of control and treated stream mesocosms on different sampling days.** $SPEAR_{pesticide}$ indices are classified to the ecological status according to the EU Water Framework Directive (Beketov et al., 2009).

| Day | Mean Control | 1.2 µg/L | 3.6 µg/L | 12 µg/L | 36 µg/L | 120 µg/L |
|---|---|---|---|---|---|---|
| -21 | 33.37 | 38.50 | 39.39 | 34.22 | 37.97 | 31.32 |
| -2 | 33.55 | 29.03 | 31.62 | 32.16 | 33.86 | 29.88 |
| 6 | 31.57 | 29.01 | 36.36 | 29.87 | 24.58 | 26.74 |
| 13 | 28.72 | 35.02 | 27.00 | 34.81 | 21.83 | 19.03 |
| 20 | 26.15 | 32.74 | 28.91 | 35.80 | 20.42 | 13.84 |
| 27 | 23.56 | 40.78 | 40.19 | 20.17 | 16.94 | 24.84 |
| 36 | 25.91 | 32.19 | 23.13 | 23.13 | 17.88 | 17.12 |
| 43 | 27.29 | 30.56 | 34.32 | 25.40 | 26.92 | 7.20 |
| 49 | 28.00 | 24.53 | 23.84 | 14.77 | 25.27 | 13.48 |
| 57 | 26.97 | 24.52 | 33.44 | 26.12 | 29.42 | 13.87 |

High (> 44)
Good (> 33 – 44)
Moderate (> 22 – 33)
Poor (>11 – 22)
Bad (0 – 11)

### 3.1.3.3 Effects of carbaryl on population level

Population dynamics of the taxa with sufficiently high control abundance (>5) are presented in Fig. 18. Regression analyses were conducted for each sampling date and taxon, but most often the response was not monotonous and did not allow the calculation of a reliable concentration response function, where a significant amount of variance is explained by the regression model. However, abundance data indicate effects due to the higher carbaryl concentrations on a few taxa. The abundance of the amphipod *Gammarus* sp. decreased to zero immediately after application in the highest carbaryl treatment and did not recover until the end of the study (Fig. 18 c). Also, at the second highest carbaryl concentration of 36 µg/L abundance of the amphipod decreased on the first sampling days after application. Likewise, the abundance of the caddisfly family Limnephilidae decreased in the highest carbaryl concentration on the first two sampling days after application (Fig. 18 f). Nevertheless, such a decrease was also observed at 3.6 µg/L and abundances of Limnephilidae decreased in all stream mesocosms including the control streams from day 20 until the end of the study down to near zero in all stream mesocosms. The abundance data of the mayfly larvae *Ephemera danica* also showed a treatment-related trend after application (Fig. 18 d). While an average of 15 mayflies of this species were found in the controls at the end of the study on day 57, only 2 individuals were found at 36 µg/L and none at 120 µg/L at the end of the study. However,

variability in the control stream was large. No treatment-related trends were found for the other taxa whose average mean abundance was higher than five in the controls on two sampling days, except for a higher abundance of *Dugesia* sp. at the end of the study in the stream treated with 120 µg/L.

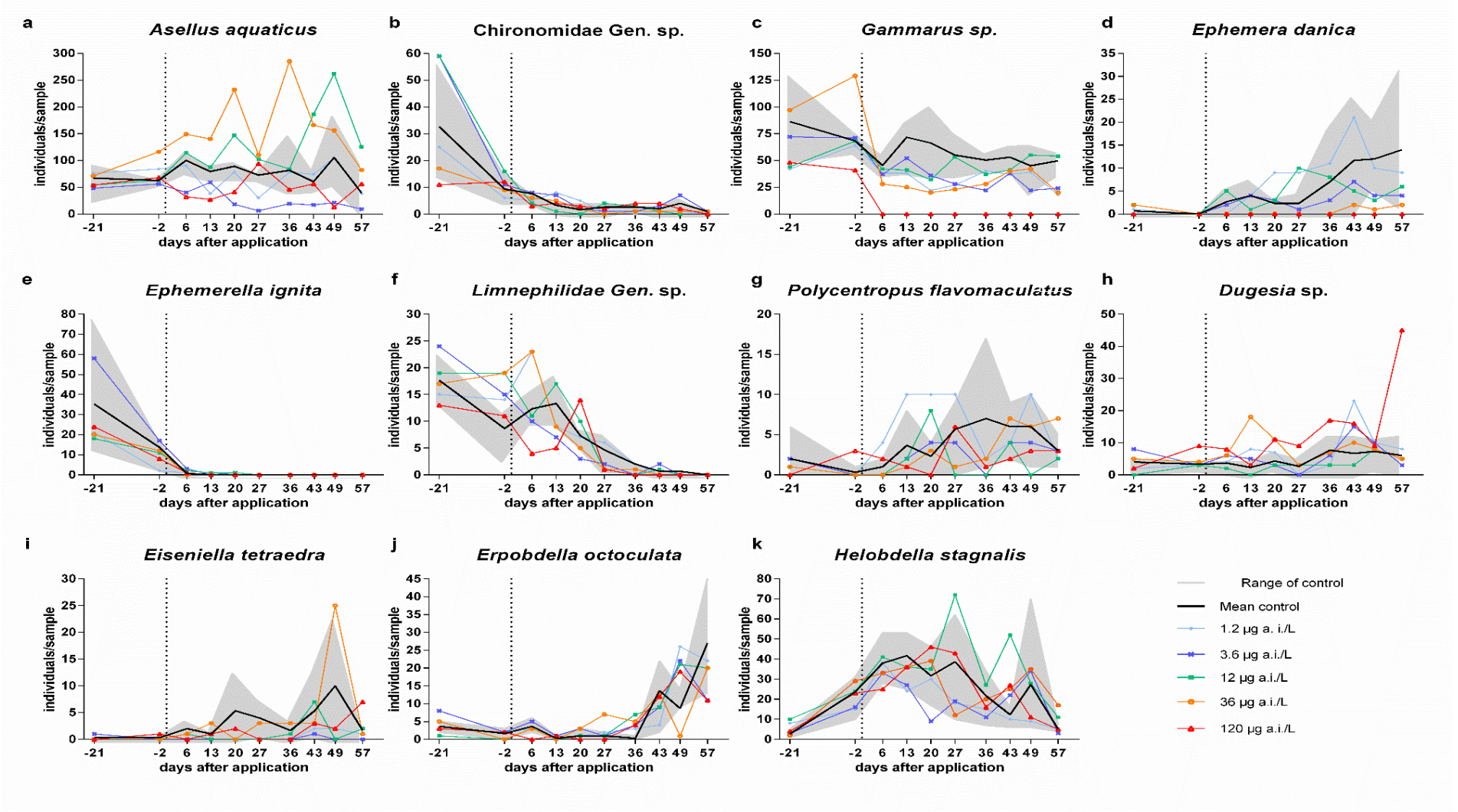


**Fig. 18: Abundance of taxa whose average abundance was more than five in the control on at least two sampling occasions in case study I: a)** ***Asellus aquaticus*****, b) Chironomidae Gen. sp, c)** ***Gammarus*** **sp., d)** ***Ephemera danica*****, e)** ***Ephemerella ignita*****, f) Limnephilidae Gen. sp., g)** ***Polycentropus flavomaculatus*****, h)** ***Dugesia*** **sp., i)** ***Erpobdella octoculata*****, k)** ***Helobdella stagnalis*****.** Dashed line: time point of application range of control: min-/ max-values of controls per time point.

### 3.1.4 Drift of invertebrates

Overall, 30 different taxa were found in the invertebrate drift samples. Thereby, ten taxa were characterised as organisms with common and/or abundant drift behaviour, in accordance with Rico & Van den Brink (2015). Invertebrate drift was clearly increased by factor eight in the highest concentration during the 4 h following carbaryl application, above the level of the controls with respect to the total number of drifted organisms (Tab. 11). Also, the number of taxa drifting was increased by more than factor two compared to the average of the control. However, the higher drift numbers found after 4 h at 120 µg/L were mainly caused by the planktonic Cladocera species *Simocephalus vetulus*. This observation was also made 8 h after carbaryl application. Regarding macroinvertebrates the amphipod *Gammarus* sp. (see Amphipoda in Tab. 11) was captured in the drift nets with more individuals in the highest carbaryl concentration (12 individuals) than in control (mean: 1.7 individuals) after 4 h. In addition, drift of mayflies (especially *E. ignita*) increased 24 h after application. While the mean number in the control stream mesocosms was at 7, the number in the carbaryl treatments ranged from 7 to 51. Hereby, a dose dependent increase in drift was not observed. For other taxa/orders similarly a dose dependent increase in drift could not be observed after 4, 8 and 24 hours of carbaryl application.

**Tab. 11: Invertebrate drift 4, 8 and 24 hours after carbaryl application in differently treated stream mesocosms.** Mean Co.: Mean of controls.

| Time | Treatment [µg/L] | Total abundance | Taxa number | Amphipoda | Basommato-phora | Coleoptera | Diptera | Ephemerop-tera | Hirudinea | Isopoda | Plecoptera | Trichoptera | *Chaetogaster* sp. | *Simocephalus vetulus* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4 hours | **Control 1** | 5 | 3 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 5 | 0 |
| | **Control 2** | 16 | 8 | 2 | 3 | 0 | 1 | 1 | 0 | 2 | 0 | 1 | 3 | 0 |
| | **Control 3** | 32 | 10 | 2 | 5 | 1 | 0 | 2 | 3 | 0 | 0 | 2 | 6 | 1 |
| | **Mean Co.** | 17.6 | 7.0 | 1.7 | 2.7 | 0.3 | 0.3 | 1.3 | 1.0 | 0.7 | 0 | 1.0 | 8.3 | 0.3 |
| | **1.2** | 13 | 4 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 2 |
| | **3.6** | 13 | 7 | 1 | 5 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 3 | 0 |
| | **12** | 17 | 5 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 13 | 1 |
| | **36** | 24 | 12 | 7 | 0 | 1 | 0 | 1 | 0 | 2 | 2 | 3 | 4 | 3 |
| | **120** | 144 | 11 | 12 | 0 | 0 | 4 | 6 | 1 | 2 | 0 | 1 | 5 | 112 |
| 8 hours | **Control 1** | 16 | 2 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 15 | 0 |
| | **Control 2** | 10 | 7 | 1 | 2 | 0 | 3 | 2 | 0 | 0 | 0 | 0 | 1 | 1 |
| | **Control 3** | 23 | 6 | 3 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 16 | 0 |
| | **Mean Co.** | 16.3 | 5 | 1.3 | 1.0 | 0 | 1.0 | 1.0 | 0.3 | 0 | 0 | 0.3 | 10.7 | 0.3 |
| | **1.2** | 17 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 11 | 6 |
| | **3.6** | 10 | 7 | 7 | 2 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 2 | 1 |
| | **12** | 15 | 4 | 4 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 11 | 2 |
| | **36** | 16 | 6 | 6 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 5 | 3 |
| | **120** | 28 | 8 | 8 | 3 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 2 | 15 |
| 24 hours | **Control 1** | 48 | 10 | 13 | 0 | 1 | 1 | 7 | 1 | 1 | 0 | 0 | 22 | 11 |
| | **Control 2** | 71 | 10 | 7 | 1 | 3 | 0 | 1 | 0 | 0 | 0 | 7 | 21 | 0 |
| | **Control 3** | 75 | 12 | 22 | 10 | 6 | 0 | 13 | 5 | 0 | 0 | 1 | 17 | 28 |
| | **Mean Co.** | 64.7 | 10.7 | 14.0 | 3.7 | 0 | 0.3 | 7.0 | 2.0 | 0.3 | 0 | 2.7 | 20.0 | 13.0 |
| | **1.2** | 218 | 11 | 2 | 0 | 2 | 1 | 7 | 2 | 2 | 0 | 5 | 67 | 130 |
| | **3.6** | 86 | 9 | 10 | 12 | 0 | 4 | 51 | 0 | 0 | 0 | 0 | 5 | 3 |
| | **12** | 82 | 11 | 8 | 5 | 0 | 8 | 11 | 2 | 3 | 0 | 1 | 43 | 0 |
| | **36** | 35 | 9 | 1 | 1 | 1 | 9 | 17 | 1 | 0 | 0 | 1 | 3 | 1 |
| | **120** | 43 | 10 | 0 | 0 | 0 | 5 | 17 | 1 | 2 | 0 | 0 | 4 | 11 |

### 3.1.5 Insect emergence

In emergence traps, a total of 29 taxa from the orders Diptera, Ephemeroptera, Odonata, Plecoptera and Trichoptera during the whole sampling was found, while a maximum of 11 taxa per sampling day was found (see taxa number, Fig. 19 a). Both taxa per sampling day in untreated and treated stream mesocosms decreased over time until the end of the study. Likewise, total abundance of emerging insects decreased from more than 200 individuals per sample before application down to less than 100 individuals until the end of the study (Fig. 19 b). A potential treatment-related decrease in total emergence and number of taxa per sample was observed 7 days after application in the highest carbaryl concentration. However, no treatment-related effects were detected with nonlinear regression analysis. Furthermore,

PRCs did not detect statistically significant differences in community structure between control and treated stream mesocosms (permutation test for first canonical axis, $p = 0.91$; permutation test for all canonical axes ($p = 0.96$); Fig. 20). Redundancy analysis and permutation tests per sampling date also revealed no significant treatment effect.

Three taxa fulfilled the criterion of more than five organisms on average in the control samples on two sampling occasion and were analysed with the regression model (Chironominae Gen. sp., Tanypodinae Gen. sp., Ephemeroptera Gen. sp.). Even so, no treatment-related effects were detected.

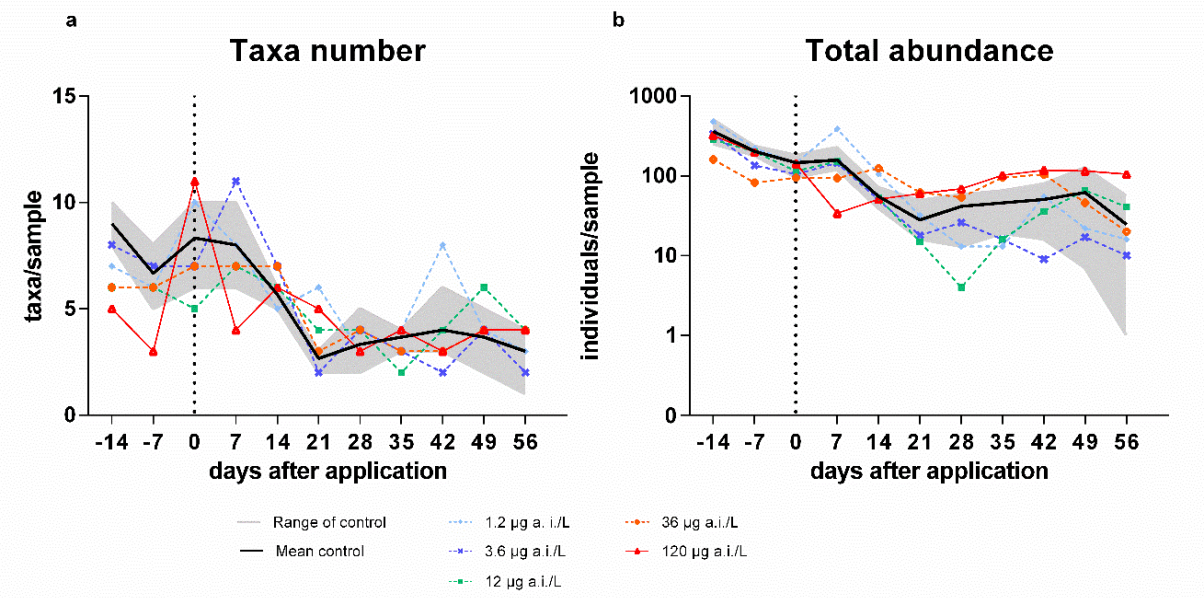


**Fig. 19: a) Number of taxa and b) total abundance in emergence traps.** Dashed line: time point of application; range of control: min-/ max-values of controls per time point.

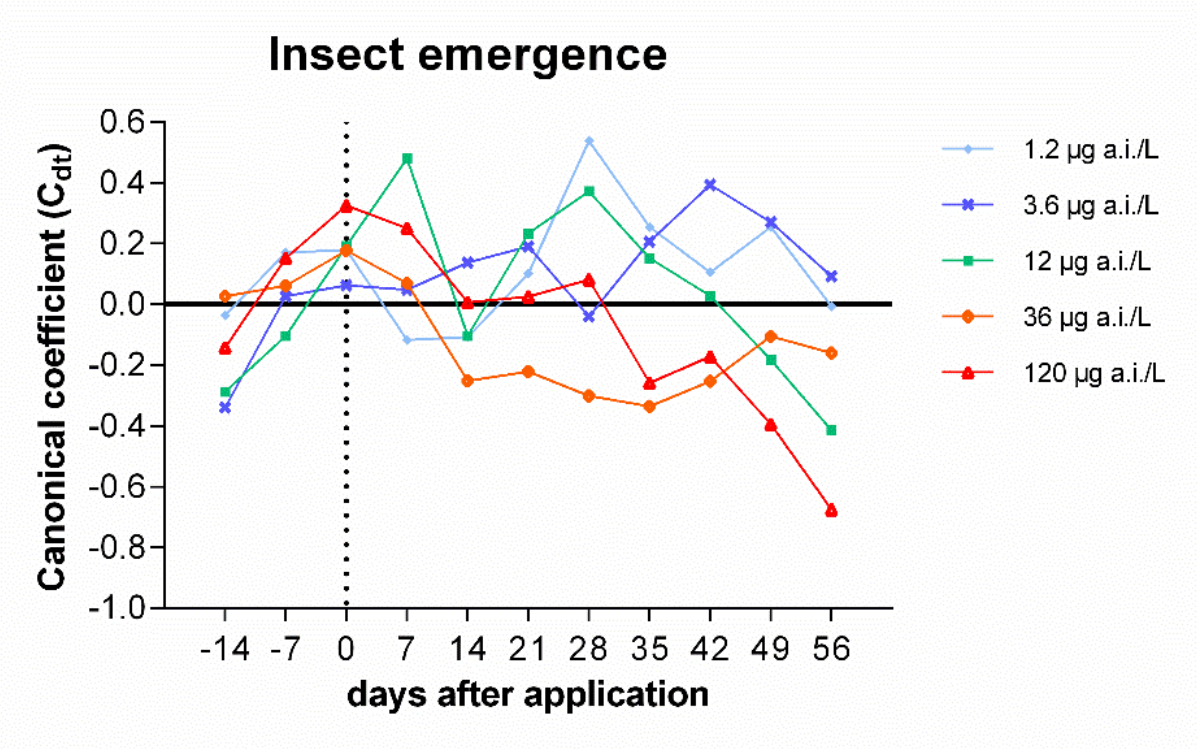


**Fig. 20: Principal response curves of the macroinvertebrate community in emergence traps (Monte Carlo permutation test, $p = 0.91$).** Dashed line: time point of application.

### 3.1.6 Suspended algae

During the study the total chlorophyll a – concentration in water increased from an average of 118 µg/L (CV: 66 %) on sampling day -3 to an average of 269 µg/L (CV: 72%; Fig. 21) until the end of the study on day 53. At this, variability between stream mesocosms before carbaryl application was rather high. In water suspended algae were dominated by green algae, with more than 70 % of total sum per sampling day on average. Diatoms were represented with average 23 % per sampling day, while blue-green algae were represented with average 6 %. Because of no clear concentration response on the different sampling days there was no indication for any effects of carbaryl on phytoplankton community and biomass.

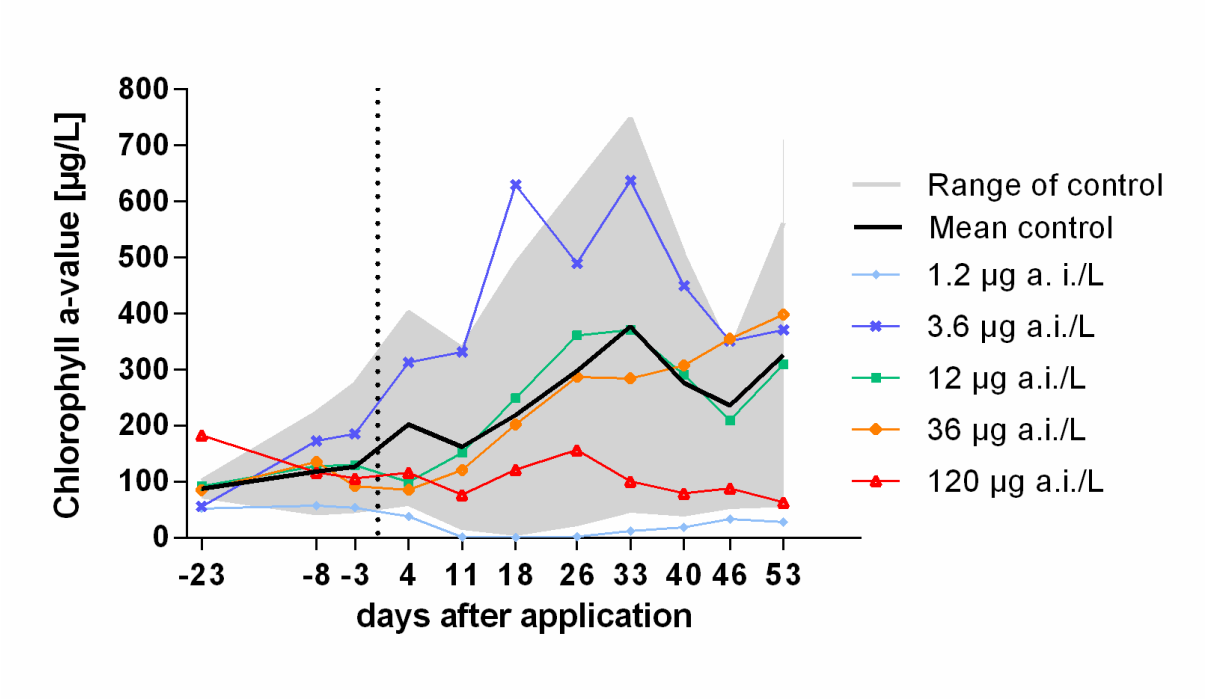


**Fig. 21: Total chlorophyll a-value of suspended algae [µg/L] from control and carbaryl-treated mesocosms.** Dashed line: time point of application, range of control: min-/ max-values of controls per time point.

### 3.1.7 Periphyton

The amount of periphyton, measured as total chlorophyll a – concentration, ranged between 129 and 1283 µg/m². During the study the total chlorophyll a – concentration decreased from average 534 µg/m² (CV: 46 %) on sampling day -2 to average 497 µg/L (CV: 50%; Fig. 22) at the end of the study on day 53. Likewise, the periphyton community was dominated by green-algae, with more than average 60 % of total sum per sampling day. Diatoms and blue-green algae were represented with average 24 and 13 %. 4 and 11 days after carbaryl application, the chlorophyll a content was increased by factor 1.2 – 2.4 (day 4) and 1.4 – 1.7 (day 11) in the treated stream mesocosms compared to the average of the controls. By contrast, 26 and 33 days after carbaryl application chlorophyll a content in the two highest carbaryl concentrations was decreased by 46 – 66 % in comparison to the average chlorophyll a content of the controls. However, there was no clear dose-response relationship and it was not possible to calculate reliable effect concentrations.

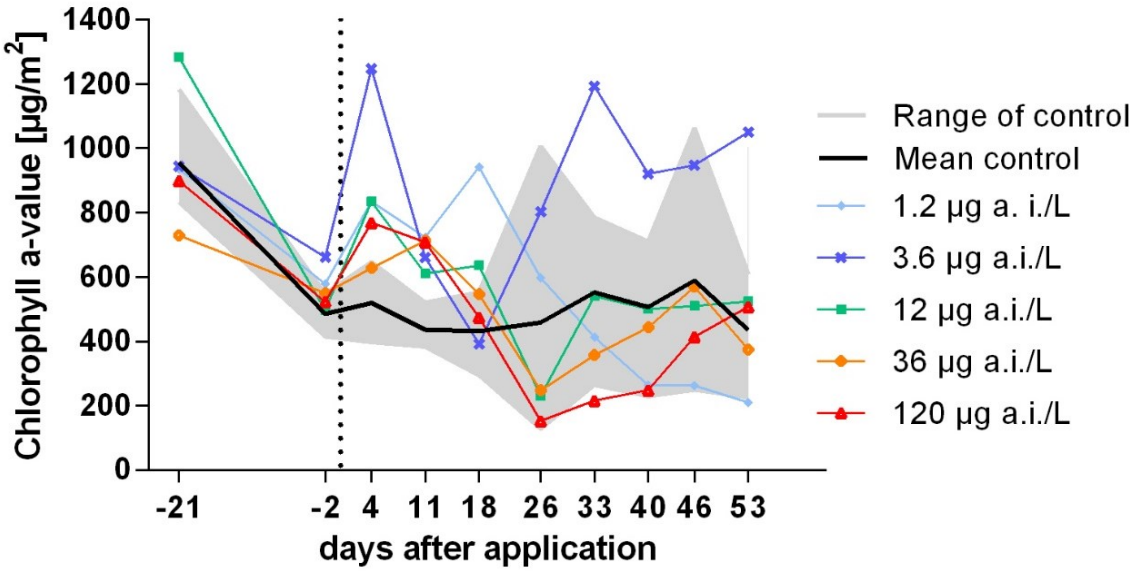


**Fig. 22: Total chlorophyll a-value of periphyton [µg/m²] from control and carbaryl-treated mesocosms.** Dashed line: time point of application, range of control: min-/ max-values of controls per time point.

## 3.2 Case study II – fungicide application

### 3.2.1 Physicochemical water parameters

During the study the temperature of the water ranged from 4.0 to 12.8 °C (8.8 °C ± 3.07 (mean+SD)), while pH ranged from 8.14 to 8.52 (8.32 ± 0.08 (mean+SD), Fig. 23 a + b). Conductivity varied between 223 and 274 µS/cm (247 µS/cm ± 11.49 (mean+SD)) and dissolved oxygen was in the range of 10.04 and 13.51 mg/L (11.55 µS/cm ± 1.02 (mean+SD), Fig. 23 c + d). Over the course of the study the mean temperature decreased, while conductivity and dissolved oxygen increased in all stream mesocosms.

The following nutritional parameters were analysed: Phosphate ranged between 0.2 – 0.4 mg/L (0.29 mg/L ± 0.04 (mean+SD)), nitrate was <0.04 mg/L, ammonium ranged between 0.02 and <0.01 mg/L and water hardness ranged between 5.8 – 8.7 °dH (7.13 °dH ± 0.93 (mean + SD)).

All physicochemical water parameters were similar in treated and untreated stream mesocosms and did not show treatment-related trends.

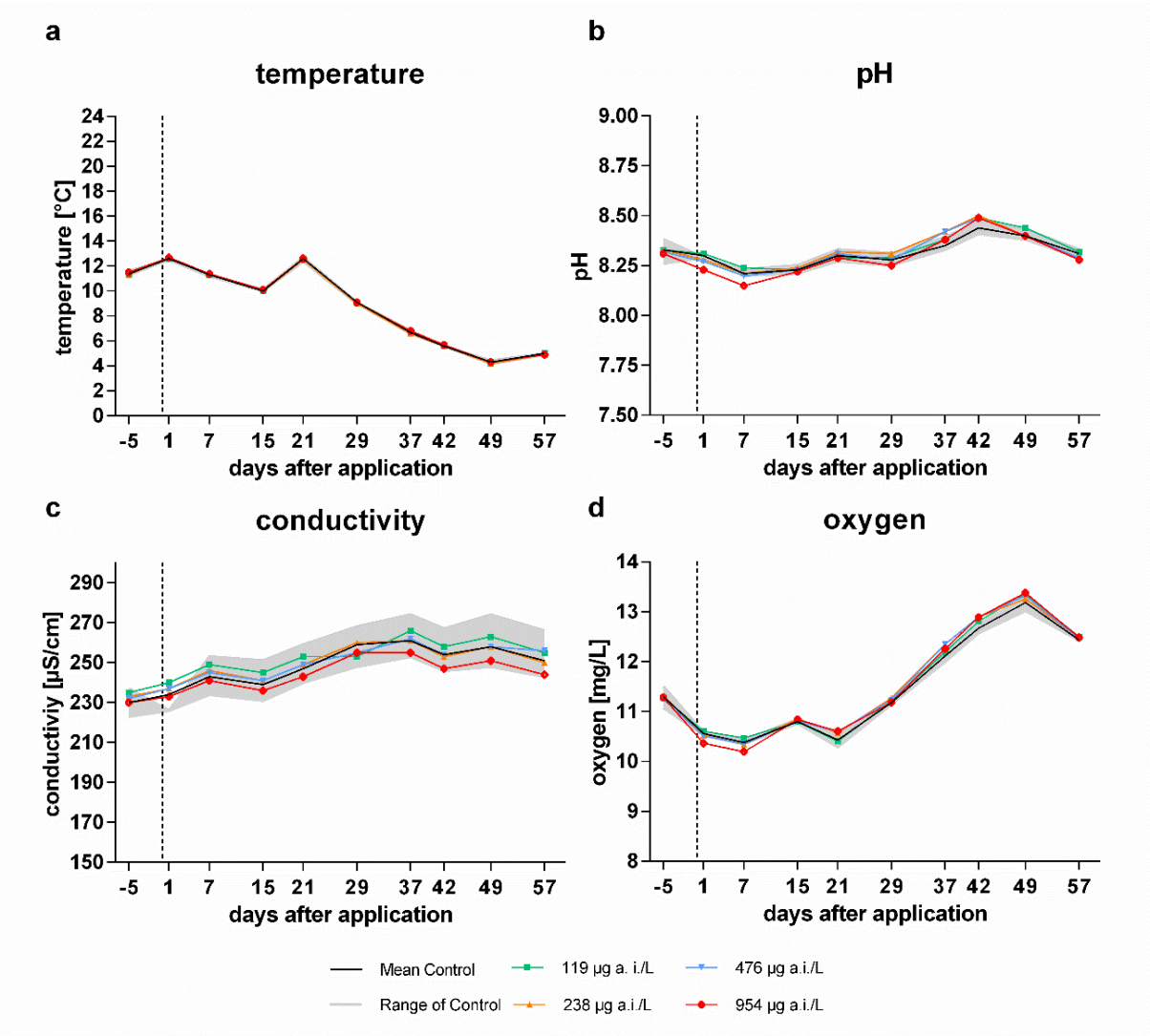


**Fig. 23: Physical parameters a) temperature, b) pH, c) conductivity and d) oxygen content from control and tebuconazole treated stream mesocosms over time.** Dashed line: time point of application; range of control: min-/ max-values of controls per time point.

## 3.2.2 Concentration of tebuconazole in water

Three hours after tebuconazole application 92 – 102 % of nominal concentrations were found in the water samples of all treated stream mesocosms. Because measured concentrations differed not more than 20 % from nominal concentrations, one can expect that intended dosing of stream mesocosms was correct. The mean dissipation time of tebuconazole amounts to 31.1 days (range 24.4 – 39.7 days, Fig. 24, A. 2). Dissipation of tebuconazole of lower nominal concentrations like 119 µg/L ($DT_{50}$ = 24.4 days) was faster in comparison to the highest nominal tebuconazole concentration ($DT_{50}$ = 39.7 days). Tebuconazole concentrations in all pooled control samples were below the LOQ, which was set to 5.00 µg a.i./L.

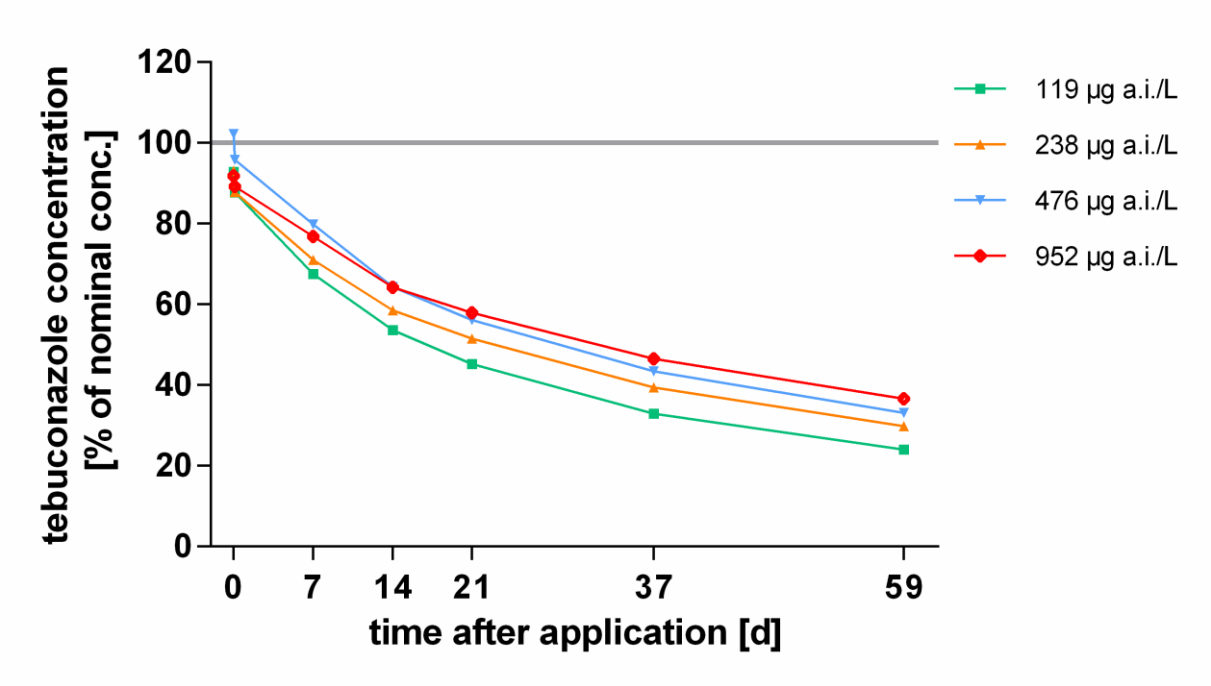


**Fig. 24: Dissipation of tebuconazole in the stream mesocosms.**

### 3.2.3 Microbial leaf decomposition

Leaf mass loss increased over time in control and all test item concentrations (Fig. 25). 57 days after application microbial leaf mass loss ranged from 60 to 95 % (81 ± 11 % (mean ± SD)), whereas leaf mass loss was lower in the highest test item concentration of 954 µg/L in comparison to control. 17 and 44 days after application leaf mass loss was statistical significantly reduced at test item concentration 954 µg/L in comparison to control (two-tailed, Williams t-test, $p < 0.05$, NOEC = 476 µg/L). Except for 4 days after application leaf mass loss was always reduced in the highest test item concentration in comparison to control on all sampling days. Leaf mass loss in the other concentrations was not significantly reduced in comparison to the control. However, no clear dose-response of leaf mass loss was observed on the different sampling days.

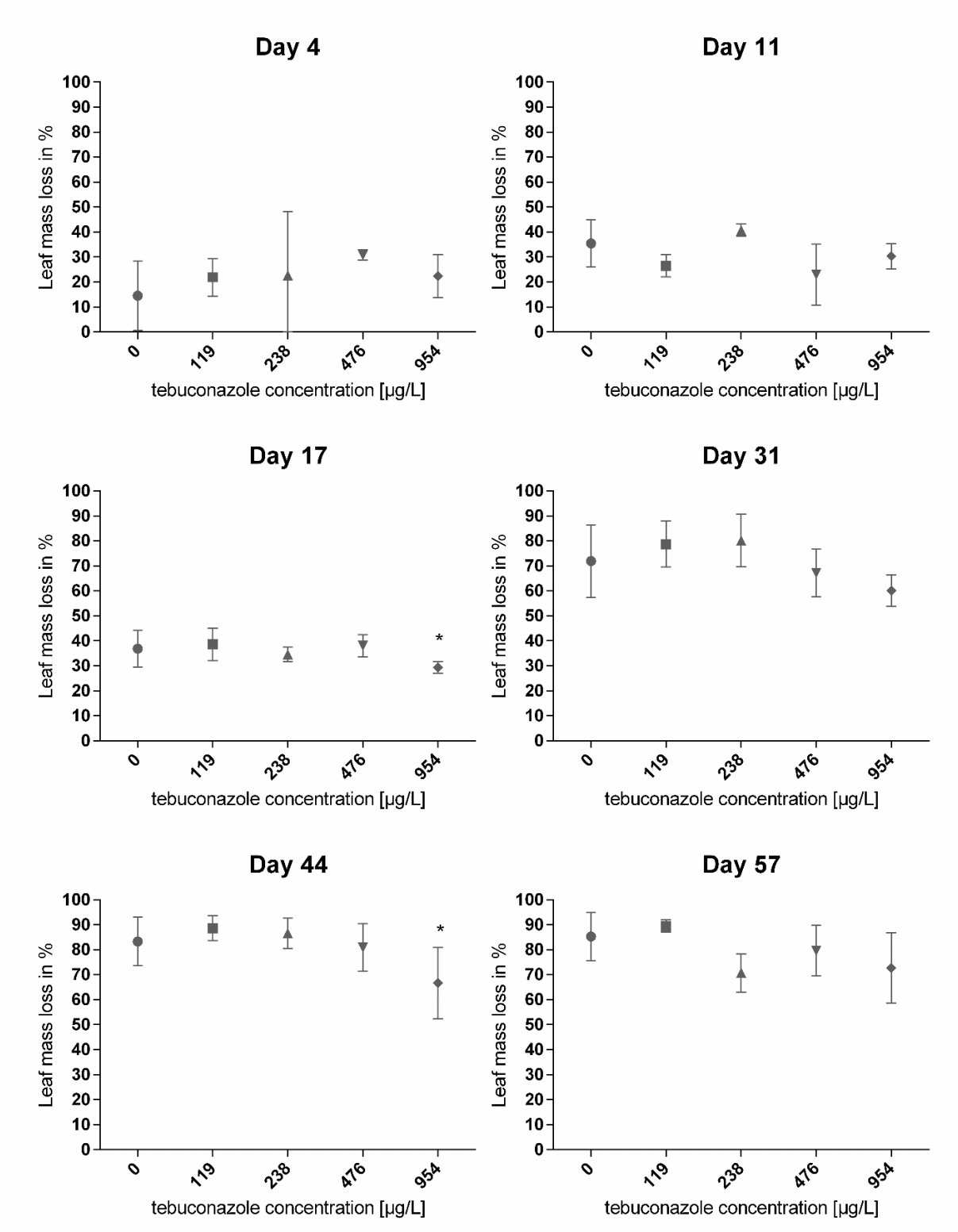


**Fig. 25: Mean (± SD) leaf mass loss of decomposing alder leaves by microbial organisms in the treated (n = 4 for every time step) and control stream (n = 8 for every time step) mesocosms.** Asterisks denote statistical differences compared to control. These are explained in the text.

### 3.2.4 Extracellular enzyme activity

The enzymes ß-glucosidase and ß-xylosidase were higher in control and all treatments four days after application in comparison to the other sampling days (Fig. 26 a, b). Four days after application ß-glucosidase ranged between 40 and 73 nmol MUF/mg dry weight/h (56 ± 8 nmol MUF/mg dry weight/h (mean ± SD)) and ß-xylosidase ranged from 9 to 18 nmol MUF/mg dry weight/h (13 ± 3 nmol MUF/mg dry weight/h (mean ± SD)). Compared to the other sampling days ß-glucosidase and ß-xylosidase activity was decreased (range ß-glucosidase sampling day 11 – 45: 2 – 40 nmol MUF/mg dry weight/h; range ß-xylosidase sampling day 11 – 45: 1 – 7 nmol MUF/mg dry weight/h). The enzyme activity of ß-glucosidase and ß-xylosidase in the different tebuconazole treatments was statistically significantly different compared to the control at various time points, but most often no clear dose-dependent response was found within the sampling days. Solely 17 days after application ß-glucosidase and ß-xylosidase activity was statistically significantly decreased in the highest tebuconazole concentration (two-tailed, Williams t-test, $p < 0.05$, NOEC = 476 µg/L) and a dose-response relation could be observed.

During the study phosphatase activity ranged from 19 to 277 nmol MUF/mg dry weight/h (126 ± 66 nmol MUF/mg dry weight/h (mean ± SD), Fig. 26 c). On sampling day 45 phosphatase activity was decreased in control and all treatments in comparison to the other four sampling days before. Regarding the tebuconazole treatment, phosphatase activity was increased in the treatments four days after application and a dose-response relation was observed. However, no statistically significant difference regarding treatments in comparison to control were observed. On the other sampling days there was no evidence of a monotone dose-response relation.

Because the degradation process of the microbial leaf discs was far gone after 31 days, there were not enough leaf discs to evaluate the phenoloxidase and peroxidase activity on sampling day 45. Therefore, the activity of these enzymes was only assessed on day 4, 11, 17 and 31 after application. The activity of the fungi specific extracellular enzymes peroxidase and phenoloxidase did not show any time-dependent differences (Fig. 26 d, e). Furthermore, no monotone dose-response relation was observed on the different sampling days.

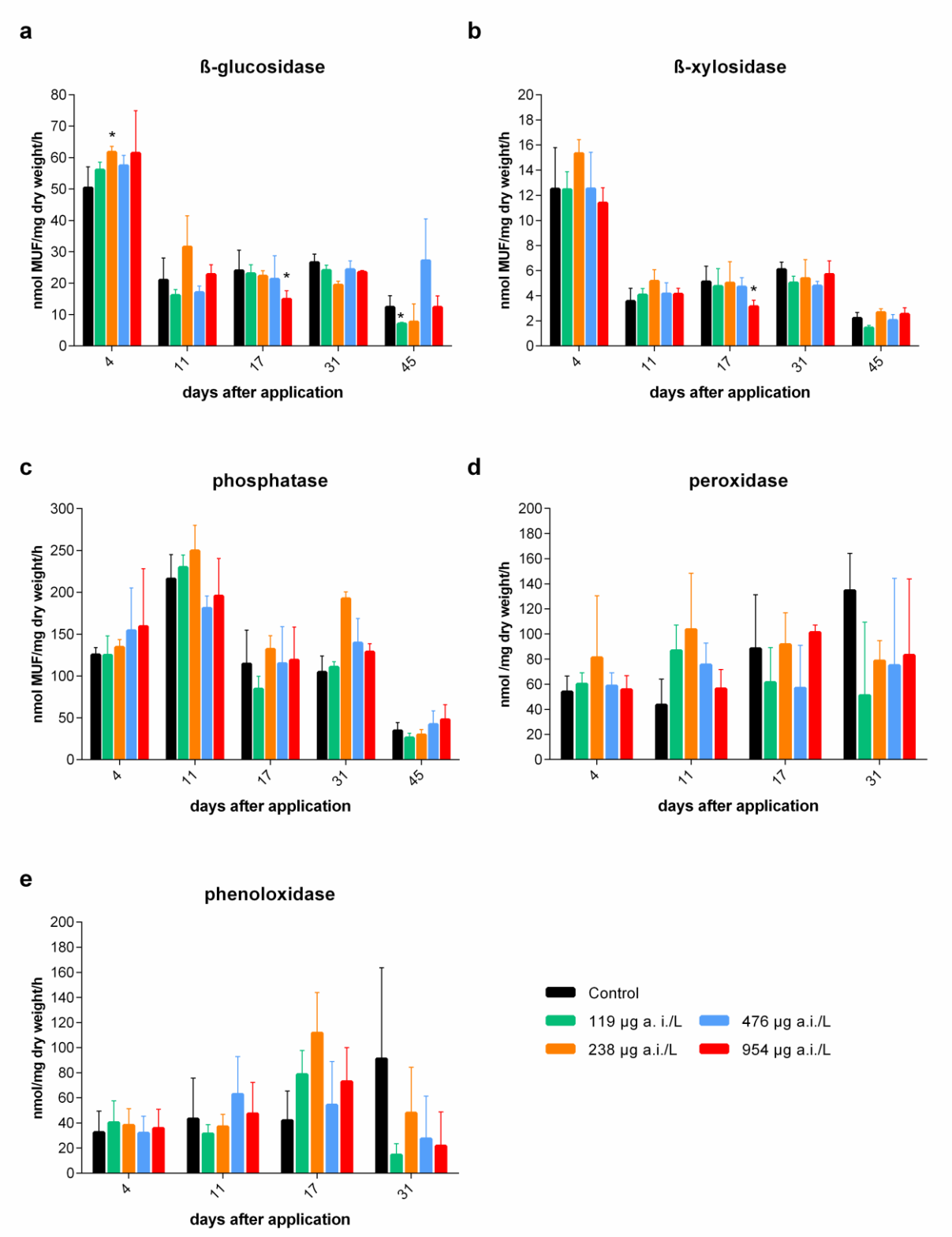


**Fig. 26: Extracellular enzyme activity (mean ± SD) of a) ß-glucosidase, b) ß-xylosidase, c) phosphatase, d) peroxidase and e) phenoloxidase of the biofilm developing in the treated (n = 4) and control (n = 8) stream mesocosms.** Asterisks denote statistical differences compared to control and are explained in the text.

### 3.2.5 Suspended algae

Six days before tebuconazole application the average amount of suspended algae measured as total chlorophyll a-concentration was 35 µg/L (CV: 11 %). During the study, suspended algae were dominated by green algae (Chlorophyta) with average 58 % and diatoms (Bacillariophyta) with 31 % on average. In the second half of the study (sampling day 23 – 59), the total chlorophyll a-concentration increased in the treated stream mesocosms in comparison to the controls (Fig. 27). On sampling day 43, 51 and 59, the chlorophyll a content was increased by factor 2.6 – 6.4 in the treated stream mesocosms compared to the average of the controls. A clear concentration response was not observed on any sampling day. However, on sampling day 38 and 51 chlorophyll a-concentration in the second lowest tebuconazole concentration of 238 µg/L was significantly increased in comparison to the control (two-sided, Multiple Sequentially-rejective Welsh t-test after Bonferroni-Holm, $p < 0.01$ (day 38) and $p = 0.003$ (day 51)). On sampling day 59 the chlorophyll-a-concentration of all treatments was significantly increased (two-sided, Williams t-test, $p < 0.01$, NOEC ≤ 119 µg/L).

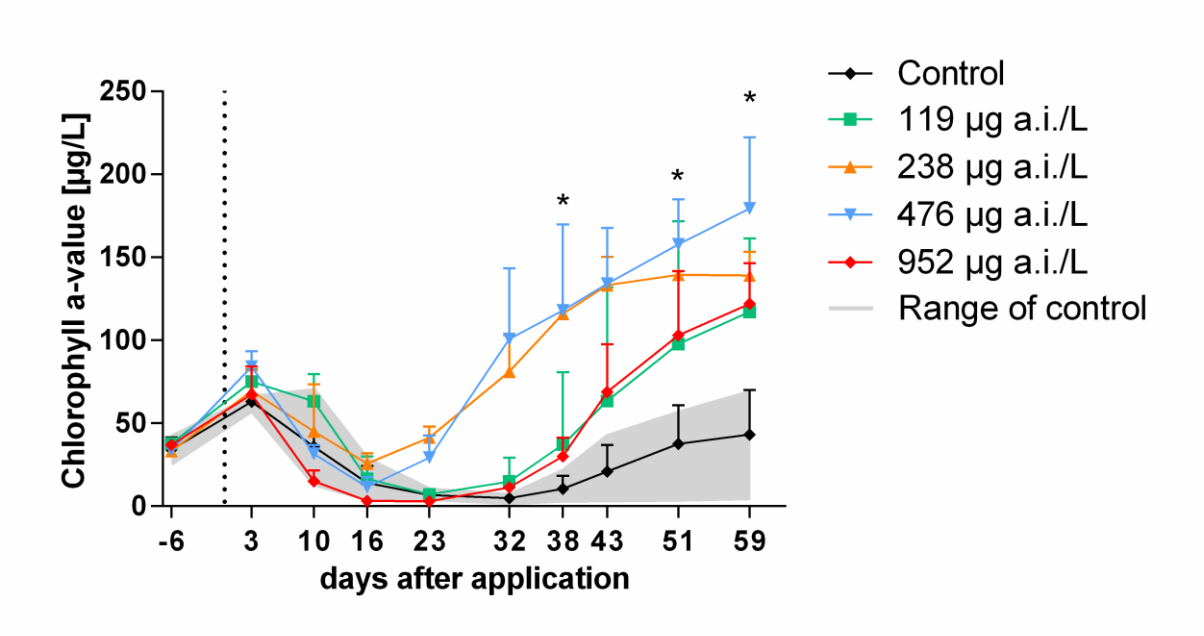


**Fig. 27: Total chlorophyll a-value (± SD) of suspended algae [µg/L] from control (n = 4) and tebuconazole (n = 2) treated mesocosms.** Dashed line: time point of application; range of control: min-/max-values of controls per time point. Asterisks denote statistical differences with control. These are explained in the text.

### 3.2.6 Periphyton

The average amount of periphyton measured as total chlorophyll a content before tebuconazole application was 2683 µg/m² (CV: 37 %). Periphyton was dominated by green algae (Chlorophyta) with on average 61 % during the study. On sampling day 16 and 23 after application the chlorophyll a-concentration was higher in the two highest tebuconazole concentrations in comparison to the control treatment, which was significant on sampling day 16 (two-sided, Williams t-test, $p < 0.01$, NOEC = 476 µg/L (Fig. 28). Furthermore, the amount of periphyton was increased in the highest test item concentration at sampling day 43, 51 and 59. No concentration-dependent response was observed on any sampling day after tebuconazole application.

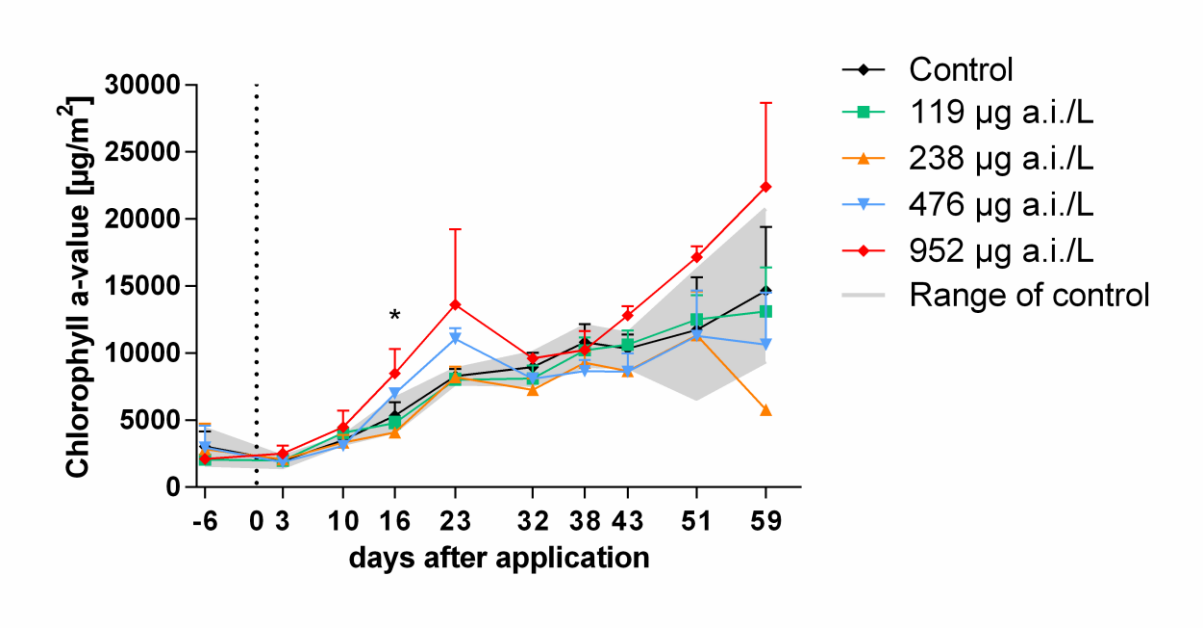


**Fig. 28: Total chlorophyll a-value (mean ± SD) of periphyton [µg/m²] from control (n = 4) and tebuconazole (n = 2) treated mesocosms.** Dashed line: time point of application; range of control: min-/max-values of controls per time point. Asterisks denote statistical differences compared to control and are explained in the text.

### 3.2.7 Fungal biomass

Fungal biomass on leaf discs expressed as ergosterol per g leaf was assessed on sampling day 4, 11 and 17 after tebuconazole application because degradation of leaf discs on sampling day 31 and 45 was far gone and there were not enough leaf discs available. Four days after application fungal biomass measured as ergosterol per g leaf was quite variable in the control and different tebuconazole treatments and ranged from 0 to 148 µg ergosterol per g leaf (mean: 36 µg ergosterol/g leaf (CV: 121 %), Fig. 29). On sampling day 17 fungal biomass was lower in all tested tebuconazole treatments (30.5 ± 16.5 µg ergosterol/g leaf (mean ± SD of all treatments)) compared to control (54.1 ± 20.2 µg ergosterol/g leaf (mean ± SD)). However, no statistically significant differences were observed.

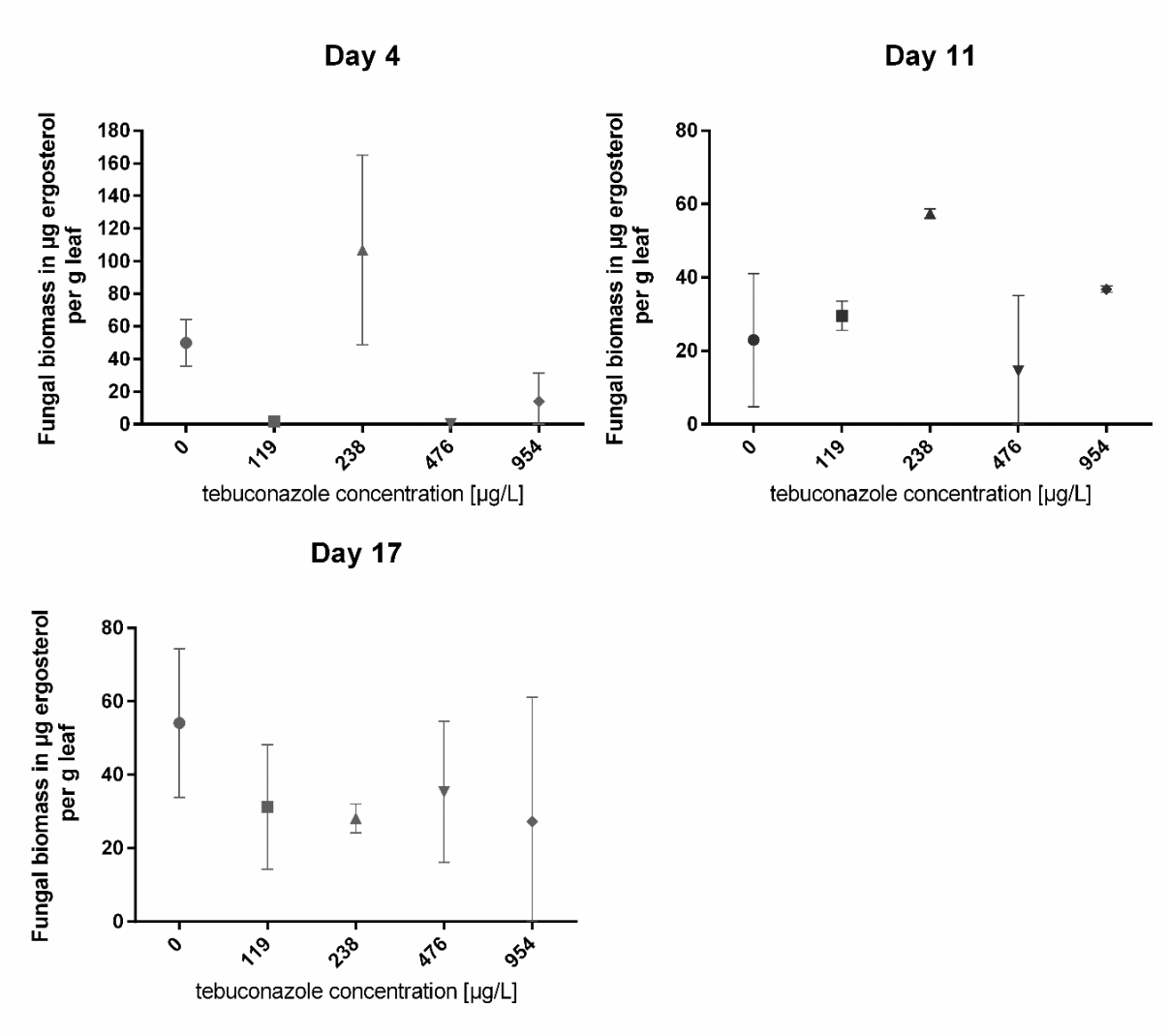


**Fig. 29: Fungal biomass expressed as µg ergosterol per g leaf (mean ± SD) of the biofilms from control (n = 4) and tebuconazole treated (n = 2) mesocosms.**

### 3.2.8 Bacterial biomass

Bacterial biomass on leaf discs was assessed on sampling day 4, 11 and 17 after tebuconazole application because degradation of leaf discs on sampling day 31 and 45 was far gone. The average amount of bacterial biomass expressed as the number of bacterial cells on leaf discs on sampling day 4, 11 and 17 was 1.13 x $10^8$ cells/mg leaf (CV: 108 %). 11 days after application bacterial biomass was 53 – 75 % decreased in the tebuconazole treatments in comparison to control. This was statistically not significant (Fig. 30). No clear dose-dependent response was observed on any sampling day.

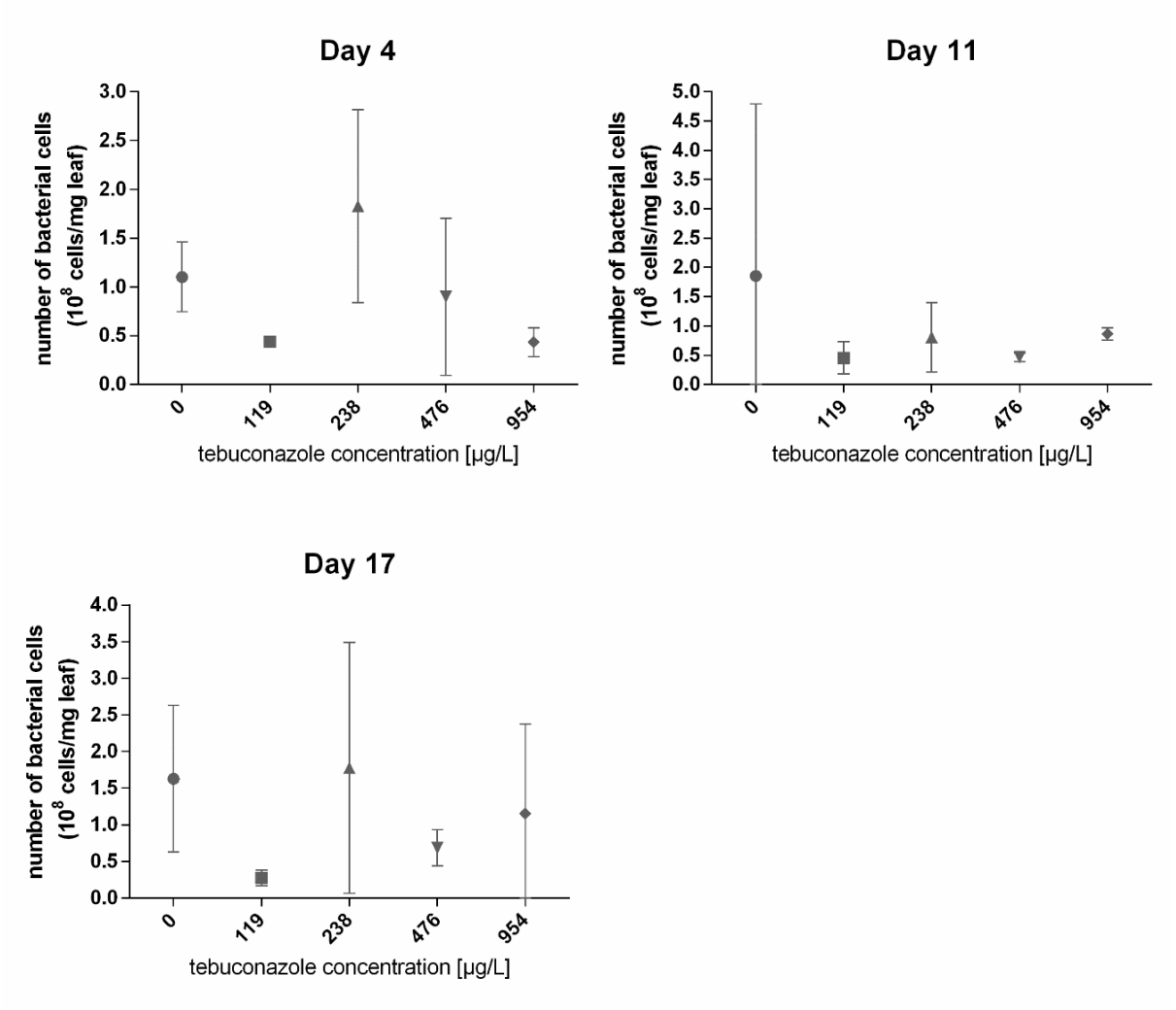


**Fig. 30: Number of bacterial cells (mean ± SD) of the biofilms from control (n = 4) and tebuconazole treated (n = 2) mesocosms.**

## 3.2.9 Effects on higher trophic level

### 3.2.9.1 Macroinvertebrate abundance and taxa richness

During the study, 14 – 22 taxa were found per sampling day. Overall, 27 taxa were present during the study. The total abundance at the beginning of the study was quite high with a mean abundance of 665 organisms in all stream mesocosms and decreased to on average 174 organisms. The total abundance was significantly decreased in the highest tebuconazole concentration of 952 µg/L on sampling day 2 (two-sided multiple sequentially-rejective Welsh t-test after Bonfrerroni-Holm, $p < 0.001$, NOEC = 476 µg/L, Fig. 31 a). On sampling day 14 the total abundance of all treatments was significantly lower in comparison to the control (two-sided Williams-test, $p < 0.001$, NOEC ≤ 119 µg/L). Overall, five taxa were present with average > 5 individuals on at least two sampling days (Fig. 31). Effects of tebuconazole on population level were observed among others for *Gammarus* sp. on sampling day 2. In all treatment groups *Gammarus* sp. was less present in comparison to the controls, which is significantly different on sampling day 2 (two-sided Williams t-test. $p < 0.001$, NOEC ≤ 119 µg/L, Fig. 31 b). Regression analysis results in an $EC_{50}$ of 122.65 µg tebuconazole/L (18.22 – 281.65 µg/L confidence limits) for *Gammarus* sp.. Abundances of the insect family Chironomidae Gen. sp. was significantly decreased in the two highest tebuconazole concentrations on sampling day 2 (two-sided Welsh t-test after Bonferroni-Holm, $p < 0.01$, NOEC = 238 µg/L). Non-linear regression reveals an $EC_{50}$ of 331.84 µg/L (124.30 – 544.18 µg/L confidence limits) for Chironomidae Gen. sp. on sampling day 2 (Fig. 31c). On sampling day 14 the abundances of Chironomidae Gen. sp. in all treatments were significantly lower in comparison to control (two-sided Williams t-test, $p < 0.001$, NOEC ≤ 119 µg/L). On sampling day 44 the abundance of chironomids was significantly higher in the highest tebuconazole concentration in comparison to the control (two-sided Williams t-test. $p < 0.001$, NOEC = 476 µg/L). No treatment-related effects were found for the other taxa whose average mean abundance was higher than five in the controls on two sampling occasions (*Asellus aquaticus*, *Erpobdella octoculata*, *Eiseniella tetraedra*; Fig. 31 d-f).

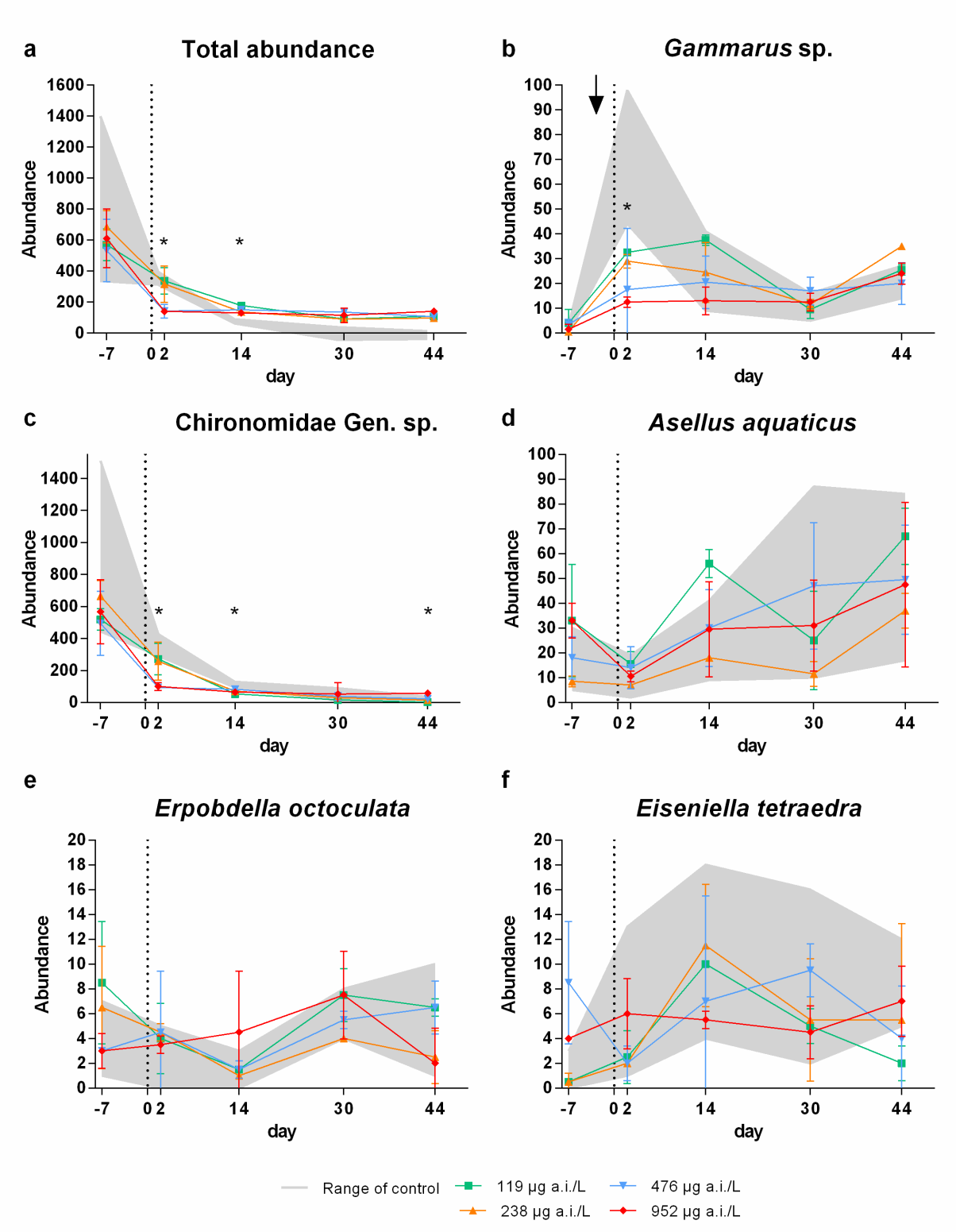


**Fig. 31: Total abundance and abundances of taxa (mean ± SD) whose average abundance was more than five in the control on at least two sampling occasions in case study II: a) Total abundance, b) *Gammarus* sp., c) Chironomidae Gen. sp., d) *Asellus aquaticus*, d) *Eiseniella tetraedra*, f) *Erpobdella octoculata*.** Dashed line: time point of application; range of control: min-/ max-values of controls per time point. Asterisks denote statistical differences with control. These are explained in the text. Arrow in b) denotes additional introduction of gammarids (150 – 300 individuals) into each stream mesocosm three days before tebuconazole application.

### 3.2.9.2 Leaf decomposition through macroinvertebrates and microorganisms

Leaf mass loss increased constantly after tebuconazole application. Leaf litter decomposition through macroinvertebrates and microorganisms was not significantly altered due to tebuconazole treatment on any sampling day (Fig. 32).

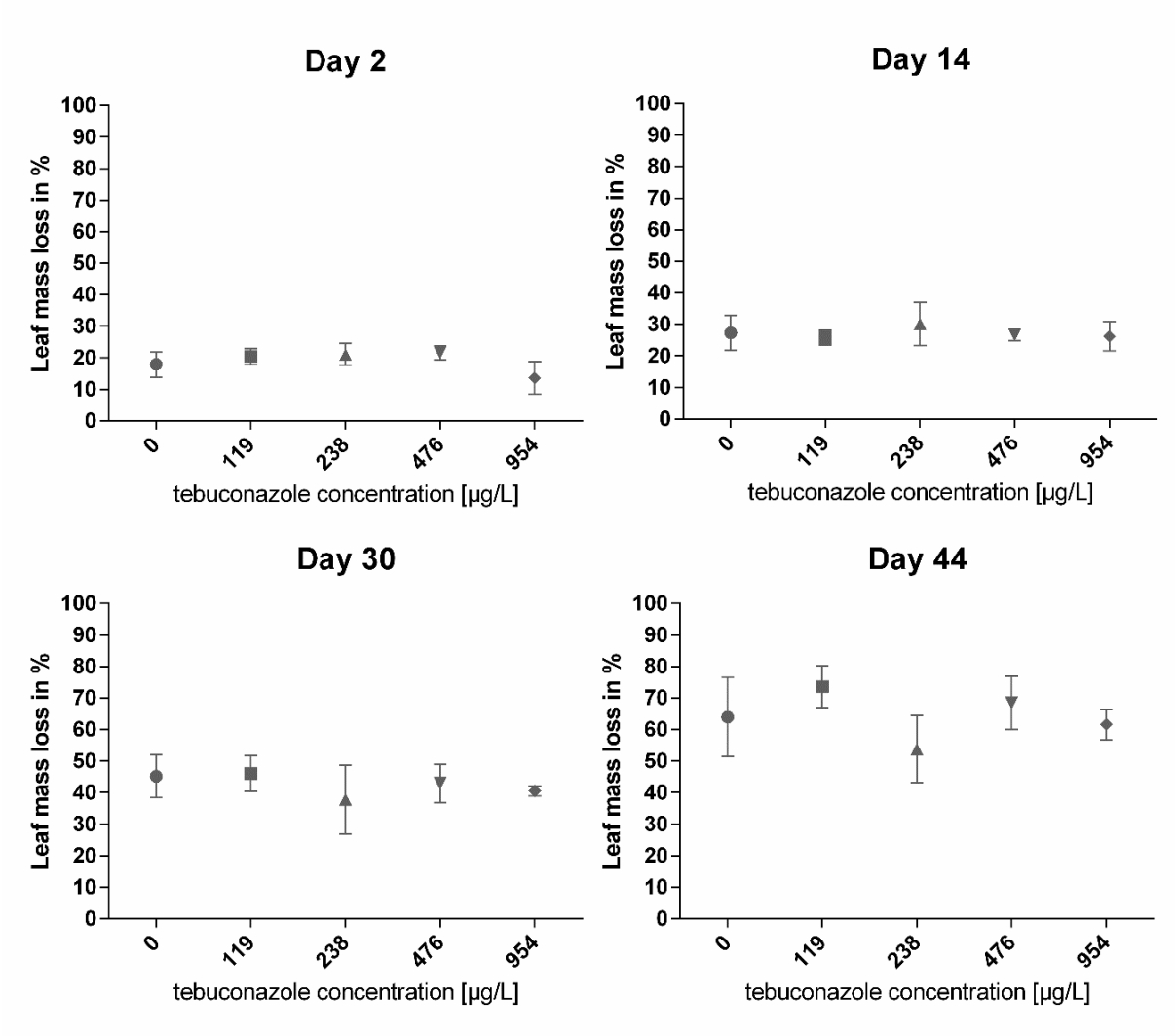


**Fig. 32: Mean (± SD) leaf mass loss of decomposing alder leaves through macroinvertebrates and microorganisms in the treated (n = 4 for every time step) and control stream (n = 8 for every time step) mesocosms.**

### *3.2.9.3 In situ* bioassay with *Gammarus pulex*

The average survival rate of the gammarids in the control stream mesocosms was between 83 and 94 % on sampling day 9, 28 and 56 (Tab. 12). In the highest test item concentration, the average survival rate ranged between 47 % on sampling day 56 and 82 % on sampling day 28. On day 56 statistically more organisms died in the highest test item concentration in comparison to the control (one-sided greater Step-down Cochran-Armitage Test Procedure,

$p < 0.001$; NOEC=476 µg/L). Regression analysis reveal $EC_{50}$ values at 865.62 µg/L (no calculated confidence intervals) on sampling day 9 and 830.20 µg/L on sampling day 56 (no calculated confidence intervals).

The mean leaf mass loss of alder leaves fed to the gammarids during the *in situ* bioassay increased over time and was significantly decreased in the highest test item concentration on every sampling day (one-sided smaller Williams t-test, $p < 0.01$, NOEC = 476 µg/L; Fig. 33 a). The mean lipid content, which can provide an indication about the energy reserves of the gammarids, was decreased by 33 % (476 µg a.i./L) and 28 % (952 µg a.i./L; Fig. 34 ) in the two highest test item concentrations 9 days after tebuconazole application compared to the control. The lipid content of gammarids exposed to 476 µg a.i./L tebuconazole was increased 28 and 56 days after application in comparison to the control. By contrast the lipid value of gammarids in the highest tebuconazole concentration was always lower in comparison to the control (38 % decreased on day 28 and 11 % on day 56). Nevertheless, these observations were substantially but not statistically significantly different.

**Tab. 12: Number of alive and dead gammarids in *in situ* bioassay with *Gammarus pulex* in the control and treated stream mesocosms.** At the beginning 30 organisms per cage were introduced.

| | **Control** | | **Tebuconazole conc. µg a.i. /L** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | **119** | | **238** | | **476** | | **952** | |
| | **alive** | **dead** | **alive** | **dead** | **alive** | **dead** | **alive** | **dead** | **alive** | **dead** |
| **Day 9** | 29 | 1 | 29 | 1 | 29 | 1 | 28 | 2 | 27 | 3 |
| [n] | 29 | 1 | 30 | 0 | 30 | 0 | 30 | 0 | 18 | 12 |
| | 26 | 4 | - | - | - | - | - | - | - | - |
| | 29 | 1 | - | - | - | - | - | - | - | - |
| **%** | **94** | **6** | **98** | **2** | **98** | **2** | **97** | **3** | **75** | **25** |
| **Day 28** | 29 | 1 | 30 | 0 | 25 | 5 | 29 | 1 | 21 | 9 |
| [n] | 24 | 6 | 30 | 0 | 29 | 1 | 25 | 5 | 28 | 2 |
| | 25 | 5 | - | - | - | - | - | - | - | - |
| | 22 | 8 | - | - | - | - | - | - | - | - |
| **%** | **83** | **17** | **100** | **0** | **90** | **10** | **90** | **10** | **82** | **18** |
| **Day 56** | 23 | 7 | 29 | 1 | 30 | 0 | 27 | 3 | 14 | 16 |
| [n] | 20 | 10 | 30 | 0 | 26 | 4 | 27 | 3 | 14 | 16 |
| | 30 | 0 | - | - | - | - | - | - | - | - |
| | 29 | 1 | - | - | - | - | - | - | - | - |
| **%** | **85** | **15** | **98** | **2** | **93** | **7** | **90** | **10** | **47** | **53** |

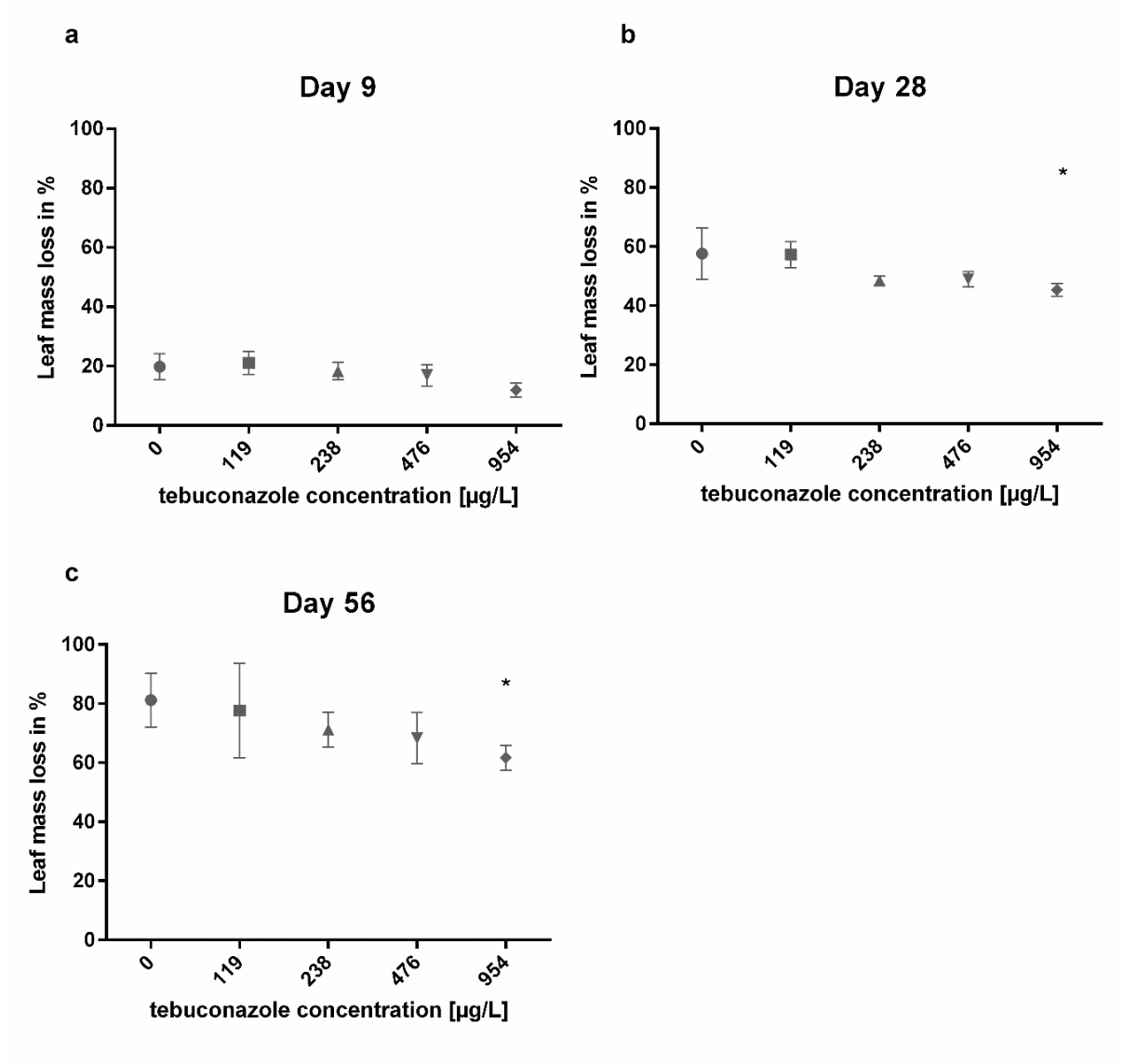


**Fig. 33: Mean leaf mass loss (± SD) of leaves fed to gammarids during the bioassay on different sampling days (a-c).** Asterisks denote statistical differences with control and are explained in the text.

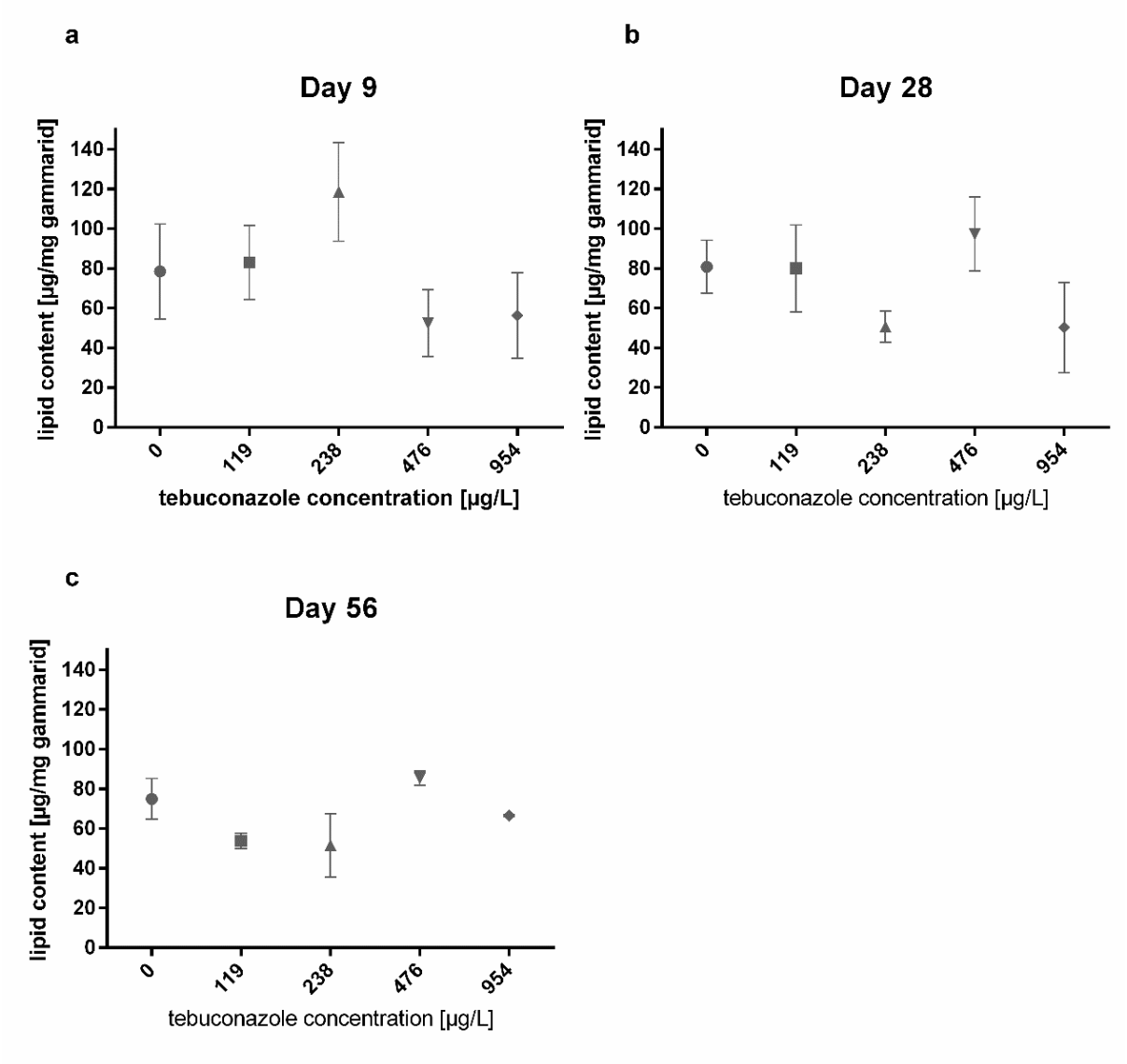


**Fig. 34: Mean lipid content (± SD) of gammarids exposed either to the control (n = 4) or the tebuconazole treatment (n = 2) on different sampling days (a-c).**

# 4 Discussion

## 4.1 Case study I – insecticide application

### 4.1.1 Establishment of a macroinvertebrate community representative for streams

One aim of the study was to establish a macroinvertebrate community in the stream mesocosms with potentially sensitive and vulnerable species typical for lotic water bodies within an agricultural landscape. Macroinvertebrates play a major role in overall biodiversity in these streams. They are of high importance as food source for predators and process organic matter (Wallace & Webster, 1996). By exposing stream biota traps in two local streams and the presence of different ponds and a stream course in the proximity to the stream mesocosms, a high diversity and abundance of macroinvertebrates was achieved in the test systems. Taxon richness in this stream mesocosm study was comparable to the found taxa in other studies with stream mesocosms (57 taxa, Wieczorek et al., 2018; 48 taxa, Mohr et al., 2012; 41 taxa, Cañedo-Argüelles et al., 2014; 40 taxa, Calapez et al., 2017). Likewise, the variability (coefficient of variation) of the macroinvertebrate communities directly before carbaryl application was in the same range as in other studies. Mohr et al. (2012) reported coefficients of variations between 31 – 40 % for crustaceans, 33 – 43 % for ephemerids, 30 – 56 % for trichopeterans and 14 – 32 % for dipterans. Arthropods, which are considered to be particularly sensitive to insecticides (Brock et al., 2000; Maltby et al., 2005) are essential for the risk assessment for insecticides and partly also fungicides ( Brock, 2009; EFSA, 2013). In case study I the number of found taxa belonging to the phylum Arthropoda (29 taxa) is quite similar to the number of taxa found in small lotic streams within an agricultural landscape in Northern Germany (27 taxa, Wogram, 2010). Most dominant taxa found in a typical lowland stream during the study time were *Gammarus pulex*, Trichoptera, Diptera and Ephemeroptera. Within the Arthropoda, crustaceans and insects are usually the most sensitive to organic pollution. Especially taxa of the insect order Ephemeroptera, Plecoptera and Trichoptera (EPT) are considered to be highly vulnerable towards insecticides and other stressors because of their partly long generation time or low dispersal ability (Rico & Van den Brink, 2015; Rubach et al., 2010). In Wogram (2010) a mean EPT taxa number of 13 was sampled, which is similar to the EPT taxa number in the recent study (15 taxa). In the stream mesocosm studies of Wieczorek et al. (2016) the EPT taxa richness ranged between 3 – 13, while Pestana et al. (2009) sampled 9 – 12 EPT taxa.

Biological traits like voltinism are an important factor for the internal recovery of impacted macroinvertebrate populations after pesticide stress (Rico & Van den Brink, 2015). In the recent study, more than half of the detected taxa (58 %) were univoltine, while a lower proportion had a semivoltine life cycle (8 %). The majority of EPT taxa and other arthropods like amphipods are uni- or semivoltine (Gergs et al., 2016). In streams and ditches across three German agricultural areas 55 – 68 % of the macroinvertebrate taxa were univoltine, while 7 – 12 % were semivoltine (Gergs et al., 2011). In the study of Wogram (2010) the amount of univoltine (61 %) and semivoltine (16 %) was a slightly higher than in the recent study. Nevertheless, the number of taxa and the presence of different types of voltinism seems to be similar and representative to what can be expected for real streams in the regional agricultural landscape.

#### 4.1.1.1 Taxa, that can be statistically analysed for effects

Due to the limited number of test systems and the selected regression design, no MDD analysis as for a typical replicated design after Brock et al. (2014) could be conducted in this study. Therefore, MDDs were estimated based on the variance of the eight mesocosms shortly before application. The estimation of MDDs was also done in Wieczorek et al. (2016) by reducing the abundance data of the control at 30, 50, 70 % in comparison to the abundance in the controls. Furthermore, Janz (2016) estimated MDDs for untreated stream mesocosms by assuming five control streams (from ten stream mesocosms overall) and five treatment groups with two replicates each. According to the EFSA Guidance Document reliable statistical analysis of effects in mesocosm studies should be possible for at least eight potentially sensitive populations in mesocosm studies, which can be evaluated by using the MDD approach (EFSA, 2013). Arthropoda taxa (especially insects and crustaceans) react quite sensitive to insecticides due to the specific mode of action (Maltby et al., 2005) and are considered as potential sensitive macroinvertebrate populations. In this study, analysis reveals small to medium effects by estimating MDDs below 70 % (MDD category III – IV) for five Arthropoda taxa (*A. aquaticus*, *Gammarus* sp., *E. ignita*, Limnephilidae Gen. n. d. and Chironomidae Gen. n. d.) and two non-Arthropoda taxa (*Dugesia* sp. and *H. stagnalis*). Four other taxa including the mayfly *E. danica* and the caddisfly *P. flavomaculatus* were less frequent at the beginning of the study but fulfilled the criteria for robust taxa on at least two sampling days after carbaryl application, which probably would have resulted in also sufficiently low MDDs.

Six of the 11 taxa belonging to Arthropoda had a univoltine life cycle from which four taxa belonged to the pollution sensitive orders Ephemeroptera and Trichoptera. Furthermore, the crustacean *Gammarus* sp. is a typical key taxon for lotic surface waters and possesses a uni-/bivoltine life cycle. Besides Arthropoda taxa as potentially sensitive species against insecticides, non-Arthropoda taxa might be relevant in case of e.g. testing a fungicide with non-specific mode of action according to the Aquatic Guidance Document (2013) and may also play an important role for the risk assessment of pesticides. By increasing the number of replicates per treatment for applying a typical test design according to EFSA (2013) it seems to be possible to detect small to medium effects for seven Arthropoda, considered potentially sensitive and some of them also vulnerable towards insecticides, and four non-Arthropoda taxa with statistical methods. However, the requirements of the Aquatic Guidance Document and based on the environmental threshold option (EFSA, 2013) that at least eight potentially sensitive populations, in the recent case study insecticides, should be present in the test systems with an appropriate minimum detectable difference to demonstrate possible treatment-related effects on population abundance were not met.

Likewise, the environmental recovery option with the recommendation of eight vulnerable species with acceptable MDD classes (< 100%;, EFSA, 2013) was not accomplished. The MDD can be narrowed and refined by three main factors (Brock et al., 2014; EFSA, 2016): 1) the number of replicates, 2) the variance of the measurement endpoints which can be separated into the inherent variability between the replicates and the variability caused by the sampling methods, and 3) the selected type I error level (an error level of 0.05 is usually selected as default). Indeed, the number of replicates in further studies with the stream mesocosms needs to be increased to allow the application of the minimum detectable difference for assessing the statistical power of a mesocosm study and to increase the statistical power of a mesocosm study. An exposure-response experimental design with preferably five or more concentrations, and at least two, preferably more, replicates per concentration is advisable (EFSA, 2006; Giddings et al., 2002). However, increasing the number of replicates per treatment is only possible to a limited extent because of practicality (in terms of manpower and costs). Another point to consider it that acceptable MDD classes (< 100%) for macroinvertebrate taxa can be achieved by improving the sampling and quantification methods while reducing the variability of invertebrate abundances between the replicates (Brock et al., 2014).

Improvements of sampling and quantification methods for the stream mesocosms will be discussed in chapter 4.1.3.

## 4.1.2 Effects of carbaryl

### 4.1.2.1 Abiotic parameters

The carbamate insecticide carbaryl did not show any treatment related trend for physical (oxygen, pH, conductivity, water temperature) and chemical water parameters (ammonium, phosphate, nitrate, water hardness). Likewise, in other experiments with carbaryl no effects regarding physical and chemical parameters were observed (Bulen & Distel, 2011; Hanazato & Yasuno, 1987; Hardersen et al., 1999; Havens, 1994).

### 4.1.2.2 Breakdown of carbaryl

Carbaryl is a fast degrading toxicant and the mean calculated half-life (38.3 h) for dissolved carbaryl in the water phase of the stream mesocosms was in accordance with previously conducted studies, where the half-life ranged between 40.0 and 139.2 h in river water (Eichelberger & Lichtenberg, 1971; Liu et al., 1981; Stanley & Trial, 1980). The slightly alkaline water milieu (pH around 8.5 on the day of application) probably conditioned the fast dissipation observed (Armbrust & Crosby, 1991; Fisher & Lohner, 1986; Wolfe et al., 1978). Fisher & Lohner (1986), for example, detected markedly higher recovery rates and increasing stability of carbaryl in water at lower pH values 4 ($DT_{50}$ = 104 days) and 6 ($DT_{50}$ = 71.6 days) in comparison to pH 8 ($DT_{50}$ = 1.4 days). Aly & El-Dib (1971) conducted hydrolysis experiments with carbaryl in aqueous media with slightly alkaline water milieu (pH =8) and calculated half-life of carbaryl in water was 43.2 h, which is in accordance with the mean half-life of the recent study. The main degradation process of carbaryl in the stream mesocosms might be caused by photolysis (Fisher & Lohner, 1986; Sharom et al., 1980; Wolfe et al., 1978). In the recent study the mean sunshine duration in the first week after application was around 9 hours per day (Attachment D) and consequently quite high.

#### 4.1.2.3 Macroinvetebrates

Carbaryl is known to be toxic to aquatic organisms, particularly insects and crustaceans (Hanazato & Yasuno, 1987; Schäfers, 2012). On the macroinvertebrate community level the PRC indicates toxic effects in the highest carbaryl concentration (120 µg/L). Even though no significant logistic regression models could be fitted for the single taxa examined in the study, the data suggest pronounced effects on several taxa. The most substantial effects were observed for the amphipod *Gammarus* sp. in the highest carbaryl concentration, where the species could no longer be detected after day 6 (Fig. 18 c). Also, in the second highest carbaryl concentration (36 µg/L), the abundance of the amphipod seemed to have been reduced for several sampling days after application. Various acute laboratory toxicity tests indicated a $LC_{50}$-value from 22 to 31 µg/L for *Gammarus* sp. after a 96 h exposure time (Sanders, 1969; Schäfers, 2012; SERA (Syracuse Environmental Research Associates), 2008). In this study the highest tested carbaryl concentrations were 36 µg/L and 120 µg/L, which were above the calculated $LC_{50}$-value in laboratory studies. Considering the fast dissipation of carbaryl in the streams compared to the constant exposure over four days in the laboratory tests, the observed decrease of *Gammarus* sp. abundance in the two highest carbaryl concentrations is probably caused by the treatment. Because *Gammarus* sp. became quasi extinct in the highest concentration, recovery would have only been possible under field conditions, e.g. by drift from uncontaminated upstream sections or other types of recolonisation (Allan, 2007; Williams & Hynes, 1976).

Besides the abundance of the amphipoda *Gammarus* sp., abundances of larvae of the caddis fly family Limnephilidae decreased in the first two weeks after application for the highest carbaryl concentration (Fig. 18 f). $LC_{50}$-values for larvae of quiver wearing caddisflies in 96 h laboratory tests ranged between 29 – 61 µg/L (Peterson, 2001; Peterson et al., 2001) suggesting that a carbaryl concentration of 120 µg/L could have indeed affected the caddisflies of the family Limnephilidae in the streams. During the study it was noticeably that abundances of Limnephilidae decreased over time in all stream mesocosms including the controls. According to the abundance data from the emergence traps it was shown that most of these caddisflies emerged during the first period of the study. Since the family Limnephilidae is widespread in Europe and a lot of species are considered to be species at risk (Kriska, 2013; Liess et al., 2018), it is recommendable to assess the potential risk for this trichopteran family in higher tier

studies like lotic stream mesocosms. To improve the test systems regarding ecological risk assessment an earlier time point of application may result in longer dwelling times as caddisfly larvae and therefore, the risk of pesticides in water can be assessed over a longer period.

The abundances of the mayfly *Ephemera danica* reached relevant numbers only on the later sampling days of the study (Day 35 – 57), but apparently depended on the test concentration (Fig. 18 d). No individuals were found in the stream treated with 120 µg/L during the whole study and at the end of the study 14, 9, 4, 6, 2 and 0 animals were found per average in the controls and the increasing test concentrations, respectively. Thus, this mayfly is perhaps more sensitive than *Gammarus* sp. In laboratory studies with sediment, the 96 h-$LC_{50}$ of carbaryl for *E. danica* was 153 µg/L (Schäfers, 2012). This result might support the theory that no effects will be expected for *E. danica* in the lower carbaryl concentrations in this study. However, it is not clear if the dissipation was similar and which stages of larvae were tested. *E. danica* is a semivoltine organism and the life cycle can last from one to three years. During the first sampling days in July and August, the larvae of the newly hatched generation were very small, which makes it difficult to detect them during these months (Svensson, 1977; Tokeshi, 1985). Younger organisms are usually more sensitive than older and larger organisms, for example because the higher surface to volume ratio results in higher uptake rates. Thus, effects on survival of the young but sensitive larvae might have been overlooked in case study I and became only visible when the survivors were larger and detected in the samples later in the study. Similar observations were also made in a stream mesocosm study by Beketov et al. (2008) on long-term effects of the insecticide thiacloprid, where the stonefly larvae *Nemoura cinerea* ant the mayfly larvae *Ephemera vulgata* were only found in control steams 27 weeks after application. Because of the fact that on every macroinvertebrate sampling day two stream biota A traps were removed from every stream mesocosm (with exception of stones and remaining leaf material), potential habitats for *E. danica* and other taxa were reduced and more concentrated on the remaining stream biota traps with every following sampling day. To avoid this possible issue, stream biota trap A should be placed back as a whole during the study.

#### 4.1.2.4 Macroinvertebrate drift

The drift behaviour of aquatic invertebrates is crucial for invertebrates themselves, as well as for the whole stream ecosystem (James et al., 2008). Various types of disturbances like chemical contamination or abiotic and biotic factors (flow velocity, predation, competition) lead to downstream drift of macroinvertebrates (Brittain & Eikeland, 1988). In the present study, drift of the amphipod *Gammarus* sp. and the cladoceran *Simocephalus vetulus* showed short-term increases (after four hours) for a carbaryl concentration of 120 µg/L (Tab. 11). The general abundance and taxa number increased with increasing carbaryl concentrations. This phenomenon was also observed by Beyers et al. (1995) and Courtemanch & Gibbs (1980), immediately after carbaryl treatment in natural streams. Gammarus sp. and the cladoceran *S. vetulus*, in particular, showed high drift in the highest carbaryl concentration. With the current sampling technique, it is not possible to determine whether both taxa were captured within the drift net because they were lethally affected or immobilised or whether they showed drift because of avoidance behaviour. In future studies, drift samples could be directly analysed for live/dead classification. However, in the case of *Gammarus* sp. lethal effects of the highest carbaryl concentration can be assumed based on the laboratory toxicity data and also the results from macroinvertebrate sampling. *Simocephalus vetulus* is a common zooplankton organism, which normally lives in litoral zones of lakes and other basins, but can also be present in lotic systems (Amoros, 1984; Amoros & Chessel, 1985). Cladocera are one of the most sensitive orders to carbamates (Rubach et al., 2010). Acute single species tests discovered that carbaryl is highly toxic to *Daphnia magna* (48 h $EC_{50}$: 5.6 µg/L), which belongs to the same family as *S. vetulus* ( EFSA, 2006). Therefore, it needs to be explored if increased numbers in drift samplers were caused by lethal effects. Visible effects of carbaryl were also observed in the drift numbers of mayflies 24 h after application. Possible mechanisms could be an increase in mortality, a knock-down effect or behavioural changes because of carbaryl (Beyers et al., 1995). Interestingly, these observations were not made until after 4 h, which indicates that it takes some hours before the internal concentration of carbaryl has reached a level to introduce drift. However, numbers of emerging mayflies were not decreased in the highest test concentration compared to control during the study suggesting that behavioural changes might play a role for the higher drift numbers.

In other ecotoxicological studies the initiation of macroinvertebrate drift for several insecticides, mostly neurotoxic insecticides, was often potentially more sensitive than survival or immobilisation in acute toxicity ($LC_{50}$) tests in laboratory (Beketov & Liess, 2008). In another stream mesocosm study, drift behaviour was increased in insect larvae and gammarids after 12-h pulses of imidacloprid, with investigators concluding that drift is a sensitive, relevant endpoint which should be considered in specific risk assessments for lotic surface waters (Berghahn et al., 2012).

#### 4.1.2.5 Insect emergence

In this study, carbaryl had no statistical effect (in the sense of significant dose response functions or on community level via PRC) on the emergence of insects. Only slight tendencies to impair the total emergence seven days after application for 120 µg/L were found. Dominant emergent insects like Chironominae, Tanypodinae and other Chironomidae, which occurred in high abundances in emerging traps, did not show any effect on larvae abundances in the macroinvertebrate sampling either, which confirms that these taxa were not affected by the treatment. Generally, emergence decreased over the course of the study. Spring and summer months are known for high emergence rates of merolimnic insects (Caquet et al., 2007; Corbet, 1964; Füreder et al., 2005). For further studies, consideration should be given for an advancement of insecticide application (e.g. start of application in April-May), but in accordance with the potential application period for the test substance on an agricultural field.

#### 4.1.2.6 Suspended algae and periphyton

For Carbaryl no clear concentration-dependent response on primary producers like suspended algae in the water phase and periphyton (both indirectly measured as the chlorophyll-a) was observed. Consequently, no direct or indirect effects on primary producers were observed.

Direct effects of carbaryl on suspended algae were not expected since carbaryl is an insecticide and laboratory studies showed suppressed growth of several species of algae only at higher carbaryl concentrations (≥ 1000 µg/L (Maly & Ruber, 1983); 96 h $E_bC_{50}$ (*Selenastrum capricornutum*) = 1370 µg/L, 120 h $EC_{50}$ (*Skeletonema* sp.) = 700 mg/L EFSA, 2006). Likewise, no effect at 1000 µg/L on phytoplankton biomass was observed in the mesocosm

study of Hanazato & Yasuno (1987). In contrast, carbaryl induced an increase in phytoplankton biomass at lower carbaryl concentrations in pond mesocosm studies, which was often observed in combination with a decline in different zooplankton communities like cladocerans (Groner & Relyea, 2011; Havens, 1995; Stoler et al., 2016). Because stream mesocosms were used for the recent case study no typical zooplankton organisms, which are often effective filter-feeders, were sampled. Former experiments with zooplankton samplings in the used stream mesocosms showed minor abundances and diversity of zooplankton organisms (Janz, 2016) and regarding that zooplankton organisms play a minor role in the food web of small lotic streams (Schwoerbel & Brendelberger, 2013), it can be assumed that less zooplankton organisms were present in the recent study. Therefore, the minor abundances of zooplankton organisms could explain why no potential indirect effect of carbaryl on the biomass of suspended algae was observed.

Similarly, no direct or indirect effects, e.g. promotion due to reduced grazing, were observed for periphyton biomass. This might be because the periphyton on the glass slides was not an attractive food source for the potentially affected taxa, such as grazers of the insect order Ephemeroptera and Trichoptera. Stoler et al. (2016) or Bulen & Distel (2011), who examined among others the effect of carbaryl on the periphyton biomass in a pond mesocosm study did not find any effect of carbaryl on periphyton biomass either (tested carbaryl conc. range: 5 - 50 µg/L (Stoler et al., 2016) and 2 – 2000 µg/L (Bulen & Distel, 2011)). In future studies, the effects on leaf litter breakdown could also be analysed to provide information on an important ecosystem function. Particularly gammarids are quite important for the utilisation process of organic substance in form of leaf litter (Cummins & Klug, 1979), which were obviously affected through carbaryl application in the recent study and an indirect effect on leaf litter breakdown could be observed.

### 4.1.3 Potential improvements for future studies

In the recent study, the taxa number with sufficiently high abundances was too small in several cases to assess the statistical reliability using the MDD concept (EFSA, 2013). One main focus for future studies should be the increase of numbers of individuals per sampling time point per test system and additionally reducing the variability between the test systems. By improving these two important driving factors, statistical power can be increased to detect whether a given difference between the means of a control and a treatment level is statistically significant. The natural colonisation ability by flying insects was possible due to natural ponds, ditches and mesocosm ponds in close proximity, but no typical streams are located nearby. Therefore, the migration ability via flight for typical stream insects was restricted. Thus, typical lotic taxa had to be introduced by passive introduction through macrophytes and substrates from undisturbed steams. Particularly, stonefly larvae (Plecoptera) were rare during the sampling time points and therefore not enough individuals were present for a reliable evaluation during the study. Species of the order Plecoptera are located most often in rheophilic areas and usually live under stones (Fochetti & Tierno De Figueroa, 2008). Consequently, the sampling technique for receiving macroinvertebrates from reference streams should be refined, e.g. by searching for stoneflies directly under stones. Through targeted introduction of stoneflies and other potentially vulnerable taxa typical for lotic streams, the number of macroinvertebrates in the stream mesocosms can be also increased. Targeted introduction of taxa into pond and stream mesocosms is a common technique which has already been used in several studies (e.g. Caquet, Thybaud, Le Bras, Jonot, & Ramade, 1992; Hickey & Golding, 2002; Kraufvelin, 1998; Van Wijngaarden et al., 1996; Wieczorek et al., 2016).

Furthermore, it could be advantageous to improve the sampling technique for macroinvertebrates within the stream mesocosms to increase the number and diversity of organisms per sampling time point. Brock et al. (2014) suggests to increase the number of sampling devices. In the recent study, three macroinvertebrate substrate samplers were evaluated per stream mesocosm per sampling time point, which is quite time-consuming. However, it could be an option to increase the sampling devices in order to receive additional data. Furthermore, the food supply in the macroinvertebrate sampler could be structured in a more diverse and delicate way by using different leaf types, which might enhance the presence of e.g. EPT taxa and other aquatic organisms. Additionally, enhancing the presence of

macrophytes and submerged vegetation seems to be beneficial to provide a habitat for aquatic invertebrates (Stang et al., 2014; Walker et al., 2013), because they serve not only as food source and substrate for periphyton, but are also frequently chosen as substrate for oviposition of merolimnic insects (Gregg & Rose, 1982). Wieczorek et al. (2016), for example, observed higher abundances of epiphytic Ephemeroptera species by providing high macrophyte densities and complex structures in stream mesocosms. Next to enhancing the presence of macrophytes and submerged vegetation in some sections of the stream mesocosms, diversity and abundance of invertebrates might be increased by providing more diverse substrates such as sediment, stones, wood and leaves.

### 4.1.4 Conclusion

Based on the results of the analysis of the response of several macroinvertebrate taxa and the macroinvertebrate community towards carbaryl in a stream mesocosm study it could be demonstrated that stream mesocosms provide suitable habitats for potentially sensitive and vulnerable taxa with traits like a long life cycle, toxicological sensitivity or poor recolonisation ability.

However, not all taxa were present in appropriate abundances to allow a reliable statistical analysis of effects. With more test systems than in this pilot study, e.g. 20 mesocosms to allow for a replicated design, statistical analysis of effects might have been possible for seven Arthropoda and four non-Arthropoda taxa. Furthermore, it is essential to increase the number of sampled organisms of sensitive and vulnerable taxa per stream mesocosm, for example, by

1) Increasing the targeted introduction by searching for organisms directly under stones and substrate.
2) Optimising the number and type of samples in the mesocosms.
3) More passive introduction of macroinvertebrates through macrophytes and substrates from undisturbed streams.
4) Start of application earlier in season e.g. April-May.

With respect to effects of carbaryl, the study showed potential effects on the macroinvertebrate community and also on specific taxa, although statistical significance was often missing due to poor statistical design of this pilot study. However, effects of carbaryl on taxa level were observed for *Gammarus* sp., Limnephilidae Gen. sp. and *Ephemera danica*, which were in line with available laboratory toxicity data.

# 4.2 Case study II – fungicide application

## 4.2.1 Effects of tebuconazole

### 4.2.1.1 Abiotic parameters

Similarly, to the insecticide carbaryl, tebuconazole did not show any treatment related trend for physical (oxygen, pH, conductivity, water temperature) and chemical water parameters (ammonium, phosphate, nitrate, water hardness). These results were to be expected as physical and chemical water parameters were not affected by tebuconazole treatment in other indoor and outdoor mesocosm experiments either (Artigas et al., 2012; Dimitrov et al., 2014; Pesce et al., 2016).

### 4.2.1.2 Breakdown of tebuconazole

In the present study tebuconazole was relatively stable with a mean calculated $DT_{50}$ of 31.1 days in water, which is quite similar with observations from other laboratory and field studies (Andreu-Sànchez, Paraiba, Jonsson, & Carrasco, 2012; Dimitrov et al., 2014; EFSA, 2014; Lv et al., 2016). In two different pond mesocosm studies, the mean water dissipation time of 50 % of tebuconazole was 32 days (range 15-39 days, Dimitrov et al., 2014 and 31 days, EFSA, 2014). Tebuconazole is a lipophilic substance (log $K_{OW}$: 3.7) and enhances the absorption to organic material like leaf or plant litter and sediment (Kahle et al., 2008). During the study the concentration of tebuconazole in sediment was not analysed. However, it can be assumed that a certain amount of tebuconazole was absorbed by organic material, while the main part of tebuconazole remained dissolved in the water (Dimitrov et al., 2014; Donnadieu et al., 2016). Although dissipation of tebuconazole in water-sediment systems is slow, it is assumed that abiotic light-induced degradation and microbial degradation takes part in the overall degradation of tebuconazole (EFSA, 2014; Lv et al., 2017, 2016; Lyu et al., 2018). For example Lv et al. (2017, 2016) and Lyu et al. (2018) showed different dissipation times for tebuconazole and imazalil in outdoor mesocosms with different wetland plant species, suggesting that plant-stimulated microbial degradation in the bed substrate or nitrifying bacteria may play an active role in the biodegradation.

Interestingly, dissipation time in the lower concentrations was faster than in the higher concentrations (Fig. 24). This phenomenon was also observed in several laboratory studies with soil, where degradation rates of tebuconazole were faster in lower tebuconazole

concentrations compared to higher concentrations (Muñoz-Leoz et al., 2011; Papadopoulou et al., 2016). Possibly, the degradative activity of microorganisms was inhibited at higher tebuconazole concentrations like it was observed for soil microorganisms for other fungicides (Chen & Edwards, 2001). Furthermore, possible adsorption by organic material (e.g. sediment) was faster and mainly limited to the beginning of the experiment and resulted in higher dissipation times for lower tebuconazole concentrations until test end (Wang et al., 2005).

#### 4.2.1.3 Microorganisms

##### 4.2.1.3.1 Functional and structural entity

Leaf litter decomposition is an important ecosystem service provided by freshwater ecosystems (Abelho, 2001; Maltby, 1992; Petersen et al., 1989). In case study II microbial leaf litter decomposition was decreased in the highest test item concentration of tebuconazole from sampling day 17 on until the end of the study. On sampling day 17 and 44 leaf mass loss was significantly decreased in comparison to control. In temperate streams, aquatic fungi, particularly aquatic hyphomycetes, are one of the main microbial colonizers of submerged litter with > 90 % to the total microbial biomass and production (Ferreira et al., 2014; Hieber & Gessner, 2002; Pascoal & Cássio, 2004; Weyers & Suberkropp, 1996). Because of the high colonisation rate of aquatic fungi it can be assumed that a tebuconazole concentration of 954 µg/L was toxic to fungi and leaf litter decomposition was impaired as an indirect effect. Potential effects of tebuconazole on leaf litter decomposition were previously assessed in other laboratory and mesocosm studies with varying results. Artigas et al. (2012) also observed a reduced leaf mass loss in the presence of tebuconazole at markedly lower concentrations of 20 – 40 µg/L (pulse exposure scenario) in laboratory microcosms and Zubrod et al. (2015) even observed a significantly higher leaf litter decomposition at a concentration of 1 µg/L. However, in several other studies leaf decomposition was not affected after treatment with tebuconazole (Dimitrov et al., 2014; Donnadieu et al., 2016; Pesce et al., 2016; Pimentão, Pascoal, Castro, & Cássio, 2019). For other fungicides tested in laboratory and field, leaf litter decomposition as a measured endpoint represents heterogeneous results. Propiconazole, likewise belonging to the group of triazole fungicides, also reduced the rate of microbial leaf decomposition over a 26 days period at concentrations of 50 and 500 µg/L (Rasmussen et al., 2012). Zubrod et al. (2015) observed a significant decrease of microbial leaf decomposition after treatment with azoxystrobin (at 100 and 500 µg/L), carbendazim (≥ 245 µg/L) and cyprodinil

(≥ 200 µg/L). Other studies observed no significant treatment effect on microbial leaf litter decomposition of fungicides with different modes of action like metiram (Lin et al., 2012) or quinoxyfen (Zubrod et al., 2015). One should keep in mind that the comparison of exposure level in different experimental systems is difficult because the response of natural microbial communities to toxicants depends most often on a combination of parameters like abiotic factors or the initial composition of the microbial community (Pesce et al., 2016). Furthermore, in several studies it was not separated between microbial and macrobial leaf litter decomposition by using e.g. fine and coarse mesh bags. If there was a separation of measured endpoint in microbial and macrobial leaf litter decomposition, the observations were also quite heterogenic like reduced leaf litter decomposition or no significant treatment related effect (Dimitrov et al., 2014; Lin et al., 2012; Rasmussen et al., 2012).

For the recent study, it was shown that microbial leaf litter decomposition as a newly developed method at the test facility is a suitable endpoint for the risk assessment of fungicides within mesocosm studies. However, the question arises if the treatment effect in microbial leaf litter decomposition in the highest tebcuconazole concentration is relevant for the risk assessment of tebuconazole. The "non-microbial" hazardous concentration (HC) of tebuconazole affecting 5 % of species derived from acute toxicity tests with fish, invertebrates and primary producers is calculated to be 238 µg/L (Maltby et al., 2009). Considering the calculated HC5 concentration of tebuconazole is protective for the majority of aquatic organisms and because the RAC for tebuconazole is 1 µg/L (FOOTPRINT, 2019), it can be assumed that a tebuconazole concentration of 954 µg/L is not environmentally relevant and the risk for microbial communities and consequently the decomposition of organic material as important ecosystem service seems to be small. Despite this fact, to improve the suitability of this method for the assessment of the microbial and macrobial leaf litter decomposition as a potential sensitive endpoint for the risk assessment, the partly high varieties between the replicates within one treatment (see partly high standard deviations per sampling day) should be diminished. This can be achieved, for example, by increasing the number of assessed leaf litter bags per treatment and per sampling day. Furthermore, it might be advantageous to increase the exposure time in a nearby waterbody without any pesticide, to increase microbial inoculum for the leaf materials and to reach a high diversity of aquatic hyphomycetes and other microbial organisms. In the recent study, the microbial leaf mesh bags were exposed 21

days in a nearby waterbody to produce microbial inoculum, while the macrobial leaf mesh bags were introduced into the stream mesocosms 4 days before tebuconazole application and without any exposition in a natural waterbody to produce microbial inoculum.

Extracellular enzymes, which are essential for the decomposition of allochthonous organic matter like leaf litter, are mainly produced by aquatic fungi and bacteria within the microbial community (Abelho, 2001; Romani et al., 2016). Leaf litter decomposition is an interplay between different trophic levels in aquatic food webs, but microorganisms are the first ones which colonize leaf litter (Battin et al., 2016). The initial steps of decomposition rely on fungi because they are the only organisms which are able to effectively decompose the recalcitrant lignin (Gulis et al., 2008; Likens, 2010; Romaní et al., 2006). Especially the lignin-modifying extracellular enzymes like lignin-peroxidase, manganese-peroxidase or phenol oxidase (laccase) are mainly produced by fungi (Beek, 2001). In the recent study the effect of tebuconazole on five extracellular enzyme activities were assessed. The extracellular enzyme activity of phenol oxidase and peroxidase, which are essential for lignin degradation in plant material, seems to be unaffected by tebuconazole treatment. This observation was unexpected since effects of tebuconazole on microbial leaf litter decomposition occurred. Initially two different explanations might be responsible for this observation. Assuming the effects of tebuconazole on microbial leaf litter decomposition were caused by indirect effects and several aquatic fungi have died in the highest test item concentration, the fungi remained alive might have compensated the depletion of others, e.g. through the production of the extracellular enzymes phenoloxidase and peroxidase. Functional redundancy among fungal species is an important way to maintain ecological functions in streams under stress (Pascoal et al., 2005). On the other side, not only fungi but also bacteria are able to produce lignin degrading enzymes (Claus, 2003; Giardina et al., 2010). It is possible that bacteria compensated the production of the enzymes and extracellular enzyme activity of peroxidase and phenoloxidase was not altered in the treated stream mesocosms in comparison to control.

Likewise, this pertains for the other extracellular enzymes ß-glucosidase, ß-xylosidase and phosphatase, which are important for the degradation of cellulose (ß-glucosidase), hemicellulose (ß-xylosidase) and phosphorus mineralisation (phosphatase; Sinsabaugh et al., 1991). Apart from the extracellular enzyme activity of ß-xylosidase on sampling day 17 no dose-dependent in- or decrease of extracellular enzyme activity was observed. Only few

studies dealt with the effect of tebuconazole on the activity of extracellular enzymes so far. In a study of Artigas et al. (2012) tebuconazole treatment altered the kinetics of the extracellular enzyme activity of ß-glucosidase and ß-xylosidase, but not those related to the breakdown of lignin (phenoloxidase). Hereby, no significant effect on the maximal velocity ($V_{max}$) of enzyme kinetic was found, but tebuconazole lead to a significant increase of $k_m$ values, indicating that tebuconazole reduced the enzyme affinity for C substrate utilisation (ß-glucosidase, ß-xylosidase). Because the enzyme kinetics ($V_{max}$ and $k_m$ values) were not assessed in the recent study, comparisons of the extracellular enzyme activities between the two studies is difficult. In another study, Artigas et al. (2017) found that laccase activity, which belongs to the group of phenoloxidases, is increased after tebuconazole treatment in leaves colonized solely by fungi (*Alatospora acuminate* populations). However, laccase activity was repressed in leaves when fungi and bacteria co-exist. In the mesocosm study of Donnadieu et al. (2016), who investigated the effect of tebuconazole (10.7 µg/L) on leaf litter decomposition in aquatic channels containing leaves or leaves mixed with sand, found out that tebuconazole temporarily reduced phenoloxidase activity in channels containing sand but not in channels containing sand mixed with leaves, assuming that a decrease in fungal biomass in the contaminated channels containing sand in the absence of leaves could be a potential explanation for this observation. In contrast, likewise in the recent study ß-glucosidase was not affected by tebuconazole treatment.

To analyse whether tebuconazole was toxic to aquatic fungi or whether bacteria may have compensated the production of the aforementioned extracellular enzymes, it is essential to check the structure of the microbial communities by assessing the leaf-associated fungal and bacterial biomass and the species of fungi and bacteria within the microbial community. In the recent study, fungal biomass (analysed by measuring the ergosterol content) and bacterial biomass (analysed by counting bacterial cells) associated with the leaf material was analysed. Fungal and bacterial biomass was not significantly altered by fungicide treatment. Other studies observed different, partially converse effects of tebuconazole on fungal and bacterial biomass. In the lentic mesocosm study of Dimitrov et al. (2014) tebuconazole (238 µg/L) had no effect on fungal biomass. Pesce et al. (2016) conducted a study in indoor recirculating channels and likewise did not observe any effects on fungal and bacterial biomass at an average tebuconazole concentration of 17.1 µg/L. In contrast, fungal biomass was significantly

affected at a tebuconazole concentration of 5 µg/L and 65 µg/L, respectively (Zubrod et al., 2011, 2015), while bacterial biomass was significantly decreased at tebuconazole concentrations of 20 – 42 µg/L (Artigas et al., 2012) and 500 µg/L (Bundschuh et al., 2011) under laboratory conditions.

In the course of this study, the leaf-associated fungal and bacterial communities were not assessed. However, a detailed characterisation of fungal and bacterial community composition can be achieved by using methods like DNA fingerprinting (Dorigo et al., 2010; Tlili et al., 2010), next-generation sequencing in combination with species- or group-specific quantitative real-time polymerase chain reaction (Bricheux et al., 2013; Hall et al., 2012; Manerkar et al., 2008) or CARD-FISH (Brümmer et al., 2000; Lawrence et al., 2007; Proia et al., 2013) and would allow taxonomic identification of microbial communities. Accordingly, by characterisation of the fungal and bacterial community composition, further insight into the dynamic adaption of the microbial community after fungicide treatment and the effects of tebuconazole on potentially sensitive aquatic fungi and bacterial species could be gained. In the recent study, fungal biomass was assessed for aquatic fungi, which produce ergosterol. Although ergosterol producing fungi are generally considered as one of the major microbial decomposers of leaf litter (Bärlocher, 1992; Hieber & Gessner, 2002), other aquatic fungi, which are present on decomposing leaf litter, might be involved in its breakdown, e.g. Chytridiomycota (Marano et al., 2011) and might be impaired through tebuconazole treatment.

The positive effect of tebuconazole on suspended algae as important primary producers is difficult to interpret. No clear dose-dependent effect on the total chlorophyll-a biomass was observed on any sampling day. Conspicuously, from sampling day 38 on chlorophyll-a concentration of suspended algae increased in all treatments compared to control. This difference was significant on the last sampling day. Indirect effects induced by a reduced grazing of zooplankton organisms or a suppression of other microorganism communities like bacteria might play a role. Considering a potential reduced grazing of zooplankton organisms, no presumptions for the recent study can be performed, because the risk of tebuconazole to zooplankton organisms was not assessed during this study. Zooplankton organisms were not actively introduced before application of tebuconazole, but zooplankton organisms had the ability to colonise the stream mesocosms with water intake. In many lentic and lotic mesocosm studies with fungicides, zooplankton organisms were the most sensitive organism group

(thiram: Bayona et al., 2014; metiram: Lin et al., 2012; cabendazim: Van Den Brink, Hattink, Bransen, Van Donk, & Brock, 2000; azoxystrobin: van Wijngaarden et al., 2014; Zafar, Belgers, Van Wijngaarden, Matser, & Van Den Brink, 2012). Therefore, it is possible that tebuconazole treatment decreased the abundances of potentially sensitive zooplankton taxa and indirect effects were observed in chlorophyll-a value of suspended algae. Dimitrov et al. (2014) also observed treatment-related decreases of Copopod nauplii and Cyclopoida on several sampling days in a mesocosm study with tebuconazole (238 µg/L), while increases of one phytoplankton population (*Trachelomonas* gr *oblonga*s ) were observed. In this study the chlorophyll-a biomass of suspended algae was not assessed. However, to get deeper insights into potential direct and indirect effects of tebuconazole on suspended algae, analysis of the algae community composition might be useful.

On the other hand it is possible that other microorganism communities like suspended bacteria were suppressed after tebuconazole treatment and lead to an increase in photosynthesis operating organisms (expressed as functional endpoint as chlorophyll-a concentration). Suspended biofilm mainly consists of diatoms, filamentous cyanobacteria and bacteria and to a lower extent of protozoans and zooplankton (Mora-Gómez et al., 2016; Simon et al., 2002). Photosynthesis operating algae like diatoms or cyanobacteria may have benefited from changes in the microorganism community composition. As both the community composition of bacteria and algae were not assessed within this study, it is difficult to decide whether this possible explanation might have resulted in higher chlorophyll-a concentrations of suspended algae. However, in the aforementioned mesocosm studies with different fungicides, no effects on phytoplankton expressed as chlorophyll-a biomass were observed after fungicide application. Therefore, the results for suspended algae in the recent study should be treated with caution. Likewise, no effect on the chlorophyll a biomass was observed for periphyton in several mesocosm studies with different fungicides (Daam et al., 2010; Gustafsson et al., 2010; Paul J. Van Den Brink et al., 2000; van Wijngaarden et al., 2014; Zafar et al., 2012). In the recent study, chlorophyll-a concentration seemed to increase with increasing study time in the highest tebuconazole concentration. Yet, the increase was only significant on sampling day 16. This increasing trend in periphyton chlorophyll-a concentration implicits the same assumptions that were done for suspended algae.

As a conclusion the recent stream mesocosm study showed that a main ecosystem service like leaf litter decomposition of microorganisms seems to be impaired in the highest tested tebuconazole concentration. Yet, no significant changes in fungal and bacterial biomass or the extracellular enzyme activity of five different enzymes was observed. However, an increasing trend of chlorophyll-a concentration of suspended algae and periphyton was observed suggesting that tebuconazole may have changed the aquatic food web structures like abundances of zooplankton communities or microorganism community composition.

#### 4.2.1.4 Higher trophic level

According to laboratory standard tests the effect of tebuconazole on aquatic organisms like algae, non-target plants, daphnia and fish is moderate (Adam, Badot, Degiorgi, & Crini, 2009; FOOTPRINT, 2019; Zubrod, Bundschuh, & Schulz, 2010). In the recent stream mesocosms study tebuconazole seemed to influence the abundance of *Gammarus* sp. and Chironomidae Gen. sp. (Fig. 31 b-c). Tebuconazole lethality for *Gammarus* sp. was assessed in laboratory tests at relatively high concentrations like an 96 h-$LC_{50}$ concentration of 1643 µg/L for *Gammarus pulex* (Adam et al., 2009) and 1347µg/L for *G. fossarum* (Zubrod et al., 2010). Since the highest tested tebuconazole concentration in the stream mesocosm study was 954 µg/L, it seems unlikely that a significant decrease of gammarid abundances in all treatment groups compared to control occurred two days after application through direct toxic effects of tebuconazole. One should keep in mind that three days before tebuconazole application, additional gammarids were introduced into the stream mesocosms. Therefore, only a rough estimation of gammarids (150 – 300) per stream mesocosm was made. This could have led to an inhomogeneous allocation of gammarids to the different stream mesocosms. Another explanation for the decreased abundance of gammarids two days after application could be that tebuconazole indirectly effected (due to food-quality related effects) the energy processing of *Gammarus* sp. Zubrod et al. (2011) observed a significant decrease in feces output while assimilation increased for acquiring energy already at a tebuconazole concentration of 65 µg/L. Furthermore, the lipid content decreased within the five-week semistatic laboratory experiment. In the absence of tebuconazole, leaf-palatability might be decreased for the typical shredder organisms *Gammarus* sp. This assumption is supported by the observed decrease in microbial leaf litter decomposition (Fig. 33) in the highest tebuconazole concentration during

this study and potential shifting in the fungal and bacterial community composition (Graça, 2001).

Due to the potential food alteration accounted for by the effect of tebuconazole treatment, leaf consumption by gammarids might be decreased. Therefore, the physiological fitness of the gammarids might be decreased and they start drifting out of the macroinvertebrate cages because of potential sublethal effects on energy processing. The aforementioned consideration is underlined by results of the *in situ* bioassay with *Gammarus pulex* (chapter 3.2.9.3). On sampling days 9 and 54 a substantial amount of gammarids in the highest tebuconazole treatment was dead in comparison to control and leaf mass loss was decreased (not corrected for remained abundance), suggesting that gammarids were affected to some extent sublethally by fungicide toxicity or nutrition-related stress. Furthermore, the lipid content of gammarids used for the bioassay was decreased, if not necessarily significant, in the highest tebuconazole concentration, supporting the theory that nutrition-related stress might occurred. A decrease in gammarid abundance and lipid content was observed in another artificial stream study with a chronic exposure to a mixture of five fungicides (sum concentration 20 µg/L; Zubrod et al., 2017). However, the assumption of nutrition-related stress should be treated with caution, since a significant decrease of *Gammarus* sp. abundance was observed on sampling day 2 only, while other parameters like microbial leaf litter decomposition or the mortality of gammarids in the bioassay and lipid content were assessed on sampling day 4 and 9, respectively. For future studies, a synchronisation of sampling time points for the different endpoints would be advantageous for connecting the meaning of the results. Furthermore, additional endpoints like fungal and bacterial community composition or assessment of the feeding rate of leaf-shredding organisms might support the assessment of the risks of fungicides on fundamental ecosystem services.

The abundance of Chironomidae Gen. sp. was decreased after tebuconazole application as well. The abundance was decreased in the two highest tebuconazole concentrations on sampling day 2 and furthermore, the abundance was decreased in all tebuconazole treatments on day 14. By contrast, abundance in the highest tebuconazole concentration was significantly higher in the highest tebuconazole treatment compared to control on the last sampling day (Fig. 31 c). Because the taxa Chironomidae Gen. sp. usually had the highest abundances during the sampling time points, significant differences observed at the total abundance level of all

macroinvertebrates are most likely due to the abundance of chironomid larvae. Lethal concentrations of tebuconazole for *Chironomus* sp. larvae tested in laboratory acute and chronic studies range between 1237 and 4618 µg/L (FOOTPRINT, 2019; Raby et al., 2019; Zhao et al., 2012) which is much higher than the test concentration range of the recent study. Therefore, it seems unlikely that direct toxic effects of tebuconazole led to a decrease of chironomid abundance on sampling days 2 and 14. Generally, chironomid larvae are generalists and ingest five kinds of food types: algae, detritus and associated microorganisms, macrophytes, woody debris and invertebrates, whereby detritus is the most commonly reported food ingested by chironomids (Berg, 1995; McLachlan, 1977; Naser & Roy, 2012; Pinder, 1992). Hereby, detritus is defined as non-living particulate organic matter and associated non-photosynthetic microorganisms (Boling et al., 1975; Cummins, 1973). In field studies the gut content of chironomids was assessed and detritus accounted for 50 – 70 % (Naser & Roy, 2012; Sephton, 1987; Titmus & Badcock, 1981). Particularly larvae, which mainly feed as scrapers and shredders and consume debris, live in flowing waters (Berg, 1995). Although chironomids are generalists and are not restricted to a single mode of feeding it is possible that the insect larvae were also affected by tebuconazole sublethally or due to nutrition-related stress similar to *Gammarus* sp.. Until the end of the study the abundances of Chironomidae Gen. sp recovered in the treated stream mesocosms and were significantly higher in the highest tebuconazole concentration compared to control. It is possible, that the available food resources were enhanced in the stream mesocosms with the highest tebuconazole treatment due to higher food availability in the form of epiphytic and suspended algae and because of a higher proportion of animal matter. In the recent study, photosynthesis capacity was enhanced in the highest tebuconazole concentration in suspended algae and epiphytic algae (Fig. 27, Fig. 28) suggesting that the amount of algae as available food source for chironomids was increased. Chironomid larvae feed on a variety of invertebrates like oligochaetes, other chironomids or diverse zooplankton organisms (Armitage, 1968; Berg, 1995; Loden, 1974). In this study abundances of zooplankton organisms and oligochaetes were not assessed and potential sublethal effects of tebuconazole might have increased the risk for predation by chironomid larvae. Thus, growth and development of larvae to adults and reproduction might be promoted.

Coarse particulate organic matter in the form of leaves fallen into small streams is an important food source for shredder organisms like amphipods, several EPT taxa, dipterans or molluscs

(Graça, 2001). To assess the microbial decomposition and the invertebrate consumption (named as macrobial leaf decomposition) coarse mesh bags were used. Macrobial leaf decomposition was not altered in stream mesocosms treated with tebuconazole compared to the control. Even though microbial leaf decomposition was reduced in the mesocosm with the highest tebuconazole concentration, possibly due to toxic effects of tebuconazole for aquatic microorganisms, invertebrate consumption did not seem to be affected. Considering that the abundances of the important detritus consumers *Gammarus* sp. and Chironomidae Gen. sp. were decreased during the study, other leaf consuming organisms like e.g. *Asellus aquaticus*, larvae of the order Ephemeroptera, Plecoptera and Trichoptera or other Diptera might have compensated and therefore decomposition of alder leaves was not altered. The same observations were made in a laboratory study of Rasmussen et al. (2012) with the fungicide propiconazole. While the microbial leaf decomposition was significantly decreased at a concentration of 50 and 500 µg/L, the macroinvertebrate shredding activity was not affected by the tested fungicide concentrations. In the mesocosm study of Dimitrov et al. (2014) no effects of tebuconazole on leaf litter decomposition conditioned by microorganisms and leaf consumption by invertebrates were observed either. In addition, other mesocosm studies with the fungicides pyrimethanil (Abelho et al., 2016) and carbendazim (Cuppen et al., 2000) also showed no effect on this functional parameter.

### 4.2.2 Implications for Ecological Risk Assessment of fungicides

Considering that the European Union´s ERA of fungicides is based on a tiered approach, starting with standard acute and chronic toxicity tests with algae, daphnia and fish (first-tier ERA; EFSA, 2013), ecotoxicity data for tebuconazole gained in laboratory tests lay between 1.96 – 4.40 mg/l for $EC_{50}/LC_{50}$ and 0.01 – 0.10 mg/L for no-observed effect concentrations (FOOTPRINT, 2019). By using a safety factor of 10 (for chronic laboratory toxicity tests) and 100 (for acute) a regulatory acceptable concentration for tebuconazole results in 1 µg/L. Within the risk assessment the RAC needs to be higher than the predicted environmental concentration. If this is the case, the protection of the majority of organisms and essential ecosystem functions should be ensured (EFSA, 2013). In this study the lowest tested tebuconazole concentration of 119 µg/L resulted in direct and indirect effects on the functional and structural entity of microorganisms and on higher trophic level. This means that

significant effects occurred at a concentration even lower than the "non-microbial" HC5 concentration (238 µg/L, L. Maltby et al., 2009). Compared to the RAC for tebuconazole, it seems unlikely that the recently tested functional and structural tested endpoints might have been sensitive at 1 µg/L, but it is possible. For example Zubrod et al. (2015) observed a higher leaf litter decomposition at a concentration of 1 µg/L, which equals the RAC of tebuconazole. Furthermore, other laboratory and semi-field studies showed that other functional and structural endpoints seem to be more sensitive, and this at partly distinct lower tebuconazole concentrations as tested in the recent study (Artigas et al., 2012; Dimitrov et al., 2014; Fernández et al., 2015; Pimentão et al., 2019).

Structural endpoints like fungal and bacterial community composition or conidial production as well as indirect effects on species interaction and ecosystem functioning (e.g. effects on higher trophic level: feeding behaviour, energy processing etc) also seem to be quite sensitive at lower fungicide concentrations (Artigas et al., 2012; Bundschuh et al., 2011; Dimitrov et al., 2014; Pimentão et al., 2019; Zubrod et al., 2010, 2011). Due to the fact that aquatic microorganisms and thereby indirectly, the whole aquatic food web seems to be affected, Puglisi (2012) suggested in his external scientific EFSA supporting publication to promote risk assessments for all pesticides considering different microbial endpoints by standard methods. These methods include e.g. enzyme activities, PCR-DGGE and colony forming units of total microbes and should be tested and promoted in the future. Zubrod et al. (2019) recommend in their review several approaches to improve the ERA procedures to assess the risk of fungicides. Among other approaches they recommend the inclusion of single-species tests in the ERA testing scheme because it has been shown in several experiments that a high risk of fungicides was indicated for aquatic fungi. Research with single-species tests with aquatic fungi is at an early stages (Dijksterhuis et al., 2011; Lategan et al., 2016; Lategan & Hose, 2014; Nagai, 2018) and more research has to be conducted prior establishing an OECD test guideline for standard toxicity testing (EFSA, 2013). Furthermore, Zubrod et al. (2019) suggest to lay the main focus on indirect effects of fungicides. By using multispecies experiments in laboratory or semi-field, e.g. with a decomposer-detritivore system (e.g. Pimentão et al., 2019; Pradhan, Seena, Pascoal, & Cássio, 2012; Zubrod, Baudy, Schulz, & Bundschuh, 2014), it is possible to gain more information about the risk of fungicides on the protection of ecosystem services (Nienstedt et al., 2012). For this approach, higher-tier studies with lentic, or, due to the fact that sensitive aquatic fungi like aquatic hyphomycetes are more present in streams than in

lentic waterbodies (Kuehn, 2016), even better lotic mesocosms, seem to be quite useful for ERA of fungicides. Indeed, further endpoints next to the commonly tested endpoints like the effects on the macroinvertebrates, zooplankton and primary producers (algae population) should be tested (e.g. leaf litter decomposition, structural entity of aquatic biofilms). The recent study displays a broad spectrum of tests to analyse the direct and indirect effects of a fungicide on microorganisms and other participants of the aquatic food web. For future ERA of fungicides, the use of more higher-tier studies might be useful for assessing the risk of fungicides on the whole aquatic food web by applying realistic exposure scenarios.

### 4.2.3 Conclusion

Within case study II a great range of newly established endpoints regarding the effects of a fungicide on the functional and structural entity of microorganisms and the effect on microorganisms-related processes was assessed in outdoor stream mesocosms.

It could be shown that the fungicide tebuconazole influenced functional endpoints of aquatic biofilms causing e.g. a decrease in leaf mass. Other endpoints showed only partially dose-dependent effects. Due to high variances within the different endpoints the number of replicates per treatment concentration should be increased, in order to increase the statistical power. It is possible that the assessment of the risk on species level of aquatic fungi and bacteria is more sensitive than the endpoints tested in this study. Furthermore, under certain circumstances the use of more sensitive microorganism-related endpoints, e.g. the conidia production of aquatic fungi, could be suitable in order to assess the risk of fungicides. These options could be tested in future studies in stream mesocosms.

## 4.3 General discussion

### 4.3.1 Experimental period during the seasons of the year

One aim of the present thesis was to establish a representative macroinvertebrate community typical for small lotic water bodies within an agricultural landscape inside a model system. In the first study with the insecticide carbaryl the macroinvertebrate community was diverse and contained potentially sensitive and vulnerable taxa.

By contrast, in the stream mesocosm study with the fungicide tebuconazole the abundances of macroinvertebrate taxa and the overall taxa number was markedly lower compared to case study I. In this study the main focus was the risk assessment of a fungicide on microorganisms and their related processes. Direct and indirect effects of tebuconazole on higher trophic level like macroinvertebrate abundances or lipid value of *Gammarus* sp. were also assessed. Considering the main focus of case study II was not the establishment of a macroinvertebrate community representative for small lotic streams but rather the stream mesocosms have representatives of typical shredder organisms like *Gammarus* sp., *Asellus aquaticus* or Chironomidae Gen. sp., no major effort was done to attract other potentially sensitive and vulnerable organisms. However, the stream mesocosms were colonised with the same method as for case study I, by laying stream biota traps filled with basalt stones and plant material as food source into two different small lotic streams. Reasons for the sparsely populated baskets with macroinvertebrates in case study II seem to be diverse. Due to the different focus of the second study, the study was purposely conducted in autumn/late autumn. Seasonal differences in abundance and diversity of macroinvertebrate communities have been observed several times, and are likely caused by changing weather conditions like e.g. precipitation and insolation (Butler, 1984). Changes in flow and temperature may cause influences in the timing of emergence, reproduction or growth and development in many aquatic macroinvertebrates (Bêche et al., 2006). During the summer months temperature is increasing and oxygen level is decreasing, which causes increases in organic matter and decreases in the diversity of the macroinvertebrate community (Šporka et al., 2006). By contrast, spring is characterized by increasing temperature, discharge, light intensity, and nutrient supply, favouring an increase in primary producers and therefore an increase in abundance of algophagous invertebrates. Whereas in autumn and winter, which is characterised by a decrease in temperature, lower illumination and a strong supply of allochthonous organic material, detritophagous

macroinvertebrates are present more often (Šporka et al., 2006). In case study II macroinvertebrate samplers were placed into the upland streams at the end of August 2017, while macroinvertebrate samplers in case study I were placed into the small lotic streams in May 2015. The different time points for attraction of macroinvertebrates from two nearby streams may have caused the differences in the abundance and diversity of the macroinvertebrate community during the studies. As an example the sampling of macroinvertebrates for the water framework directive is recommended for February/March because high abundances and diversities can be assessed (EU, 2000).

Another point to consider is that heavy rain events in June and July 2017 (see daily weather data, attachment A. 5) caused flood in the Pferdsbach and Gleenbach in the subsequent weeks. The alteration of natural flow regimes in running water ecosystems is among others different due to the season but also altered through anthropogenic activities (dam building, flood-control projects etc.). Aquatic organisms developed three modes of adaptation against floods and droughts (life history, behavioural and morphological, Lytle & Poff, 2004). It is possible that the flow velocity caused by the heavy rain events increased and therefore behavioural changes of invertebrates caused moving/drifting to areas with lower flow velocity. This might be an additional reason for the sparse population success in the macroinvertebrate sampler.

Considering the seasonal differences in macroinvertebrate community and acute changes due to natural flow regimes in combination with the improvements for sampling discussed in chapter 4.1.3, the colonisation of the stream mesocosms with macroinvertebrates should improve in regard to abundance and diversity of the macroinvertebrate community. However, the main focus should be on which organism group (e.g. plants, macroinvertebrates, algae, microorganisms) an experimental outdoor study is based on and choose the best suitable season of the year to assess the effects. For both case studies the time period of application start was chosen based on the main focus of the risk assessment of an insecticide and a fungicide.

### 4.3.2 Comparison of the newly constructed stream mesocosms with other stream mesocosms for ecological risk assessment of plant protection products

In Europe and worldwide, only a few test facilities have stream mesocosms which are similar to the newly constructed stream mesocosms used for both case studies (S. Berger & Nejstgaard, 2020). At this time, stream mesocosms are not routinely used for the risk assessment of insecticides, herbicides and fungicides in higher-tier studies (EFSA, 2013). In Germany the Federal Environmental Agency in Berlin possesses 16 modular streams which can be prolonged up to 106 m (water level 45 cm) per stream mesocosm (Silvia Mohr et al., 2005). They can be used in recirculating mode or in flow-through mode. Likewise, the stream mesocosm facility at the Landau Campus of the university of Landau has 16 independent stream channels (each channel: 45 m length, 0.5 m depth, 0.4 width), which can be run in a flow-through or recirculating mode (Elsaesser et al., 2013). Both test facilities use their stream mesocosms mainly for research issues, but not typically for the conduction of higher tier studies within the risk assessment of pesticides. The newly constructed stream mesocosm at the test facility are unique, since they can be used routinely for risk assessment of plant protection products at an acceptable cost-benefit-ratio due to their size. Although the stream mesocosms are smaller compared to other test facility stream mesocosms, risk assessment of pesticides investigating the effect on several different endpoints on aquatic food web can be conducted and, for example, abundances of macroinvertebrates are comparable to the larger stream mesocosms in the test facility of university Landau or the test facility in Berlin (see chapter 4.1.1). Within this thesis it was shown that higher-tier studies can be conducted successfully.

### 4.3.3 Realistic exposure scenarios of plant protection products and other chemicals- further possible applications for stream mesocosms

Small, lotic, edge-of-field waters usually receive temporary entries of plant protection products due to run-off, drainage or drift events. The entered substance is diluted and transported downstream in the lotic water body, which often results in pulsed local exposure events expected for the organisms in a stream. The most realistic exposure scenario for edge-of-field surface waters seems to be hour-scale pulse exposures (Bakanov et al., 2020; Rabiet et al., 2010; Wieczorek, Bakanov, Stang, et al., 2016). Artificial stream mesocosms are able to simulate both different exposure scenarios and a realistic natural environment. This type of exposure scenario was already applied in experimental facilities by using flow-through conditions (Bayona et al., 2014, 2015; Harris et al., 2007; Wieczorek et al., 2018). A great benefit of open linear channels is the potential immigration of organisms from untreated upstream sections (Ippolito et al., 2012), which can facilitate the recovery potential (Liess & Von Der Ohe, 2005). The disadvantage of the test systems is the enormous need of water and the huge amount of wastewater. Furthermore, there are stream mesocosm designs, for which different pesticide pulses can be simulated by exchanging treated water with uncontaminated water at a specific time following application (Berghahn et al., 2012; Wieczorek, Bakanov, Lagadic, et al., 2016). The technical effort is big and plankton organisms cannot be monitored consequently. In both conducted case studies with the insecticide carbaryl and fungicide tebuconazole, a worst-case scenario with recirculating mode was conducted. This type of stream mesocosm study was also used in studies with insecticides, herbicides and heavy metals to examine the effects under worst case conditions (M. A. Beketov et al., 2008; Clements, 1999; S. Mohr et al., 2007). To simulate more realistic, shorter exposure events expected in real streams, it is possible to replace part of the contaminated water through submersible pumps with uncontaminated water of similar quality and temperature, like it was performed e.g. in the study of Berghahn et al. (Berghahn et al., 2012). Because the stream mesocosms are in close proximity to lentic mesocosms it is possible to conduct comparative analysis of the risk of a substance on the biodiversity of both lentic and lotic ecosystems at the same time point. Hereby, same weather conditions can provide additional and helpful data. Furthermore, different exposure scenarios in lentic and lotic mesocosms can supply data regarding the fate

of a substance and provide additional data for the FOCUS modelling approach, which is used within the regulatory risk assessment of the European Union.

Moreover, stream mesocosms can also be used for additional aquatic ecological research questions like consequences of climate change (e.g. increase of water temperature or drought events; Whitehead et al., 2009), other anthropogenic stressors (e.g. whether the runoff of fertilizers from agricultural fields into freshwaters alters geochemical cycles of carbon, nitrogen and phosphorous, Le Moal et al., 2019; Wurtsbaugh et al., 2019) or the influence of invasive species on the aquatic community (Dextrase & Mandrak, 2006; Havel et al., 2015).

Taken together, important insights regarding the influence of anthropogenic stressors on freshwater habitats can be gained from experiments by using these stream mesocosms. Especially the water management within the Water Framework Directive can gain further information for their ecosystem-based objectives and planning processes at the freshwater level in the European Union (Carvalho et al., 2019).

# 5 Conclusion and outlook

The present work provides new insights for the risk assessment of insecticides and fungicides within higher-tier testing in outdoor stream mesocosms. By using carbaryl as a model insecticide and tebuconazole as a model fungicide a whole spectrum of different endpoints for the assessment of direct and indirect effects on the biocenosis of small lotic water bodies was tested. In general, it could be shown that stream mesocosms have the ability to represent a macroinvertebrate community typical for small lotic waterbodies near agricultural areas. Not only can direct effects of pesticides and other chemicals on population level be tested, but also indirect effects on population and community level can be assessed over a time period of several months. Especially the amount of EPT taxa and gammarids, which are considered to be ecologically vulnerable macroinvertebrates, is higher in lotic waterbodies compared to lentic ones (Biggs et al., 2007). Therefore, it is a further option to conduct mesocosm studies for higher-tier risk assessment of insecticides also in lotic mesocosms.

For fungicides, the test system provides realistic model ecosystem characteristics, as potentially sensitive microorganisms like aquatic hyphomycetes occur more often in lotic waterbodies (Kuehn, 2016). It is therefore desirable to conduct mesocosm studies in lotic test systems to assess risk of fungicides on microorganism communities and related ecosystem functions. A whole bandwidth of different endpoints regarding the risk assessment of fungicides was tested during the study. However, the measured endpoints need to be improved to be used for future risk assessment

The number of stream mesocosms was enlarged from eight (for case study I) to twelve (for case study II). This was acceptable based on the aim of the studies to develop sufficient methods and realistic model ecosystems. For future studies in risk assessment of chemicals, the number of replicates per concentration should be even further increased. The EFSA guidance document suggests a “minimal” design with five replicates for the control and five different concentration levels with two replicates each (EFSA, 2013). However, three replicates per concentration level could increase statistical power for the tested endpoints further, without raising the cost-benefit ratio too much.

By using the recommended replicated design the use of the stream mesocosm test systems can be recommended for insecticides. For fungicides, further development of sufficient endpoints needs to be explored.

The test system also offers further exposure scenario options:

In the present work, two potential worst-case scenarios were tested. With respect to realistic exposure scenarios for pesticides, further realistic exposure scenarios like hour-scale pulse exposure scenarios can be tested with stream mesocosms by replacing treated water with uncontaminated water at a specific time following application. Hereby, further information about the fate of a substance can be gained and can support additional data for the FOCUS modelling approach, which is used within the regulatory risk assessment of the European Union. Moreover, the stream mesocosms were used as a semi-closed system, where a recolonisation was possible due to flying insects, but not due to drifting organisms. For future studies, long-term effects of pesticides and other chemicals can also be assessed by using stream mesocosms. For this, targeted introduction into stream mesocosms after certain time periods can simulate a potential immigration of organisms from untreated upstream sections.

# 6 References

Abelho, M. (2001). From litterfall to breakdown in streams: a review. *The Scientific World*, *1*, 656–680. https://doi.org/10.1100/tsw.2001.103

Abelho, M., Martins, T. F., Shinn, C., Moreira-Santos, M., & Ribeiro, R. (2016). Effects of the fungicide pyrimethanil on biofilm and organic matter processing in outdoor lentic mesocosms. *Ecotoxicology*, *25*(1), 121–131. https://doi.org/10.1007/s10646-015-1574-x

Abhilash, P. C., & Singh, N. (2009). Pesticide use and application: An Indian scenario. *Journal of Hazardous Materials*, *165*(1–3), 1–12. https://doi.org/10.1016/j.jhazmat.2008.10.061

Adam, O., Badot, P. M., Degiorgi, F., & Crini, G. (2009). Mixture toxicity assessment of wood preservative pesticides in the freshwater amphipod Gammarus pulex (L.). *Ecotoxicology and Environmental Safety*, *72*(2), 441–449. https://doi.org/10.1016/j.ecoenv.2008.07.017

Allan, J. D. (2007). Chapter 10: Drift. In *Stream Ecology: Structure and Function of Running Waters* (pp. 221–377). Springer.

Allan, J. D., & Castillo, M. M. (2007). Stream Ecology: Structure and Function of Running Waters. In *Springer*. Springer.

Aly, O. M., & El-Dib, M. A. (1971). Studies on the persistence of some carbamate insecticides in the aquatic environment-I. Hydrolysis of sevin, baygon, pyrolan and dimetilan in waters. *Water Research*, *5*(12), 1191–1205. https://doi.org/10.1016/0043-1354(71)90084-4

Amiard-Triquet, C. (2015). How to Improve Toxicity Assessment? From Single-Species Tests to Mesocosms and Field Studies. In C. Amiard-Triquet, J. C. Amiard, & C. Mouneyrac (Eds.), *Aquatic exotoxicology: advancing tools for dealing with emerging risks* (pp. 127–182). Elsevier Academic Press. https://doi.org/10.1017/CBO9781107415324.004

Amoros, C. (1984). Crustacés cladocères. *Ectrait Du Bulletin Mensuel de La Société Linnéenne de Lyon*, *5*, 1–63.

Amoros, C., & Chessel, D. (1985). Populations of Cladocera (Crustacea), as describers of the hydrological functioning of stagnant waterways. *Annales de Limnologie*, *21*, 227–240.

Andreu-Sànchez, O., Paraiba, L. C., Jonsson, C. M., & Carrasco, J. M. (2012). Acute Toxicity and Bioconcentration of Fungicide Tebuconazole in Zebrafish (Danio rerio). *Environmental Toxicology*, *27*(2), 109–116. https://doi.org/10.1002/tox

Angelier, E. (2019). *Ecology of streams and rivers*. CRC Press.

Armbrust, K. L., & Crosby, D. G. (1991). Fate of Carbaryl, 1-Naphthol, and Atrazine in Seawater. *Pacific Science*, *45*(3), 314–320.

Armitage, P. D. (1968). Some notes on the food of the chironomid larvae of a shallow woodland lake in South Finland. *Finnish Zoological and Botanical Publishing Board*, *5*(1), 6–13.

Artigas, J., Majerholc, J., Foulquier, A., Margoum, C., Volat, B., Neyra, M., & Pesce, S. (2012). Effects of the fungicide tebuconazole on microbial capacities for litter breakdown in streams. *Aquatic Toxicology*, *122–123*, 197–205. https://doi.org/10.1016/j.aquatox.2012.06.011

Artigas, J., Rossi, F., Gerphagnon, M., & Mallet, C. (2017). Sensitivity of laccase activity to the fungicide tebuconazole in decomposing litter. *Science of the Total Environment*, *584–585*, 1084–1092. https://doi.org/10.1016/j.scitotenv.2017.01.167

Azimonti, G., Galimberti, F., Marchetto, F., Menaballi, L., Ullucci, S., Pellicioli, F., Allessandra, C., Lidia, C., Allesio, I., Angelo, M., de Boer, W., & van der Voet, H. (2015). Comparison of NOEC values to EC10/EC20 values , including confidence intervals , in aquatic and terrestrial ecotoxicological risk assessment. *EFSA Supporting Publications*, *12*(12), 906E.

Bakanov, N., Wieczorek, M. V., & Schulz, R. (2020). The role of organic matrices in the fate of hydrophobic pesticides: An outdoor stream mesocosm study. *Chemosphere*, *259*(127459). https://doi.org/https://doi.org/10.1016/j.chemosphere.2020.127459

Balian, E. V., Segers, H., Lévèque, C., & Martens, K. (2008). The Freshwater Animal Diversity Assessment: An overview of the results. *Hydrobiologia*, *595*(1), 627–637. https://doi.org/10.1007/s10750-007-9246-3

Bärlocher, F. (1985). The role of fungi in the nutrition of stream invertebrates. *Botanical Journal of the Linnean Society*, *91*(1–2), 83–94. https://doi.org/10.1111/j.1095-8339.1985.tb01137.x

Bärlocher, F. (1992). Community organization. In F. Bäerlocher (Ed.), *The Ecology of Aquatic Hyphomycetes* (pp. 39–76). Springer-Verlag.

Bärlocher, F. (2005). Leaching. In M. A. S. Graca, F. Bärlocher, & M. O. Gessner (Eds.), *Methods to Study Litter Decomposition: A Practical Guide* (pp. 27–30). Kluwer Academic Publishers.

Bärlocher, F., & Kendrick, B. (1975). Leaf-conditioning by microorganisms. *Oecologia*, *20*(4), 359–362. https://doi.org/10.1007/BF00345526

Battin, T. J., Besemer, K., Bengtsson, M. M., Romani, A. M., & Packmann, A. I. (2016). The ecology and biogeochemistry of stream biofilms. *Nature Reviews Microbiology*, *14*(4), 251–263. https://doi.org/10.1038/nrmicro.2016.15

Bayona, Y., Roucaute, M., Cailleaud, K., Lagadic, L., Basseres, A., & Caquet, T. (2015). Effect of thiram and of a hydrocarbon mixture on freshwater macroinvertebrate communities in outdoor stream and pond mesocosms: II. Biological and ecological trait responses and leaf litter breakdown. *Ecotoxicology*, *24*(9), 1933–1946. https://doi.org/10.1007/s10646-015-1531-8

Bayona, Y., Roucaute, M., Cailleaud, K., Lagadic, L., Bassères, A., & Caquet, T. (2014). Structural and biological trait responses of diatom assemblages to organic chemicals in outdoor flow-through mesocosms. *Environmental Pollution*, *192*, 186–195. https://doi.org/10.1016/j.envpol.2014.05.023

Bêche, L. A., McElravy, E. P., & Resh, V. H. (2006). Long-term seasonal variation in the biological traits of benthic-macroinvertebrates in two Mediterranean-climate streams in California, U.S.A. *Freshwater Biology*, *51*(1), 56–75. https://doi.org/10.1111/j.1365-2427.2005.01473.x

Beek, B. (2001). *Biodegradation and persistence*. Springer.

Beketov, M. A., Foit, K., Schäfer, R. B., Schriever, C. A., Sacchi, A., Capri, E., Biggs, J., Wells, C., & Liess, M. (2009). SPEAR indicates pesticide effects in streams – Comparative use of species- and family-level biomonitoring data. *Environmental Pollution*, *157*, 1841–1848. https://doi.org/10.1016/j.envpol.2009.01.021

Beketov, M. A., Schäfer, R. B., Marwitz, A., Paschke, A., & Liess, M. (2008). Long-term stream invertebrate community alterations induced by the insecticide thiacloprid: Effect concentrations and recovery dynamics. *Science of the Total Environment*, *405*(1–3), 96–108. https://doi.org/10.1016/j.scitotenv.2008.07.001

Beketov, M., Kefford, B. J., Schäfer, R. B., & Liess, M. (2013). Pesticides reduce regional biodiversity of stream invertebrates. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(27), 11039–11043. https://doi.org/10.1073/pnas.1305618110

Beketov, M., & Liess, M. (2008). Potential of 11 pesticides to initiate downstream drift of stream macroinvertebrates. *Archives of Environmental Contamination and Toxicology*, *55*(2), 247–253. https://doi.org/10.1007/s00244-007-9104-3

Benfield, E. F. (1997). Comparison of litterfall input to streams. *Journal of the North American Benthological Society*, *16*, 104–108.

Berenzen, N., Kumke, T., Schulz, H. K., & Schulz, R. (2005). Macroinvertebrate community structure in agricultural streams: Impact of runoff-related pesticide contamination. *Ecotoxicology and Environmental Safety*, *60*(1), 37–46. https://doi.org/10.1016/j.ecoenv.2003.10.010

Berg, H. B. (1995). Larval food and feeding behavior. In P. D. Armitage, P. S. Cranston, & L. C. V. Pinder (Eds.), *The Chironomidae: Biology and ecology of non-biting midges* (p. 584p.). Chapman and Hall.

Berger, E., Haase, P., Schäfer, R. B., & Sundermann, A. (2018). Towards stressor-specific macroinvertebrate indices: Which traits and taxonomic groups are associated with vulnerable and tolerant taxa? *Science of the Total Environment*, *619–620*(November 2017), 144–154. https://doi.org/10.1016/j.scitotenv.2017.11.022

Berger, S., & Nejstgaard, J. (2020). *Mesocosm- An open virtual network for aquatic mesocosm facilities worldwide*. http://mesocosm.org/

Berghahn, R., Mohr, S., Hübner, V., Schmiediche, R., Schmiedling, I., Svetich-Will, E., & Schmidt, R. (2012). Effects of repeated insecticide pulses on macroinvertebrate drift in indoor stream mesocosms. *Aquatic Toxicology*, *122–123*, 56–66. https://doi.org/10.1016/j.aquatox.2012.05.012

Beuter, L.-K., Dören, L., Hommen, U., Kotthoff, M., Schäfers, C., & Ebke, K. P. (2019). Testing effects of pesticides on macroinvertebrate communities in outdoor stream mesocosms using carbaryl as example test item. *Environmental Sciences Europe*, *31*(1), 1–17. https://doi.org/10.1186/s12302-019-0185-1

Beyers, D. W., Farmer, M. S., & Sikoski, P. J. (1995). Effects of rangeland aerial application of Sevin-4-Oil® on fish and aquatic invertebrate drift in the Little Missouri River, North Dakota. *Archives of Environmental Contamination and Toxicology*, *28*(1), 27–34. https://doi.org/10.1007/BF00213965

Biggs, J., Nicolet, P., Mlinaric, M., & Lalanne, T. (2014). Report of the Workshop on the Protection and Management of Small Water Bodies, Brussels, 14th November 2013. *The European Environmental Bureau (EEB) and the Freshwater Habitats Trust*, 23 p.

Biggs, J., Williams, P., Whitfield, M., Nicolet, P., Brown, C., Hollis, J., Arnold, D., & Pepper, T. (2007). The freshwater biota of British agricultural landscapes and their sensitivity to pesticides. *Agriculture, Ecosystems and Environment*, *122*(2), 137–148. https://doi.org/10.1016/j.agee.2006.11.013

Boesten, J. J. T. I., Köpp, H., Adriaanse, P. I., Brock, T. C. M., & Forbes, V. E. (2007). Conceptual model for improving the link between exposure and effects in the aquatic risk assessment of pesticides. *Ecotoxicology and Environmental Safety*, *66*(3), 291–308. https://doi.org/10.1016/j.ecoenv.2006.10.002

Boling, R. H., Goodman, E. D., Van Sickle, J. A., Zimmer, J. O., Cummings, K. W., Petersen, R. C., & Reice, S. R. (1975). Toward A Model of Detritus Processing in a Woodland Stream. *Ecology*, *56*(1), 141–151. https://doi.org/10.2307/1935306

Brabec, K., Zahrádková, S., Nemejcová, D., Paril, P., Kokes, J., & Jarkovský, J. (2004). Assessment of organic effect considering differences between lotic and lentic stream habitats. *Hydrobiologia*, *516*, 331–346. https://doi.org/10.1023/B

Breuer, F., Janz, P., Farrelly, E., & Ebke, K. P. (2017). Environmental and structural factors influencing algal communities in small streams and ditches in central Germany. *Journal of Freshwater Ecology*, *32*(1), 65–83. https://doi.org/10.1080/02705060.2016.1241954

Bricheux, G., Morin, L., Le Moal, G., Coffe, G., Balestrino, D., Charbonnel, N., Bohatier, J., & Forestier, C. (2013). Pyrosequencing assessment of prokaryotic and eukaryotic diversity in biofilm communities from a French river. *MicrobiologyOpen*, *2*(3), 402–414. https://doi.org/10.1002/mbo3.80

Brittain, J. E., & Eikeland, T. J. (1988). Invertebrate drift - A review. *Hydrobiologia*, *166*(1), 77–93. https://doi.org/10.1007/BF00017485

Brock, T. C. M. (2009). Linking Aquatic Exposure and Effects: Risk Assessment of Pesticides. In *Linking Aquatic Exposure and Effects*. CRC Press. https://doi.org/10.1201/9781439813492

Brock, T. C. M. (2013). Priorities to improve the ecological risk assessment and management for pesticides in surface water. *Integrated Environmental Assessment and Management*, *9*(3), 64–74. https://doi.org/10.1002/ieam.1429

Brock, T. C. M., Alix, A., Brown, C., Capri, E., Gottesbüren, B., Heimbach, F., Lythgo, C., Schulz, R., & Streloke, M. (2010). *Linking aquatic exposure and effects- Risk assessment of pesticides*. CRC Press, Society of Environmental Toxicology and Chemistry (SETAC) Press.

Brock, T. C. M., Hammers-Wirtz, M., Hommen, U., Preuss, T. G., Ratte, H. T., Roessink, I., Strauss, T., & Van den Brink, P. J. (2014). The minimum detectable difference (MDD) and the interpretation of treatment-related effects of pesticides in experimental ecosystems. *Environmental Science and Pollution Research*, *22*(2), 1160–1174. https://doi.org/10.1007/s11356-014-3398-2

Brock, T. C. M., Van Wijngaarden, P. . P. A., & Van Geest, G. J. (2000). *Ecological risk of pesticides in freshwater ecosystems. Part 2: Insecticides*.

Brümmer, I. H. M., Fehr, W., & Wagner-Döbler, I. (2000). Biofilm community structure in polluted rivers: Abundance of dominant phylogenetic groups over a complete annual cycle. *Applied and Environmental Microbiology*, *66*(7), 3078–3082. https://doi.org/10.1128/AEM.66.7.3078-3082.2000

Buesing, N. (2005). Bacterial counts and biomass determination by epifluorescence microscopy. In M. A. S. Graca, F. Bärlocher, & M. O. Gessner (Eds.), *Methods to Study Litter Decomposition: A Practical Guide* (pp. 203–208). Springer Netherlands. https://doi.org/10.1007/1-4020-3466-0_27

Bulen, B. J., & Distel, C. A. (2011). Carbaryl concentration gradients in realistic environments and their influence on our understanding of the tadpole food web. *Archives of Environmental Contamination and Toxicology*, *60*(2), 343–350. https://doi.org/10.1007/s00244-010-9630-2

Bundschuh, M., Zubrod, J. P., Kosol, S., Maltby, L., Stang, C., Duester, L., & Schulz, R. (2011). Fungal composition on leaves explains pollutant-mediated indirect effects on amphipod feeding. *Aquatic Toxicology*, *104*(1–2), 32–37. https://doi.org/10.1016/j.aquatox.2011.03.010

Butler, M. G. (1984). Life histories of aquatic insects. In V. H. Resh & D. M. Rosenberg (Eds.), *The Ecology of Aquatic Insects* (pp. 24–55). Praeger.

BVL (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit). (2019). *Absatz an Pflanzenschutzmitteln in der Bundesrepublik Deutschland Ergebnisse der Meldungen gemäß § 64 Pflanzenschutzgesetz für das Jahr 2018*. www.bvl.bund.de/psmstatistiken

Calapez, A. R., Branco, P., Santos, J. M., Ferreira, T., Hein, T., Brito, A. G., & Feio, M. J. (2017). Macroinvertebrate short-term responses to flow variation and oxygen depletion: A mesocosm approach. *Science of the Total Environment*, *599–600*, 1202–1212. https://doi.org/10.1016/j.scitotenv.2017.05.056

Cañedo-Argüelles, M., Bundschuh, M., Gutiérrez-Cánovas, C., Kefford, B. J., Prat, N., Trobajo, R., & Schäfer, R. B. (2014). Effects of repeated salt pulses on ecosystem structure and functions in a stream mesocosm. *Science of the Total Environment*, *476–477*, 634–642. https://doi.org/10.1016/j.scitotenv.2013.12.067

Caquet, T. (2013). Aquatic Mesocosms in Ecotoxicology. In J.-F. Férard & C. Blaise (Eds.), *Encyclopedia of Aquatic Ecotoxicology* (p. 99). Springer Netherlands. https://doi.org/10.1007/978-94-007-5704-2

Caquet, T., Hanson, M. L., Graham, D. W., Babin, E., Azam, D., Coutellec, M.-A., Knapp, C. W., Lagadic, L., & Caquet, T. (2007). Influence of isolation on the recovery of pond mesocosms from the application of an insecticide. I. Study design and planktonic community responses. *Environmental Toxicology and Chemistry*, *26*(6), 1280–1290. https://doi.org/10.1897/06-250r.1

Caquet, T., Lagadic, L., Jonot, O., Baturo, W., Kilanda, M., Simon, P., Le Bras, S., Echaubard, M., & Ramade, F. (1996). Outdoor Experimental Ponds (Mesocosms) Designed for Long-Term Ecotoxicological Studies in Aquatic Environment. *Ecotoxicology and Environmental Safety*, *34*(34), 125–133. https://doi.org/10.1006/eesa.1996.0053

Caquet, T., Lagadic, L., & Sheffield, S. R. (2000). Mesocosms in ecotoxicology. 1. Outdoor Auqatic Systems. *Reviews of Environmental Contamination and Toxicology*, *165*, 1–38.

Caquet, Thierry, Thybaud, E., Le Bras, S., Jonot, O., & Ramade, F. (1992). Fate and biological effects of lindane and deltamethrin in freshwater mesocosms. *Aquatic Toxicology*, *23*(3–4), 261–277. https://doi.org/10.1016/0166-445X(92)90057-T

Carvalho, L., Mackay, E. B., Cardoso, A. C., Baattrup-Pedersen, A., Birk, S., Blackstock, K. L., Borics, G., Borja, A., Feld, C. K., Ferreira, M. T., Globevnik, L., Grizzetti, B., Hendry, S., Hering, D., Kelly, M., Langaas, S., Meissner, K., Panagopoulos, Y., Penning, E., … Solheim, A. L. (2019). Protecting and restoring Europe's waters: An analysis of the future development needs of the Water Framework Directive. *Science of the Total Environment*, *658*, 1228–1238. https://doi.org/10.1016/j.scitotenv.2018.12.255

Chamier, A. (1985). Cell-wall-degrading enzymes of aquatic hyphomycetes: a review. *Botanical Journal of the Linnean Society*, *91*(1–2), 67–81. https://doi.org/10.1111/j.1095-8339.1985.tb01136.x

Chen, S. K., & Edwards, C. A. (2001). A microcosm approach to assess the effects of fungicides on soil ecological processes and plant growth: Comparisons of two soil types. *Soil Biology and Biochemistry*, *33*(14), 1981–1991. https://doi.org/10.1016/S0038-0717(01)00132-8

Claus, H. (2003). Laccases and their occurrence in prokaryotes. *Archives of Microbiology*, *179*(3), 145–150. https://doi.org/10.1007/s00203-002-0510-7

Clements, W. H. (1999). Metal tolerance and predator-prey interactions in benthic macroinvertebrate stream communities. *Ecological Applications*, *9*(3), 1073–1084. https://doi.org/doi:10.1890/1051-0761(1999)009[1073:MTAPPI]2.0.CO;2

Corbet, P. S. (1964). Temporal Patterns of Emergence in Aquatic Insects. *Canadian Entomologist*, *96*(1–2), 264–279.

Courtemanch, D. L., & Gibbs, E. K. (1980). Short-and long-term effects of forest spraying of carbaryl (Sevin-4-oil®) on stream invertebrates. *The Canadian Entom*, *112*(03), 271–276.

Cummins, K. (1973). Trophic Relations of Aquatic Insects. *Annual Review of Entomology*, *18*, 183–206.

Cummins, K. W. (1973). Trophic relations of aquatic insects. *Annual Review of Entomology*, *18*, 183–206.

Cummins, K. W., & Klug, M. T. (1979). Feeding Ecology of Stream Invertebrates. *Annual Review of Ento*, *10*, 147–172.

Cuppen, J. G. M., Van Den Brink, P. J., Camps, E., Uil, K. F., & Brock, T. C. M. (2000). Impact of the fungicide carbendazim in freshwater microcosms. I. Water quality, breakdown of particulate organic matter and responses of macroinvertebrates. *Aquatic Toxicology*, *48*(2–3), 233–250. https://doi.org/10.1016/S0166-445X(99)00036-3

Daam, M. A., Satapornvanit, K., Van den Brink, P. J., & Nogueira, A. J. A. (2010). Direct and indirect effects of the fungicide carbendazim in tropical freshwater microcosms. *Archives of Environmental Contamination and Toxicology*, *58*(2), 315–324. https://doi.org/10.1007/s00244-009-9367-y

De, A., Bose, R., & Mozumdar, S. (2014). Worldwide pesticide use. In A. De, R. Bose, & S. Mozumdar (Eds.), *Targeted delivery using biodegradable polymeric nanoparticles* (pp. 5–6). Springer Berlin. https://doi.org/10.1007/978-1-4614-0881-9_10

Dextrase, A. J., & Mandrak, N. E. (2006). Impacts of alien invasive species on freshwater fauna at risk in Canada. *Biological Invasions*, *8*(1), 13–24. https://doi.org/10.1007/s10530-005-0232-2

Dijksterhuis, J., Van Doorn, T., Samson, R., & Postma, J. (2011). Effects of seven fungicides on non-target aquatic fungi. *Water, Air, and Soil Pollution*, *222*(1–4), 421–425. https://doi.org/10.1007/s11270-011-0836-3

Dimitrov, M. R., Kosol, S., Smidt, H., Buijse, L., Van den Brink, P. J., Van Wijngaarden, R. P., Brock, T. C. M., & Maltby, L. (2014). Assessing effects of the fungicide tebuconazole to heterotrophic microbes in aquatic microcosms. *Science of the Total Environment*, *490*, 1002–1011. https://doi.org/10.1016/j.scitotenv.2014.05.073

DIN 38.412, T. 16. (1985). *Bestimmung des Chlorophyll-a-Gehaltes von Oberflächenwasser*.

Donnadieu, F., Besse-Hoggan, P., Forestier, C., & Artigas, J. (2016). Influence of streambed substratum composition on stream microbial communities exposed to the fungicide tebuconazole. *Freshwater Biology*, *61*(12), 2026–2036. https://doi.org/10.1111/fwb.12679

Dorigo, U., Berard, A., Rimet, F., Bouchez, A., & Montuelle, B. (2010). In situ assessment of periphyton recovery in a river contaminated by pesticides. *Aquatic Toxicology*, *98*(4), 396–406. https://doi.org/10.1016/j.aquatox.2010.03.011

Dudgeon, D., Arthington, A. H., Gessner, M. O., Kawabata, Z.-I., Knowler, D. J., Lévêque, C., Naiman, R. J., Prieur-Richard, A.-H., Soto, D., Stiassny, M. L. J., & Sullivan, C. a. (2006). Freshwater biodiversity: Importance, threats, status and conservation challenges. *Biological Reviews*, *81*(02), 163–182. https://doi.org/10.1017/S1464793105006950

DWD (German Weather Station). (2019). *Weather data measuring station Neu-Ulrichstein (ID 7410)*. www.dwd.de

EC. (2009). *Commission Regulation No 1107/2009, OJ L 309 of 24.11.2009*.

EFSA. (2019). Outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology. *EFSA Supporting Publications*, *EN-1673*, 117 pp. https://doi.org/10.2903/sp.efsa.2019.en-1673

EFSA, Brown, K., Tomlinson, J., Duncan, J., Hinchcliffe, A., & Palmquist, K. (2009). Critical comparison of available and potential higher tier testing approaches for the risk assessment of plant protection products, considering at least field and semi-field experimental designs, extrapolation from dose-response relationships, and increas. *EFSA Supporting Publications*, *6*(8), 16E.

EFSA Scientific Committee. (2016). Scientific opinion on recovery in environmental risk assessment at EFSA. *EFSA Journal*, *14*(2), 4313. 85 pp. https://doi.org/10.2903/j.efsa.2016.4313

Eichelberger, J. W., & Lichtenberg, J. J. (1971). Persistence of pesticides in river water. *Environmental Science & Technology*, *5*(6), 541–544. https://doi.org/10.1021/es60053a002

Elsaesser, D., Stang, C., Bakanov, N., & Schulz, R. (2013). The Landau stream mesocosm facility: Pesticide mitigation in vegetated flow-through streams. *Bulletin of Environmental Contamination and Toxicology*, *90*(6), 640–645. https://doi.org/10.1007/s00128-013-0968-9

EU. (2000). *Directive 2000/60/EC of the European Parliament and of the Council establishing a framework for the Community action in the field of water policy*.

European Food Safety Authority (EFSA). (2013). Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters. *EFSA Journal*, *11*(7), 3290,268 pp. https://doi.org/10.2903/j.efsa.2013.3290

European Food Safety Authority (EFSA). (2014). *Conclusion on the peer review of the pesticide risk assessment of the active substance tebuconazole*.

European Food Safety Authority (EFSA). (2016). Recovery in environmental risk assessments at EFSA. *EFSA Journal*, *14*(2). https://doi.org/10.2903/j.efsa.2016.4313

Eurpean Food Safety Authority (EFSA). (2006). *Final addendum to the Draft Assessment Report (DAR) Carbaryl*.

Feckler, A., Goedkoop, W., Zubrod, J. P., Schulz, R., & Bundschuh, M. (2016). Exposure pathway-dependent effects of the fungicide epoxiconazole on a decomposer-detritivore system. *Science of the Total Environment*, *571*, 992–1000. https://doi.org/10.1016/j.scitotenv.2016.07.088

Fernández, D., Voss, K., Bundschuh, M., Zubrod, J. P., & Schäfer, R. B. (2015). Effects of fungicides on decomposer communities and litter decomposition in vineyard streams. *Science of the Total Environment*, *533*, 40–48. https://doi.org/10.1016/j.scitotenv.2015.06.090

Ferreira, V., Gulis, V., Pascoal, C., & Graça, M. A. S. (2014). Stream pollution and fungi. In E. B. G. Jones, K. D. Hyde, & K.-L. Pang (Eds.), *Freshwater Fungi: And Fungal-like Organisms.* (pp. 441-). Walter de Truyter GmbH & Co KG. https://doi.org/10.1515/9783110333480.389

Fisher, S. W., & Lohner, T. W. (1986). Studies on the Environmental Fate of Carbaryl as a Function of pH. *Archives of Environmental Contamination and Toxicology*, *667*(15), 661–667.

Fleeger, J. W., Carman, K. R., & Nisbet, R. M. (2003). Indirect effects of contaminants in aquatic ecosystems. *Science of the Total Environment*, *317*(1–3), 207–233. https://doi.org/10.1016/S0048-9697(03)00141-4

Fochetti, R., & Tierno De Figueroa, J. M. (2008). Global diversity of stoneflies (Plecoptera; Insecta) in freshwater. *Hydrobiologia*, *595*(1), 365–377. https://doi.org/10.1007/s10750-007-9031-3

FOCUS (Forum for the Coordination of Pesticide Fate Models and Their Use). (2001). *FOCUS Surface Water Scenarios in the EU Evaluation Process under 91/414/EEC. Report of the FOCUS Working Group on Surface Water Scenarios. EC Document Reference SANCO/4802/2001-rev.2*.

FOCUS (Forum for the Coordination of Pesticide Fate Models and Their Use). (2007). *Landscape and mitigation factors in aquatic risk assessment. Volume 1. Extended summary and recommendations. Report of the FOCUS Working Group on Landscape and Mitigation Factors in Ecological Risk Assessment. EC Document Reference SANCO/10422/2005v2.0*.

FOOTPRINT. (2019). *The FOOTPRINT Pesticide Properties Database. Database collated by the university of Hertfordshire as part of the EU-funded FOOTPRINT project (FP6-SSP-022704)*. http://www.eu-footprint.org/ppdb.html

Füreder, L., Wallinger, M., & Burger, R. (2005). Longitudinal and seasonal pattern of insect emergence in alpine streams. *Aquatic Ecology*, *39*(1–2), 67–78. https://doi.org/10.1007/s10452-004-2969-6

Gergs, A., Classen, S., Hommen, U., & Preuss, T. G. (2011). Identification of realistic worst case aquatic macroinvertebrate species for prospective risk assessment using the trait concept. *Environmental Science and Pollution Research*, *18*(8), 1316–1323. https://doi.org/10.1007/s11356-011-0484-6

Gergs, A., Classen, S., Strauss, T., Ottermanns, R., Brock, T. C. M., Ratte, H. T., Hommen, U., & Preuss, T. G. (2016). Ecological Recovery Potential of Freshwater Organisms: Consequences for Environmental Risk Assessment of Chemicals. In P. de Voogt (Ed.), *Reviews of Environmental Contamination and Toxicology* (Vol. 236, pp. 259–294). Springer International Publishing Switzerland. https://doi.org/10.1007/978-3-319-20013-2

Gerhardt, V., & Bodemer, U. (1998). Delayed Fluorescence Excitation Spectroscopy: A Method for Automatic Determination of Phytoplankton Composition of Freshwaters and Sediments (Interstitial) and of Algal Composition of Benthos. *Limnologica*, *28*(3), 313–322.

Gerhardt, V., & Bodemer, U. (2000). Delayed fluorescence excitation spectroscopy: a method for determining phytoplankton composition. *Advances in Limnology*, *55*, 101–120.

Gessner, M. O. (2005). Ergosterol as a measure of fungal biomass. In M. A. A. Graca, F. Bärlocher, & M. O. Gessner (Eds.), *Methods to Study Litter Decomposition: A Practical Guide* (pp. 189–195). Springer Netherlands. https://doi.org/10.1007/1-4020-3466-0_25

Gessner, M. O., & Chauvet, E. (1993). Ergosterol-to -Biomass Conversion Factors for Aquatic Hyphomycetes. *Applied and Environmental Microbiology*, *59*(2), 502–507.

Gessner, M. O., Chauvet, E., & Dobson, M. (1999). A Perspective on Leaf Litter Breakdown in Streams All use subject to JSTOR Terms and Conditions A perspective on leaf litter breakdown in streams. *Nordic Society Oikos*, *85*, 377–384. http://www.jstor.org/stable/3546505

Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S., & Sannia, G. (2010). Laccases: A never-ending story. *Cellular and Molecular Life Sciences*, *67*(3), 369–385. https://doi.org/10.1007/s00018-009-0169-1

Giddings, J. M., Brock, T. C. M., Heger, W., Heimbach, F., Maund, S. J., Norman, S., Ratte, H.-T., Schäfers, C., & Steloke, M. (2002). Community-level aquatic system studies-interpretation criteria. (CLASSIC). *SETAC*, 44p.

Graça, M. A. S. (2001). The role of invertebrates on leaf litter decomposition in streams - A review. *International Review of Hydrobiology*, *86*(4–5), 383–393. https://doi.org/10.1002/1522-2632(200107)86:4/5<383::AID-IROH383>3.0.CO;2-D

Gregg, W. W., & Rose, F. R. E. D. L. (1982). The effects of aquatic macrophytes environment on the stream microenvironment. *Aquatic Botany*, *14*, 309–324.

Groner, M. L., & Relyea, R. A. (2011). A tale of two pesticides: How common insecticides affect aquatic communities. *Freshwater Biology*, *56*(11), 2391–2404. https://doi.org/10.1111/j.1365-2427.2011.02667.x

Gulis, V., Suberkropp, K., & Rosemond, A. D. (2008). Comparison of fungal activities on wood and leaf litter in unaltered and nutrient-enriched headwater streams. *Applied and Environmental Microbiology*, *74*(4), 1094–1101. https://doi.org/10.1128/AEM.01903-07

Gustafsson, K., Blidberg, E., Elfgren, I. K., Hellström, A., Kylin, H., & Gorokhova, E. (2010). Direct and indirect effects of the fungicide azoxystrobin in outdoor brackish water microcosms. *Ecotoxicology*, *19*(2), 431–444. https://doi.org/10.1007/s10646-009-0428-9

Hall, E. K., Besemer, K., Kohl, L., Preiler, C., Riedel, K., Schneider, T., Wanek, W., & Battin, T. J. (2012). Effects of resource chemistry on the composition and function of stream hyporheic biofilms. *Frontiers in Microbiology*, *3*(FEB), 1–14. https://doi.org/10.3389/fmicb.2012.00035

Hamilton, P. B., Cowx, I. G., Oleksiak, M. F., Griffiths, A. M., Grahn, M., Stevens, J. R., Carvalho, G. R., Nicol, E., & Tyler, C. R. (2016). Population-level consequences for wild fish exposed to sublethal concentrations of chemicals – a critical review. *Fish and Fisheries*, *17*(3), 545–566. https://doi.org/10.1111/faf.12125

Hanazato, T., & Yasuno, M. (1987). Effects of a carbamate insecticide, carbaryl, on the summer phyto- and zooplankton communities in ponds. *Environmental Pollution*, *48*(2), 145–159. https://doi.org/10.1016/0269-7491(87)90093-5

Hanlon, R. D. G. (1982). The breakdown and decomposition of allochthonous and autochthonous plant litter in an oligotrophic lake (Llyn Frongoch). *Hydrobiologia*, *88*, 281–288.

Hardersen, S., Wratten, S. D., & Frampton, C. M. (1999). Does carbaryl increase fluctuating asymmetry in damselflies under field conditions? A mesocosm experiment with Xanthocnemis zealandica (Odonata: Zygoptera). *Journal of Applied Ecology*, *36*(4), 534–543. https://doi.org/10.1046/j.1365-2664.1999.00417.x

Harris, R. M. L., Armitage, P. D., Milner, A. M., & Ledger, M. E. (2007). Replicability of physicochemistry and macroinvertebrate assemblages in stream mesocosms: Implications for experimental research. *Freshwater Biology*, *52*(12), 2434–2443. https://doi.org/10.1111/j.1365-2427.2007.01839.x

Hauer, F. R., & Resh, V. H. (2007). Macroinvertebrates. In F. R. Hauer & G. A. Lamberti (Eds.), *Methods in Stream Ecology.* (2nd edn., pp. 435–463). Elsevier Academic Press.

Havel, J. E., Kovalenko, K. E., Thomaz, S. M., Amalfitano, S., & Kats, L. B. (2015). Aquatic invasive species: challenges for the future. *Hydrobiologia*, *750*(1), 147–170. https://doi.org/10.1007/s10750-014-2166-0

Havens, K. E. (1994). An experimental comparison of the effects of two chemical stressors on a freshwater zooplankton assemblage. *Environmental Pollution*, *84*(3), 245–251. http://www.ncbi.nlm.nih.gov/pubmed/15091695

Havens, K. E. (1995). Insecticide (Carbaryl, 1-Napthyl-N-Methylcarbamate) effects on a freshwater plankton community: zooplankton size, biomass, and algal abundance. *Water, Air and Soil Pollution*, *84*, 1–10.

Hawkes, H. A. (1975). River zonation and classification. *River Ecology*, 312–374.

Hellawell. (1986). *Biological Indicators of Freshwater Pollution and Environmental Management*. Elsevier.

Hendel, B. (1999). *Der mikrobielle Abbau von Holz und Laub im Breitenbach unter besonderer Berücksichtigung der Bedeutung extrazellulärer Enzyme*. Justus-Liebig Universität Gießen.

Hendel, B., Sinsabaugh, R. L., & Marxsen, J. (2005). Lignin-degrading enzymes: Phenoloxidase and peroxidase. In M. A. S. Graca, F. Bärlocher, & M. O. Gessner (Eds.), *Methods to Study Litter Decomposition: A Practical Guide* (pp. 273–278). Springer Netherlands. https://doi.org/10.1007/1-4020-3466-0_37

Hickey, C. W., & Golding, L. A. (2002). Response of macroinvertebrates to copper and zinc in a stream mesocosm. *Environmental Toxicology and Chemistry*, *21*(9), 1854–1863. https://doi.org/10.1002/etc.5620210913

Hieber, M., & Gessner, M. O. (2002). Contribution of stream detrivores, fungi, and bacteria to leaf breakdown based on biomass estimates. *Ecology*, *83*(4), 1026–1038. https://doi.org/10.1890/0012-9658(2002)083[1026:COSDFA]2.0.CO;2

HLNUG. (2018). *WRRL-Viewer des HLNUG (Hessisches Landesamt für Naturschutz, Umwelt und Geologie), Umweltbehörde im Geschäftsbereich des Hessischen Ministerium für Umwelt, Klimaschutz,Landwirtschaft und Verbraucherschutz.* http//:wrrl.hessen.de

Hoppe, H.-G. (1983). Significance of exoenzymatic activities in the ecology of brackish water: measurements by means of methylumbelliferyl-substrates. *Marine Ecology-Progress Series*, *11*, 299–308.

Horwitz, R. J. (1978). Temporal Variability Patterns and the Distributional Patterns of Stream Fishes. *Ecological Monographs*, *48*(3), 307–321. https://doi.org/10.2307/2937233

Hose, G. C., & Van Den Brink, P. J. (2004). Confirming the species-sensitivity distribution concept for endosulfan using laboratory, mesocosm, and field data. *Archives of Environmental Contamination and Toxicology*, *47*(4), 511–520. https://doi.org/10.1007/s00244-003-3212-5

Hussain, Q., & Pandit, A. K. (2012). Macroinvertebrates in streams: A review of some ecological factors. *International Journal of Fisheries and Aquaculture*, *4*(7), 114–123. https://doi.org/10.5897/IJFA11.045

Illies, J., & Botosaneanu, L. (1963). Problems and methods for the classification and ecological zoning of these currents, considering the faunistic point of view. *Mitteilungen-Internationale Vereinigung Fuer Theoretische Und Angewandte Limnologie*, *12*, 1–57.

Ippolito, A., Carolli, M., Varolo, E., Villa, S., & Vighi, M. (2012). Evaluating pesticide effects on freshwater invertebrate communities in alpine environment: A model ecosystem experiment. *Ecotoxicology*, *21*(7), 2051–2067. https://doi.org/10.1007/s10646-012-0957-5

Ittner, L. D., Junghans, M., & Werner, I. (2018). Aquatic Fungi: A Disregarded Trophic Level in Ecological Risk Assessment of Organic Fungicides. *Frontiers in Environmental Science*, *6*(September). https://doi.org/10.3389/fenvs.2018.00105

James, A. B. W., Dewson, Z. S., & Death, R. G. (2008). The effect of experimental flow reductions on macroinvertebrate drift in natural and streamside channels. *River Research and Applications*, *24*, 22–35. https://doi.org/10.1002/rra

Janz, P. (2016). *Risikobewertung von Chemikalien mit Fließgewässermodellökosystemen. Dissertation*. Justus-Liebig University of Gießen.

Jekel, H., Arle, J., Bartel, H., Baumgarten, K., Blondzi, U., Claussen, U., Damian, H. P., Döscher, K., Dubbert, W., Eggers, H.-H., Fricke, K., Fuß, F., Galander, C., & Gast, M. (2013). Water Resource Management in Germany, Part 1: Fundamentals. *Federal Environmental Agency (Umweltbundesamt), 150 pp.* https://doi.org/10.1177/0019556119990201

Kahle, M., Buerge, I. J., Hauser, A., Müller, M. D., & Poiger, T. (2008). Azole fungicides: Occurrence and fate in wastewater and surface waters. *Environmental Science and Technology*, *42*(19), 7193–7200. https://doi.org/10.1021/es8009309

Kattwinkel, M., Römbke, J., & Liess, M. (2012). Ecological recovery of populations of vulnerable species driving the risk assessment of pesticides. *Supporting Publications EN-338*, 98 pp. https://doi.org/10.2903/sp.efsa.2012.EN-338

Kennedy, J. H., LaPoint, T. W., Balci, P., Stanley, J. K., & Johnson, Z. B. (2003). Model Aquatic Ecosystems in Ecotoxicological Research: Considerations of Design, Implementation, and Analysis. In D. J. Hoffman, B. A. Rattner, G. A. Burton, & J. Cairns (Eds.), *Handbook of Ecotoxicology, Second Edition* (2nd ed., pp. 45–74). CRC Press. https://doi.org/10.1201/9781420032505.ch23

Kolkewitz, R., & Marsson, M. (1902). Grundsätze für die biologische Beurteilung des Wassers nach seiner Floa und Fauna. *Mjitt. Aus d. Kgl. Prüfungsanstalt Für Wasserversorgung u. Abwasserbeseitigung*, *1*, 33–72.

Koop, J. H. E., Schäffer, M., Ortmann, C., & Winkelmann, C. (2008). Towards environmental assessment of river ecosystems by analyzing energy reserves of aquatic invertebrates. *Limnologica*, *38*, 378–387. https://doi.org/10.1016/j.limno.2008.05.004

Kraufvelin, P. (1998). Model ecosystem replicability challenged by the "soft" reality of a hard bottom mesocosm. *Journal of Experimental Marine Biology and Ecology*, *222*(1–2), 247–267. https://doi.org/10.1016/S0022-0981(97)00143-3

Kriska, G. (2013). *Freshwater Invertebrates in Central Europe*. Sringer-Verlag Wien 2013.

Kuehn, K. A. (2016). Lentic and lotic habitats as templets for fungal communities: Traits, adaptations, and their significance to litter decomposition within freshwater ecosystems. *Fungal Ecology*, *19*, 135–154. https://doi.org/10.1016/j.funeco.2015.09.009

Lamberti, G. A. (1996). The role of periphyton in benthic food webs. In R. J. Stevenson, M. L. Bothwell, & R. I. Lowe (Eds.), *Algal ecology: freshwater benthic ecosystems*. Elsevier Academic Press.

Lamberti, G. A., Feminella, J. W., & Pringle, C. M. (2007). Primary producer-consumer interactions. In F. R. Hauer & G. A. Lamberti (Eds.), *Methods in Stream Ecology.* (2nd edn., pp. 537–559). Elsevier Academic Press.

Lategan, M. J., & Hose, G. C. (2014). Development of a groundwater fungal strain as a tool for toxicity assessment. *Environmental Toxicology and Chemistry*, *33*(12), 2826–2834. https://doi.org/10.1002/etc.2748

Lategan, M. J., Klare, W., Kidd, S., Hose, G. C., & Nevalainen, H. (2016). The unicellular fungal tool RhoTox for risk assessments in groundwater systems. *Ecotoxicology and Environmental Safety*, *132*(February), 18–25. https://doi.org/10.1016/j.ecoenv.2016.05.019

Lawrence, J. R., Swerhone, G. D. W., Topp, E., Korber, D. R., Neu, T. R., & Wassenaar, L. I. (2007). Structural and functional responses of river biofilm communities to the nonsteroidal anti-inflammatory diclofenac. *Environmental Toxicology and Chemistry*, *26*(4), 573–582. https://doi.org/10.1897/06-340R.1

Le Moal, M., Gascuel-Odoux, C., Ménesguen, A., Souchon, Y., Étrillard, C., Levain, A., Moatar, F., Pannard, A., Souchu, P., Lefebvre, A., & Pinay, G. (2019). Eutrophication: A new wine in an old bottle? *Science of the Total Environment*, *651*(February), 1–11. https://doi.org/10.1016/j.scitotenv.2018.09.139

Lewis, M. A. (1991). Chronic and sublethal toxicities of surfactants to aquatic animals: A review and risk assessment. *Water Research*, *25*(1), 101–113. https://doi.org/10.1016/0043-1354(91)90105-Y

Liber, K., Kaushik, N. K., Solomon, K. R., & Carey, J. H. (1992). Experimental Designs for Aquatic Mesocosm Studies: A Comparison of the Anova and Regression Design for Assessing the Impact of Tetrachlorophenol on Zooplankton Populations in Limnocorrals. *Environmental Toxicology and Chemistry*, *11*(1), 61–77. https://doi.org/10.1002/etc.5620110107

Liess, M., & Von Der Ohe, P. C. (2005). Analyzing effects of pesticides on invertebrate communities in streams. *Environmental Toxicology and Chemistry / SETAC*, *24*(4), 954–965.

Liess, M., Von Der Ohe, P. C., Schriever, C. A., Schäfer, R. B., & Beketov, M. A. (2018). *Online database of species at risk (SPEAR database)*. Http://Www.Systemecology.Eu/Spear/.

Likens, G. E. (2010). *Plankton of inland waters*. Academic Press.

Lin, R., Buijse, L., Dimitrov, M. R., Dohmen, P., Kosol, S., Maltby, L., Roessink, I., Sinkeldam, J. A., Smidt, H., Van Wijngaarden, R. P. A., & Brock, T. C. M. (2012). Effects of the fungicide metiram in outdoor freshwater microcosms: Responses of invertebrates, primary producers and microbes. *Ecotoxicology*, *21*(5), 1550–1569. https://doi.org/10.1007/s10646-012-0909-0

Liu, D., Thomson, K., & Strachan, W. M. J. (1981). Biodegradation of carbaryl in simulated aquatic environment. *Bulletin of Environmental Contamination and Toxicology*, *27*(1), 412–417. https://doi.org/10.1007/BF01611041

Loden, M. S. (1974). Predation by chironomid (Diptera) larvae on oligochaetes. *Limnology and Oceanography*, *19*(1), 156–159. https://doi.org/10.4319/lo.1974.19.1.0156

Lorenz, S., Rasmussen, J. J., Süß, A., Kalettka, T., Golla, B., Horney, P., Stähler, M., Hommel, B., & Schäfer, R. B. (2017). Specifics and challenges of assessing exposure and effects of pesticides in small water bodies. *Hydrobiologia*, *793*(1), 213–224. https://doi.org/10.1007/s10750-016-2973-6

Lv, T., Zhang, Y., Carvalho, P. N., Zhang, L., Button, M., Arias, C. A., Weber, K. P., & Brix, H. (2017). Microbial community metabolic function in constructed wetland mesocosms treating the pesticides imazalil and tebuconazole. *Ecological Engineering*, *98*, 378–387. https://doi.org/10.1016/j.ecoleng.2016.07.004

Lv, T., Zhang, Y., Zhang, L., Carvalho, P. N., Arias, C. A., & Brix, H. (2016). Removal of the pesticides imazalil and tebuconazole in saturated constructed wetland mesocosms. *Water Research*, *91*, 126–136. https://doi.org/10.1016/j.watres.2016.01.007

Lytle, D. A., & Poff, N. L. R. (2004). Adaptation to natural flow regimes. *Trends in Ecology and Evolution*, *19*(2), 94–100. https://doi.org/10.1016/j.tree.2003.10.002

Lyu, T., Zhang, L., Xu, X., Arias, C. A., Brix, H., & Carvalho, P. N. (2018). Removal of the pesticide tebuconazole in constructed wetlands: Design comparison, influencing factors and modelling. *Environmental Pollution*, *233*, 71–80. https://doi.org/10.1016/j.envpol.2017.10.040

Maltby, L. (1992). Detritus processing. In P. Calow & G. E. Petts (Eds.), *The Rivers Handbook-Hydrological and ecological principles* (Vol. I, pp. 331–353). Blackwell Scientific Publications.

Maltby, L., Blake, N., Brock, T. C. M., & Van den Brink, P. J. (2005). Insecticide species sensitivity distributions: importance of test species selection and relevance to aquatic ecosystems. *Environmental Toxicology and Chemistry*, *24*(2), 379–388. https://doi.org/10.1897/04-025R.1

Maltby, L., Brock, T. C. M., & Van Den Brink, P. J. (2009). Fungicide risk assessment for aquatic ecosystems: Importance of interspecific variation, toxic mode of action, and exposure regime. *Environmental Science and Technology*, *43*(19), 7556–7563. https://doi.org/10.1021/es901461c

Maly, M., & Ruber, E. (1983). Effects of pesticides on pure and mixed species cultures of salt marsh pool algae. *Bulletin of Environmental Contamination and Toxicology*, *30*(1), 464–472. https://doi.org/10.1007/BF01610161

Manerkar, M. A., Seena, S., & Bärlocher, F. (2008). Q-RT-PCR for assessing archaea, bacteria, and fungi during leaf decomposition in a stream. *Microbial Ecology*, *56*(3), 467–473. https://doi.org/10.1007/s00248-008-9365-z

Marano, A. V., Pires-Zottarelli, C. L. A., Barrera, M. D., Steciow, M. M., & Gleason, F. H. (2011). Diversity, role in decomposition, and succession of zoosporic fungi and straminipiles on submerged decaying leaves in a woodland stream. *Hydrobiologia*, *659*(1), 93–109. https://doi.org/10.1007/s10750-009-0006-4

Mason, H. S. (1948). The Chemistry of Melanin 3. Mechanism of the oxidation of Dihydrophenylanlanine by Tyrosinase. *Journal of Biological Chemistry*, *172*(1), 83–100.

McLachlan, A. J. (1977). Some Effects of Tube Shape on the Feeding of Chironomus plumosus L. (Diptera: Chironomidae). *Journal of Animal Ecology*, *46*(1), 139–146. https://doi.org/10.1002/9780470057339.vab033

Mead, J. A. R., Smith, J. N., & Williams, R. T. (1955). The biosynthesis of the glucoronides of umbelliferone and 4-methylumbellifreone and their use in fluorimetric determination of ß-glucoronidase. *Biochemical Journal*, 569–574.

Merritt, R. W., & Cummins, K. W. (Eds.). (1996). *An Introduction to the Aquatic Insects of North America* (3rd et.). Kendall/Hunt.

Merritt, R. W., & Cummins, K. W. (2007). Trophic Relationships of Macroinvertebrates. In F. R. Hauer & G. A. Lamberti (Eds.), *Methods in Stream Ecology.* (2nd edn., pp. 585–609). Elsevier Academic Press.

Millennium Ecosystem Assessment. (2005). *Ecosystem and human Well-Being: Biodiversity synthesis.* Island Press.

Mohr, S., Berghahn, R., Feibicke, M., Meinecke, S., Ottenströer, T., Schmiedling, I., Schmiediche, R., & Schmidt, R. (2007). Effects of the herbicide metazachlor on macrophytes and ecosystem function in freshwater ponds and stream mesocosms. *Aquatic Toxicology*, *82*(2), 73–84. https://doi.org/10.1016/j.aquatox.2007.02.001

Mohr, Silvia, Berghahn, R., Schmiediche, R., Hübner, V., Loth, S., Feibicke, M., Mailahn, W., & Wogram, J. (2012). Macroinvertebrate community response to repeated short-term pulses of the insecticide imidacloprid. *Aquatic Toxicology*, *110–111*, 25–36. https://doi.org/10.1016/j.aquatox.2011.11.016

Mohr, Silvia, Feibicke, M., Ottenströer, T., Meinecke, S., Berghahn, R., & Schmidt, R. (2005). Enhanced experimental flexibility and control in ecotoxicological mesocosm experiments - A new outdoor and indoor pond and stream system. *Environmental Science and Pollution Research*, *12*(1), 5–7. https://doi.org/10.1065/espr2004.08.217

Mora-Gómez, J., Freixa, A., Perujo, N., & Barral-Fraga, L. (2016). Limits of the Biofilm Concept and Types of Aquatic Biofilms. In A. Romani, H. Guasch, & M. D. Baqlaguer (Eds.), *Aquatic Biofilms* (1st ed., p. 200). Caister Academic Press.

Muñoz-Leoz, B., Ruiz-Romera, E., Antigüedad, I., & Garbisu, C. (2011). Tebuconazole application decreases soil microbial biomass and activity. *Soil Biology and Biochemistry*, *43*(10), 2176–2183. https://doi.org/10.1016/j.soilbio.2011.07.001

Nabi, Z., Youssouf, M., & Manzoor, J. (2019). Impact of Pesticides on Aquatic Life. In *handbook of Research on the Adverse Effects of Pesticide Pollution in Aquatic Ecosystems* (pp. 170–181). IGI Global.

Nagai, T. (2018). A novel, efficient, and ecologically relevant bioassay method using aquatic fungi and fungus-like organisms for fungicide ecological effect assessment. *Environmental Toxicology and Chemistry*, *37*(7), 1980–1989. https://doi.org/10.1002/etc.4138

Naser, M. N., & Roy, D. (2012). Feeding Ecology of Chironomus Larvae (Insecta: Diptera) collected from different habitat of Dhaka, Bangladesh. *Bangladesh Journal Zoology*, *51*(4), 484–493.

Nienhuis, P. H. (1993). Nutrient cycling and foodwebs in Dutch estuaries. *Hydrobiologia*, *265*(1–3), 15–44. https://doi.org/10.1007/BF00007261

Nienstedt, K. M., Brock, T. C. M., van Wensem, J., Montforts, M., Hart, A., Aagaard, A., Alix, A., Boesten, J., Bopp, S. K., Brown, C., Capri, E., Forbes, V., Köpp, H., Liess, M., Luttik, R., Maltby, L., Sousa, J. P., Streissl, F., & Hardy, A. R. (2012). Development of a framework based on an ecosystem services approach for deriving specific protection goals for environmental risk assessment of pesticides. *Science of the Total Environment*, *415*, 31–38. https://doi.org/10.1016/j.scitotenv.2011.05.057

Nordberg, M., Templeton, D. M., Andersen, O., & Duffus, J. H. (2009). Glossary of terms used in ecotoxicology (IUPAC Recommendations 2009). *Pure and Applied Chemistry*, *81*(5), 829–970. https://doi.org/10.1351/PAC-REC-08-07-09

OECD. (2006). *Guidance Document on Simulated Freshwater Lentic Field Tests (outdoor microcosms and mesocosms). Series on Testing and Assessment,ENV/JM/MONO(2006)17* (Vol. 53, Issue ENV/JM/MONO(2006)17, p. 37pp.). OECD Environmental Directorate. 37 pp.

OECD. (2012). *Test No. 211: Daphnia magna Reproduction Test*. https://doi.org/https://doi.org/10.1787/9789264185203-en

OECD. (2019a). *Guidance Document on Aquatic Toxicity Testing of Difficult Substance Mixtures*. https://doi.org/https://doi.org/10.1787/0ed2f88e-en

OECD. (2019b). *Test No. 203: Fish, Acute Toxocity Test*. https://doi.org/https://doi.org/10.1787/9789264069961-en

Papadopoulou, E. S., Karas, P. A., Nikolaki, S., Storck, V., Ferrari, F., Trevisan, M., Tsiamis, G., Martin-Laurent, F., & Karpouzas, D. G. (2016). Dissipation and adsorption of isoproturon, tebuconazole, chlorpyrifos and their main transformation products under laboratory and field conditions. *Science of the Total Environment*, *569–570*, 86–96. https://doi.org/10.1016/j.scitotenv.2016.06.133

Pascoal, C., & Cássio, F. (2004). Contribution of Fungi and Bacteria to Leaf Litter Decomposition in a Polluted River. *Applied and Environmental Microbiology*, *70*(9), 5266–5273. https://doi.org/10.1128/AEM.70.9.5266

Pascoal, C., Cássio, F., & Marvanová, L. (2005). Anthropogenic stress may affect aquatic hyphomycete diversity more than leaf decomposition in a low-order stream. *Archiv Fur Hydrobiologie*, *162*(4), 481–496. https://doi.org/10.1127/0003-9136/2005/0162-0481

Pereira Maduenho, L., & Martinez, C. B. R. (2008). Acute effects of diflubenzuron on the freshwater fish Prochilodus lineatus. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *148*(3), 265–272. https://doi.org/10.1016/j.cbpc.2008.06.010

Pesce, S., Zoghlami, O., Margoum, C., Artigas, J., Chaumot, A., & Foulquier, A. (2016). Combined effects of drought and the fungicide tebuconazole on aquatic leaf litter decomposition. *Aquatic Toxicology*, *173*, 120–131. https://doi.org/10.1016/j.aquatox.2016.01.012

Pestana, J. L. T., Alexander, A. C., Culp, J. M., Baird, D. J., Cessna, A. J., & Soares, A. M. V. M. (2009). Structural and functional responses of benthic invertebrates to imidacloprid in outdoor stream mesocosms. *Environmental Pollution*, *157*(8–9), 2328–2334. https://doi.org/10.1016/j.envpol.2009.03.027

Petersen, R. C., & Cummins, K. W. (1974). Leaf processing in a woodland stream. *Freshwater Biology*, *4*, 343–368.

Petersen, R. C., Cummins, K. W., & Ward, G. M. (1989). Microbial and animal processing of detritus in a woodland stream. *Ecological Monographs*, *59*, 21–39.

Peterson, J. L. (2001). *The Use of Native Macroinvertebrates to Assess Pesticide Risk to Oregon Streams.Dissertation*. Oregon State University.

Peterson, J. L., Jepson, P. C., & Jenkins, J. J. (2001). A test system to evaluate the susceptibility of Oregon, USA, native stream invertebrates to triclopyr and carbaryl. *Environmental Toxicology and Chemistry / SETAC*, *20*(10), 2205–2214. https://doi.org/10.1897/1551-5028

Pimentão, A. R., Pascoal, C., Castro, B. B., & Cássio, F. (2019). Fungistatic effect of agrochemical and pharmaceutical fungicides on non-target aquatic decomposers does not translate into decreased fungi- or invertebrate-mediated decomposition. *Science of the Total Environment*, *xxxx*. https://doi.org/10.1016/j.scitotenv.2019.135676

Pinder, L. C. V. (1992). Biology of epiphytic Chironomidae (Diptera:Nematocera) in chalk streams. *Hydrobiologia*, *248*(1), 39–51. https://doi.org/10.1007/BF00008884

Pohlon, E., Marxsen, J., & Küsel, K. (2010). Pioneering bacterial and algal communities and potential extracellular enzyme activities of stream biofilms. *FEMS Microbiology Ecology*, *71*(3), 364–373. https://doi.org/10.1111/j.1574-6941.2009.00817.x

Pozo, J., Gonzáles, E., Díez, J., Molinero, J., & Elosegi, A. (1997). Inputs of particulate organic matter to streams with different riparian vegetation. *Journal of the North American Benthological Society*, *16*, 602–611.

PPDB (Pesticide properties database). (2018). *Carbaryl (REF: UC 7744)*. https://sitem.herts.ac.uk/aeru/ppdb/en/Reports/115.htm

Pradhan, A., Seena, S., Pascoal, C., & Cássio, F. (2012). Copper oxide nanoparticles can induce toxicity to the freshwater shredder Allogamus ligonifer. *Chemosphere*, *89*(9), 1142–1150. https://doi.org/10.1016/j.chemosphere.2012.06.001

Proia, L., Lupini, G., Osorio, V., Pérez, S., Barceló, D., Schwartz, T., Amalfitano, S., Fazi, S., Romaní, A. M., & Sabater, S. (2013). Response of biofilm bacterial communities to antibiotic pollutants in a Mediterranean river. *Chemosphere*, *92*(9), 1126–1135. https://doi.org/10.1016/j.chemosphere.2013.01.063

Puglisi, E. (2012). Response of microbial organisms (aquatic and terrestrial) to pesticides. *EFSA Supporting Publications*, EN-359. https://doi.org/10.2903/sp.efsa.2012.en-359

Rabiet, M., Margoum, C., Gouy, V., Carluer, N., & Coquery, M. (2010). Assessing pesticide concentrations and fluxes in the stream of a small vineyard catchment - Effect of sampling frequency. *Environmental Pollution*, *158*(3), 737–748. https://doi.org/10.1016/j.envpol.2009.10.014

Raby, M., Maloney, E., Poirier, D. G., & Sibley, P. K. (2019). Acute Effects of Binary Mixtures of Imidacloprid and Tebuconazole on 4 Freshwater Invertebrates. *Environmental Toxicology and Chemistry*, *38*(5), 1093–1103. https://doi.org/10.1002/etc.4386

Rasmussen, J. J., Monberg, R. J., Baattrup-Pedersen, A., Cedergreen, N., Wiberg-Larsen, P., Strobel, B., & Kronvang, B. (2012). Effects of a triazole fungicide and a pyrethroid insecticide on the decomposition of leaves in the presence or absence of macroinvertebrate shredders. *Aquatic Toxicology*, *118–119*, 54–61. https://doi.org/10.1016/j.aquatox.2012.03.015

Rawer-Jost, C., Böhmer, J., Blank, J., & Rahmann, H. (2000). Macroinvertebrate functional feeding group methods in ecological assessment. *Hydrobiologia*, *422–423*, 225–232. https://doi.org/10.1007/978-94-011-4164-2_18

Richter, S., Völker, J., Borchardt, D., & Mohaupt, V. (2013). The Water Framework Directive as an approach for Integrated Water Resources Management: Results from the experiences in Germany on implementation, and future perspectives. *Environmental Earth Sciences*, *69*(2), 719–728. https://doi.org/10.1007/s12665-013-2399-7

Rico, A., Brock, T. C. M., & Daam, M. (2019). Is the Effect Assessment Approach for Fungicides as Laid Down in the European Food Safety Authority Aquatic Guidance Document Sufficiently Protective for Freshwater Ecosystems? *Environmental Toxicology and Chemistry*, *38*(10), 2279–2293. https://doi.org/10.1002/etc.4520

Rico, A., & Van den Brink, P. J. (2015). Evaluating aquatic invertebrate vulnerability to insecticides based on intrinsic sensitivity, biological traits, and toxic mode of action. *Environmental Toxicology and Chemistry*, *34*(8), 1907–1917. https://doi.org/10.1002/etc.3008

Rinke, K., Keller, P. S., Kong, X., Borchardt, D., & Weitere, M. (2019). Ecosystem Services from Inland Waters and Their Aquatic Ecosystems. In M. Schröter, A. Bonn, S. Klotz, & C. Baessler (Eds.), *Atlas of Ecosystem Services* (pp. 191–195). Springer, Cham. https://doi.org/https://doi.org/10.1007/978-3-319-96229-0_30

Rolauffs, P., Stubauer, I., Zahrádkova, S., Brabec, K., & Moog, O. (2004). Integration of the saprobic system into the European Union Water Framework Directive: Case Studies in Austria, Germany and Czech Republic. *Hydrobiologia*, *516*, 285–298.

Romaní, A. M., Fischer, H., Mille-Lindblom, C., & Tranvik, L. J. (2006). Interactions of bacteria and fungi on decomposing litter: Differential extracellular enzyme activities. *Ecology*, *87*(10), 2559–2569. https://doi.org/10.1890/0012-9658(2006)87[2559:IOBAFO]2.0.CO;2

Romani, A. M., Guasch, H., & Balaguer, M. D. (2016). *Aquatic Biofilms-Ecology, Water Quality and Wastewater Treatment* (1.). Caister Academic Press.

Rosenberg, D. M., & Resh, V. H. (1993). Introduction. In D. M. Rosenverg & V. H. Resh (Eds.), *Freshwater Biomonitoring and Benthic Macroinvertebrates* (pp. 1–9). Chapman and Hall.

Rubach, M. N., Baird, D. J., & Van Den Brink, P. J. (2010). A new method for ranking mode-specific sensitivity of freshwater arthropods to insecticides and its relationship to biological traits. *Environmental Toxicology and Chemistry*, *29*(2), 476–487. https://doi.org/10.1002/etc.55

Sánchez-Bayo, F., & Wyckhuys, K. A. G. (2019). Worldwide decline of the entomofauna: A review of its drivers. *Biological Conservation*, *232*(September 2018), 8–27. https://doi.org/10.1016/j.biocon.2019.01.020

Sanders, H. O. (1969). *Toxicity of Pesticides to the Crustacean Gammarus lacustris*.

Schäfer, R. B., Liess, M., & Sch, R. B. (2013). *Species at Risk ( SPEAR ) biomonitoring indicators*. *January*. https://doi.org/10.1007/978-94-007-5704-2

Schäfer, R. B., Von Der Ohe, P. C., Rasmussen, J., Kefford, B. J., Beketov, M. A., Schulz, R., & Liess, M. (2012). Thresholds for the effects of pesticides on invertebrate communities and leaf breakdown in stream ecosystems. *Environmental Science and Technology*, *46*(9), 5134–5142. https://doi.org/10.1021/es2039882

Schäfers, C. (2012). *Ecological approaches to aquatic ecotoxicology challenged by the needs of risk assessment*. Habilitation, University Koblenz-Landau.

Schulz, R., & Liess, M. (1999). A field study of the effects of agriculturally derived insecticide input on stream macroinvertebrate dynamics. *Aquatic Toxicology*, *46*(3–4), 155–176. https://doi.org/10.1016/S0166-445X(99)00002-8

Schwoerbel, J., & Brendelberger, H. (2013). *Einführung in die Limnologie* (10th ed.). Elsevier GmbH, Spektrum Akademischer Verlag.

Secretariat of the Convention on Biological Diversity. (2005). *Handbook of the Convention on Biological Diversity Including its Cartagena Protocol on Biosafety* (3rd ed.). https://doi.org/10.4324/9781315071770

Sephton, T. W. (1987). Some observations on the food of larvae of Procladius bellus (Diptera: Chironomidae). *Aquatic Insects*, *9*(4), 195–202. https://doi.org/10.1080/01650428709361296

SERA (Syracuse Environmental Research Associates). (2008). Carbaryl- Human Health and Ecological Risk Assessment Revised Final Report SERA Internal TR 52-01-05a. In *USDA/Forest Service, Southern Region* (Issue 315).

Sharma, A., Kumar, V., Shahzad, B., Tanveer, M., Sidhu, G. P. S., Handa, N., Kohli, S. K., Yadav, P., Bali, A. S., Parihar, R. D., Dar, O. I., Singh, K., Jasrotia, S., Bakshi, P., Ramakrishnan, M., Kumar, S., Bhardwaj, R., & Thukral, A. K. (2019). Worldwide pesticide usage and its impacts on ecosystem. *SN Applied Sciences*, *1*(11). https://doi.org/10.1007/s42452-019-1485-1

Sharom, M. S., Miles, J. R. W., Harris, C. R., & McEwen, F. L. (1980). Persistence of 12 insecticides in water. *Water Research*, *14*(8), 1089–1093. https://doi.org/10.1016/0043-1354(80)90157-8

Simon, M., Grossart, H. P., Schweitzer, B., & Ploug, H. (2002). Microbial ecology of organic aggregates in aquatic ecosystems. *Aquatic Microbial Ecology*, *28*(2), 175–211. https://doi.org/10.3354/ame028175

Sinsabaugh, R. L., Antibus, R. K., & Linkins, A. E. (1991). An enzymic approach to the analysis of microbial activity during plant litter decomposition. *Agriculture, Ecosystems and Environment"*, *34*(1), 43–54. http://www.sciencedirect.com/science/article/pii/016788099190092C

Sinsabaugh, R. L., & Linkins, A. E. (1990). Enzymic and chemical analysis of particulate organic matter from a boreal river. *Freshwater Biology*, *23*, 301–309. https://doi.org/10.1111/j.1365-2427.1990.tb00273.x

Šporka, F., Vlek, H. E., Bulánková, E., & Krno, I. (2006). Influence of seasonal variation on bioassessment of streams using macroinvertebrates. *Hydrobiologia*, *566*(1), 543–555. https://doi.org/10.1007/s10750-006-0073-8

Stang, C., Wieczorek, M. V., Noss, C., Lorke, A., Scherr, F., Goerlitz, G., & Schulz, R. (2014). Role of submerged vegetation in the retention processes of three plant protection products in flow-through stream mesocosms. *Chemosphere*, *107*, 13–22. https://doi.org/10.1016/j.chemosphere.2014.02.055

Stanley, J. G., & Trial, J. G. (1980). Disappearance Constants of Carbaryl from Streams Contaminated by Forest Spraying. *Bulletin of Environmental Contamination and Toxicology*, *25*, 771–776.

Steinman, A. D., Lamberti, G. A., & Leavit, P. R. (2007). Biomass and Pigments of Benthic Algae. In F. R. Hauer & G. A. Lamberti (Eds.), *Methods in Stream Ecology*. (2nd edn., pp. 357–379). Elsevier Academic Press.

Stoler, A. B., Walker, B. M., Hintz, W. D., Jones, D. K., Lind, L., Mattes, B. M., Schuler, M. S., & Relyea, R. A. (2016). Combined effects of road salt and an insecticide on wetland communities. *Environmental Toxicology and Chemistry*, *9999*(9999), 1–9. https://doi.org/10.1002/etc.3639

Svensson, B. (1977). Life cycle, energy fluctuations and sexual differentiation in Ephemera danica (Ephemeroptera), a stream-living mayfly. *Nordic Society Oikos*, *29*(1), 78–86.

Tada, M., & Shiraishi, H. (1994). Changes in abundance of benthic macroinvertebrates in a pesticide-contaminated river. *Japan Journal of Limnology*, *55*, 159–164.

Ter Braak, C. J. F., & Smilauer, P. (2002). *Canoco Reference manual and CanoDraw for Windows User´s guide: Software for Canonical Community Ordination (version 4.5).* (p. 500 pp.). Microcomputer Power. 500 pp.

Titmus, G., & Badcock, R. M. (1981). Distribution and feeding of larval Chironomidae in a gravel-pit lake. *Freshwater Biology*, *11*(3), 263–271. https://doi.org/10.1111/j.1365-2427.1981.tb01259.x

Tlili, A., Bérard, A., Roulier, J. L., Volat, B., & Montuelle, B. (2010). PO43- dependence of the tolerance of autotrophic and heterotrophic biofilm communities to copper and diuron. *Aquatic Toxicology*, *98*(2), 165–177. https://doi.org/10.1016/j.aquatox.2010.02.008

Tokeshi, M. (1985). Life-Cycle and Production of the Burrowing Mayfly , Ephemera danica : A New Method for Estimating Degree-Days Required for Growth. *Journal of Animal Ecology*, *54*(3), 919–930.

Van Den Brink, P. J., Blake, N., Brock, T. C. M., & Maltby, L. (2006). Predictive value of species sensitivity distributions for effects of herbicides in freshwater ecosystems. *Human and Ecological Risk Assessment*, *12*(4), 645–674. https://doi.org/10.1080/10807030500430559

Van Den Brink, P. J., Hattink, J., Bransen, F., Van Donk, E., & Brock, T. C. M. (2000). Impact of the fungicide carbendazim in freshwater microcosms. II. Zooplankton, primary producers and final conclusions. *Aquatic Toxicology*, *48*(2–3), 251–264. https://doi.org/10.1016/S0166-445X(99)00037-5

Van den Brink, P. J., & Ter Braak, C. J. F. (1999). Principal Response Curves : Analysis of Time-Dependent Multivariate Responses of Biological Community To Stress. *Environmental Toxicology and Chemistry*, *18*(2), 138–148. https://doi.org/10.1002/etc.5620180207

Van Den Brink, P. J., & Ter Braak, C. J. F. (1998). Multivariate analysis of stress in experimental ecosystems by principal responses curves and similarity analysis. *Aquatic Ecology*, *32*, 163–178. https://doi.org/10.1023/a:1009944004756

Van den Brink, P. J., Van Donk, E., Gylstra, R., Crum, S. J. H., & Brock, T. C. M. (1995). Effects of chronic low concentrations of the pesticides Chlorpyrifos and Atrazine in indoor freshwater microcosms. *Chemosphere*, *31*(5), 3181–3200.

Van Handel, E. (1985). Rapid determination of total lipids in mosquitoes. *Journal of the American Mosquito Control Association*, *1*(3), 302–304.

Van Wijngaarden, R. P. A., Belgers, D. J. M., Zafar, M. I., Matser, A. M., Boerwinkel, M. C., & Arts, G. H. P. (2014). Chronic aquatic effect assessment for the fungicide azoxystrobin. *Environmental Toxicology and Chemistry*, *33*(12), 2775–2785. https://doi.org/10.1002/etc.2739

Van Wijngaarden, René P.A., Vand den Brink, P. J., Crum, S. J. H., Oude Voshaar, J. H., Brock, T. C. M., & Leeuwangh, P. (1996). Effects of the insecticide Dursban 4E (active ingredient chlorpyrifos) in outdoor experimental ditches. 1. Comparison of short-term toxicity between the laboratory and the field. *Environmental Toxicology and Chemistry*, *15*(7), 1133–1142. https://doi.org/10.1897/1551-5028(1996)015<1133:eotida>2.3.co;2

Vannote, R. L., Minshall, G. W., Cummins, K. W., Sedell, J. R., & Cushing, C. E. (1980). River Continuum Concept. *Canadian Journal of Fisheries and Aquatic Sciences*, *44*(1–2), 112–129.

Verdonschot, P. F. M., & Nijboer, R. C. (2004). Testing the European stream typology of the Water Framework Directive for macroinvertebrates. *Hydrobiologia*, *516*(1–3), 35–54. https://doi.org/10.1023/B:HYDR.0000025257.30311.b7

Von der Ohe, P. C., & Liess, M. (2004). Relative Sensitivity Distribution of aquatic invertebrates to organic and metal compounds. *Environmental Toxicology and Chemistry*, *23*(1), 150–156.

Walker, P. D., Wijnhoven, S., & van der Velde, G. (2013). Macrophyte presence and growth form influence macroinvertebrate community structure. *Aquatic Botany*, *104*, 80–87. https://doi.org/10.1016/j.aquabot.2012.09.003

Wallace, J. B., Eggert, S. L., Meyer, J. L., & Webster, J. R. (1999). Effects of resource limitation on a detrital-based ecosystem. *Ecological Monographs*, *69*(4), 409–442.

Wallace, J. B., & Webster, J. R. (1996). The Role of Macroinvertebrates in Stream Ecosystem Function. *Annual Review of Entomology*, *41*(1), 115–139. https://doi.org/10.1146/annurev.ento.41.1.115

Wang, N., Besser, J. M., Buckler, D. R., Honegger, J. L., Ingersoll, C. G., Johnson, B. T., Kurtzweil, M. L., MacGregor, J., & McKee, M. J. (2005). Influence of sediment on the fate and toxicity of a polyethoxylated tallowamine surfactant system (MON 0818) in aquatic microcosms. *Chemosphere*, *59*(4), 545–551. https://doi.org/10.1016/j.chemosphere.2004.12.009

Webster, J. R., & Benfield, E. F. (1986). Vascular plant breakdown in freshwater ecosystems. *Annual Review of Ecology and Systematics*, *17*, 567–594.

Wetzel, R. G. (2001). *Limnology: Lake and River Ecosystems* (3 rd Editi). gulf professional publishing.

Weyers, H. S., & Suberkropp, K. (1996). Fungal and Bacterial Production during the Breakdown of Yellow Poplar Leaves in 2 Streams. *Journal of the North American Benthological Society*, *15*(4), 408–420. https://doi.org/10.2307/1467795

Whitehead, P. G., Wilby, R. L., Battarbee, R. W., Kernan, M., & Wade, A. J. (2009). A review of the potential impacts of climate change on surface water quality. *Hydrological Sciences Journal*, *54*(1), 101–123. https://doi.org/10.1623/hysj.54.1.101

Wieczorek, M. V., Bakanov, N., Bilancia, D., Szöcs, E., Stehle, S., Bundschuh, M., & Schulz, R. (2018). Structural and functional effects of a short-term pyrethroid pulse exposure on invertebrates in outdoor stream mesocosms. *Science of the Total Environment*, *610–611*, 810–819. https://doi.org/10.1016/j.scitotenv.2017.08.048

Wieczorek, M. V., Bakanov, N., Lagadic, L., Bruns, E., & Schulz, R. (2016). Response and recovery of the macrophytes Elodea canadensis and Myriophyllum spicatum following a pulse exposure to the herbicide iofensulfuron-sodium in outdoor stream mesocosms. *Environmental Toxicology and Chemistry*, *36*(4), 1–11. https://doi.org/10.1002/etc.3636

Wieczorek, M. V., Bakanov, N., Stang, C., Bilancia, D., Lagadic, L., Bruns, E., & Schulz, R. (2016). Reference scenarios for exposure to plant protection products and invertebrate communities in stream mesocosms. *Science of the Total Environment*, *545–546*, 308–319. https://doi.org/10.1016/j.scitotenv.2015.12.048

Williams, A. D. D., & Hynes, H. B. N. (1976). The Recolonization Mechanisms of Stream Benthos. *OIKOS*, *27*(2), 265–272.

Wogram, J. (2010). Ecological Characterization of small streams in northern and central Germany. In T. C. M. Brock, A. Alix, C. Brownet, B. F. F. Göttesbüren, F. Heimbach, C. M. Lythgo, R. Schulz, & M. Streloke (Eds.), *Linking aquatic exposure and effects-Risk assessment of pesticides* (pp. 250–268). CRC Press, Societay of Environmental Toxicology and Chemistry (SETAC) Press.

Wolfe, N. L., Zepp, R. G., & Paris, D. F. (1978). Carbaryl, propham and chlorpropham: A comparison of the rates of hydrolysis and photolysis with the rate of biolysis. *Water Research*, *12*(8), 565–571. https://doi.org/10.1016/0043-1354(78)90134-3

Wurtsbaugh, W. A., Paerl, H. W., & Dodds, W. K. (2019). Nutrients, eutrophication and harmful algal blooms along the freshwater to marine continuum. *Wiley Interdisciplinary Reviews: Water*, *6*(5), 1–27. https://doi.org/10.1002/wat2.1373

Wurzbacher, C., Kerr, J. L., & Grossart, H.-P. (2011). Aquatic fungi. In *The Dynamical Process of Biodiversity-Case Studies of Evolution and Spatial Distribution* (pp. 125–149). InTech.

WWF. (2016). Living Planet Report 2016: Risk and resilience in a new era. In *World wide fund for nature*. https://doi.org/978-2-940529-40-7

Zafar, M. I., Belgers, J. D. M., Van Wijngaarden, R. P. a, Matser, A., & Van Den Brink, P. J. (2012). Ecological impacts of time-variable exposure regimes to the fungicide azoxystrobin on freshwater communities in outdoor microcosms. *Ecotoxicology*, *21*(4), 1024–1038. https://doi.org/10.1007/s10646-012-0856-9

Zhang, W. (2018). Global pesticide use: Profile, trend, cost / benefit and more. *Proceedings of the International Academy of Ecology and Environmental Sciences*, *8*(1), 1–27. www.iaees.org

Zhao, Y., Tang, B. H., Cai, L. M., Zhao, Y. Y., Wu, Y. H., & Wang, J. (2012). The acute toxicity of tebuconazole to Chironomus kiiensis larval and its responses of antioxidant enzyme system. *Agrochemicals*, *51*(6), 445–448.

Zubrod, J. P., Baudy, P., Schulz, R., & Bundschuh, M. (2014). Effects of current-use fungicides and their mixtures on the feeding and survival of the key shredder Gammarus fossarum. *Aquatic Toxicology*, *150*, 133–143. https://doi.org/10.1016/j.aquatox.2014.03.002

Zubrod, J. P., Bundschuh, M., Arts, G., Brühl, C. A., Imfeld, G., Knäbel, A., Payraudeau, S., Rasmussen, J. J., Rohr, J., Scharmüller, A., Smalling, K., Stehle, S., Schulz, R., & Schäfer, R. B. (2019). Fungicides: An Overlooked Pesticide Class? *Environmental Science and Technology*, *53*(7), 3347–3365. https://doi.org/10.1021/acs.est.8b04392

Zubrod, J. P., Bundschuh, M., Feckler, A., Englert, D., & Schulz, R. (2011). Ecotoxicological impact of the fungicide tebuconazole on an aquatic decomposer-detritivore system. *Environmental Toxicology and Chemistry*, *30*(12), 2718–2724. https://doi.org/10.1002/etc.679

Zubrod, J. P., Bundschuh, M., & Schulz, R. (2010). Effects of subchronic fungicide exposure on the energy processing of Gammarus fossarum (Crustacea; Amphipoda). *Ecotoxicology and Environmental Safety*, *73*(7), 1674–1680. https://doi.org/10.1016/j.ecoenv.2010.07.046

Zubrod, J. P., Englert, D., Feckler, A., Koksharova, N., Konschak, M., Bundschuh, R., Schnetzer, N., Englert, K., Schulz, R., & Bundschuh, M. (2015). Does the Current Fungicide Risk Assessment Provide Sufficient Protection for Key Frivers in Aquatic Ecosystem Functioning? *Environmental Science and Technology*, *49*(2), 1173–1181. https://doi.org/10.1021/es5050453

Zubrod, J. P., Englert, D., Wolfram, J., Rosenfeldt, R. R., Feckler, A., Bundschuh, R., Seitz, F., Konschak, M., Baudy, P., Lüderwald, S., Fink, P., Lorke, A., Schulz, R., & Bundschuh, M. (2017). Long-term effects of fungicides on leaf-associated microorganisms and shredder populations—an artificial stream study. *Environmental Toxicology and Chemistry*, *36*(8), 2178–2189. https://doi.org/10.1002/etc.3756

# 7 Appendix

## Attachment A

**A. 1: Recovery of carbaryl in stream mesocosms**

| | | Carbaryl concentration | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1.2 | | 3.6 | | 12 | | 36 | | 120 | |
| Time after application [h] | Con-trol | µg a.i./L | Rec. [%] | µg a.i./L | Rec. [%] | µg a.i./L | Rec. [%] | µg a.i./L | Rec. [%] | µg a.i./L | Rec. [%] |
| **1** | n.d | 1.15 | 96 | 3.19 | 89 | 9.65 | 80 | 32.46 | 90 | 105.14 | 88 |
| **2** | n.d | 1.52 | 126 | 3.10 | 86 | 9.13 | 76 | 31.01 | 86 | 97.80 | 82 |
| **24** | n.d | 0.82 | 69 | 2.32 | 65 | 6.29 | 52 | 21.79 | 61 | 70.16 | 58 |
| **48** | n.d | 0.51 | 42 | 1.79 | 50 | 3.53 | 29 | 12.64 | 35 | 40.72 | 34 |
| **120** | n.d | 0.19 | 16 | 0.45 | 13 | 0.75 | 6 | 2.39 | 7 | 8.14 | 7 |
| **168** | n.d | 0.18 | 15 | 0.22 | 6 | 0.31 | 3 | 0.68 | 2 | 2.36 | 2 |

LOQ was set to be 0.15 µg a. i./L; n.d.: not detected, Rec.: Recovery.

**A. 2: Recovery of tebuconazole in stream mesocosms**

| | | Tebuconazole concentration | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 119 | | | | 238 | | | | 476 | | | | 954 | | | |
| | | F 1 | | F 10 | | F 6 | | F 11 | | F 3 | | F 8 | | F 4 | | F 7 | |
| Time after application [days] | Con-trol | µg a.i./L | Rec. [%] | µg a.i./L | Rec. [%] | µg a.i./L | Rec. [%] | µg a.i./L | Rec. [%] | µg a.i./L | Rec. [%] | µg a.i./L | Rec. [%] | µg a.i./L | Rec. [%] | µg a.i./L | Rec. [%] |
| **0.125** | n.d | 106 | 89 | 115 | 96 | 228 | 96 | 216 | 91 | 452 | 95 | 522 | 95 | 869 | 91 | 880 | 92 |
| **1** | n.d | 102 | 86 | 107 | 90 | 209 | 88 | 209 | 88 | 418 | 88 | 495 | 88 | 842 | 88 | 858 | 90 |
| **7** | n.d | 77.0 | 65 | 83.7 | 70 | 172 | 72 | 166 | 70 | 342 | 72 | 418 | 72 | 750 | 79 | 714 | 75 |
| **14** | n.d | 61.3 | 52 | 66.5 | 56 | 143 | 60 | 135 | 57 | 279 | 59 | 334 | 59 | 608 | 64 | 616 | 65 |
| **21** | n.d | 51.8 | 44 | 56.0 | 47 | 126 | 53 | 119 | 50 | 239 | 50 | 295 | 50 | 557 | 59 | 547 | 57 |
| **37** | n.d | 37.9 | 32 | 40.5 | 34 | 97.1 | 41 | 90.7 | 38 | 189 | 40 | 225 | 40 | 452 | 47 | 435 | 46 |
| **59** | n.d | 28.4 | 24 | 28.7 | 24 | 72.9 | 31 | 69.0 | 29 | 146 | 31 | 169 | 31 | 348 | 37 | 350 | 37 |

LOQ was set to be 5.00 µg a. i./L; n.d.: not detected, Rec.: Recovery.

# Attachment B

**A. 3: List of macroinvertebrate taxa in case study I with dominance [%], generation time in years, voltinisms and classification as Species at Risk for Pesticides,** a: Liess M, Von Der Ohe PC, Schriever CA, Schäfer RB, Beketov MA (2018) "Online database of species at risk (SPEAR database)." In: http://www.systemecology.eu/spear/.

| Phylum | Class | Subclass | Order | Family | Taxon | Dominance [%] | Generation time (years) | Voltinism | $SPEAR_{Pesticide}$ from trait database [a] |
|---|---|---|---|---|---|---|---|---|---|
| Annelida | Clitellata | Hirudinea | Arhynchobdellida | Erpobdellidae | *Erpobdella octoculata* | 2.671 | 1.00 | univoltine | 0 |
| | | | Hirudinida | Haemopidae | *Haemopis sanguisuga* | 0.010 | 1.00 | univoltine | 0 |
| | | | Rhynchobdellida | Glossiphoniidae | *Glossiphonia complanata* | 1.103 | 1.00 | univoltine | 0 |
| | | | | Glossiphoniidae | *Glossiphonia heteroclita* | 0.234 | 0.50 | multivoltine | 0 |
| | | | | Glossiphoniidae | *Helobdella stagnalis* | 9.485 | 0.50 | multivoltine | 0 |
| | | Oligochaeta | Crassiclitellata | Lumbricidae | *Eiseniella tetraedra* | 1.278 | 0.33 | multivoltine | 0 |
| | | | | Lumbricidae | *Lumbriculus variegatus* | 0.154 | 0.33 | multivoltine | 0 |
| | | | Haplotaxida | Naididae | Naididae Gen. sp. | 0.383 | 0.33 | multivoltine | 0 |
| | | | | Naididae | *Stylaria lacustris* | 0.063 | 0.50 | multivoltine | 0 |
| | | | | Tubificidae | Tubificidae Gen. sp. | 0.957 | 0.30 | multivoltine | 0 |
| | | | | | Oligochaeta Gen. sp. | 0.174 | 0.33 | multivoltine | 0 |
| Arthropoda | Arachnida | Acari | | | Acari Gen. sp. | 0.056 | 0.50 | multivoltine | 0 |
| | Crustacea | Malacostrata | Isopoda | Asellidae | *Asellus aquaticus* | 32.555 | 0.33 | multivoltine | 0 |
| | | | Amphipoda | Gammaridae | *Gammarus* sp. | 27.700 | 0.75 | uni/bivoltine | 0 |
| | Insecta | Pterygota | Coleoptera | | Coleoptera Gen. sp. | 0.026 | 1.07 | univoltine | 0 |
| | | | Diptera | Athericidae | *Atherix ibis* | 0.148 | 1.00 | univoltine | 1 |
| | | | | Chironomidae | Chironomidae Gen. sp. | 4.544 | 0.33 | multivoltine | 0 |
| | | | | Simuliidae | *Simulium sp.* | 0.000 | 0.25 | multivoltine | 0 |
| | | | | Chironomidae | Tanypodinae | 0.018 | 0.33 | multivoltine | 0 |
| | | | | Tipulidae | *Tipula sp.* | 0.009 | 0.50 | multivoltine | 0 |
| | | | Ephemeroptera | Baetidae | Baetidae Gen. sp. | 0.000 | 0.60 | uni/bivoltine | 1 |

| Phylum | Class | Subclass | Order | Family | Taxon | Dominance [%] | Generation time (years) | Voltinism | SPEAR$_{Pesticide}$ from trait database [a] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Baetidae | *Baetis sp.* | 0.715 | 0.65 | uni/bivoltine | 1 |
| | | | | Baetidae | *Cloeon dipterum* | 0.079 | 0.30 | multivoltine | 0 |
| | | | | Ephemeridae | *Ephemera danica* | 2.328 | 2.00 | semivoltine | 0 |
| | | | | Ephemerellidae | *Ephemerella ignita* | 3.329 | 1.00 | univoltine | 0 |
| | | | | n.b. | Ephemeroptera Gen. sp. | 0.089 | 0.82 | uni/bivoltine | 1 |
| | | | | Leptophlebiidae | *Habrophlebia fusca* | 0.401 | 1.00 | univoltine | 0 |
| | | | | Heptageniidae | Heptageniidae Gen. sp. | 0.096 | 0.66 | uni/bivoltine | 1 |
| | | | Megaloptera | Sialidae | *Sialis lutaria* | 1.897 | 1.00 | univoltine | 1 |
| | | | Odonata | Aeschnidae | Aeshnidae Gen. sp. | 0.017 | 2.00 | semivoltine | 0 |
| | | | | n.b. | Anisoptera Gen. sp. | 0.010 | 1.72 | semivoltine | 0 |
| | | | | Calopterygidae | *Calopteryx splendens* | 0.012 | 2.00 | semivoltine | 0 |
| | | | | Coenagrionidae | Coenagrionidae Gen. sp. | 0.154 | 1.00 | univoltine | 1 |
| | | | | n.b. | Zygoptera Gen. sp. | 0.089 | 1.05 | univoltine | 0 |
| | | | Plecoptera | n.b. | Plecoptera Gen. sp. | 0.349 | 1.00 | univoltine | 1 |
| | | | Trichoptera | Lepidostomatidae | *Lepidostoma hirtum* | 0.013 | 1.00 | univoltine | 1 |
| | | | | Leptoceridae | Leptoceridae Gen. sp. | 0.013 | 1.00 | univoltine | 0 |
| | | | | Leptophlebidae | Leptophlebiidae Gen. sp. | 0.000 | 1.00 | univoltine | 0 |
| | | | | Limnephilidae | Limnephilidae Gen. sp. | 3.893 | 1.00 | univoltine | 1 |
| | | | | Phryganeidae | Phryganeidae Gen. sp. | 0.013 | 1.00 | univoltine | 1 |
| | | | | Polycentropodidae | *Polycentropus flavomaculatus* | 1.579 | 1.00 | univoltine | 1 |
| Cnidaria | Hydrozoa | Hydroidolina | Hydroidaea | Hydridae | *Hydra* sp. | 0.000 | n.d. | n.d. | 0 |
| Mollusca | Gastropoda | Heterobranchia | Hygrophila | Planorbidae | *Ancylus fluviatilis* | 0.000 | 1.00 | univoltine | 0 |
| | | | | Lymnaeidae | Lymnaeidae Gen. sp. | 0.047 | 0.94 | univoltine | 0 |
| | | | | Lymnaeidae | *Lymnea stagnalis* | 0.041 | 1.00 | univoltine | 0 |

| Phylum | Class | Subclass | Order | Family | Taxon | Dominance [%] | Generation time (years) | Voltinism | SPEAR$_{Pesticide}$ from trait database [a] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Planorbidae | *Planorbis planorbis* | 0.324 | 0.50 | multivoltine | 0 |
| | | | | Lymnaeidae | *Radix ovata* | 0.237 | 0.50 | multivoltine | 0 |
| | | | | Lymnaeidae | *Radix* sp. | 0.055 | 1.00 | univoltine | 0 |
| | Bivalvia | Heterodonta | Voneroida | Spaeriidae | *Musculium lacustre* | 0.011 | 1.00 | univoltine | 0 |
| | | | | Sphaeriidae | *Pisidium* sp. | 0.354 | 1.00 | univoltine | 0 |
| Plathel-minthes | Turbellaria | | Tricladida | Dugesiidae | *Dugesia* sp. | 2.279 | 1.00 | univoltine | 0 |

# Attachment C

**A. 4: List of macroinvertebrate taxa in case study II.**

| Phylum | Class | Subclass | Order | Family | Taxon |
|---|---|---|---|---|---|
| Annelida | Clitellata | Hirudinea | Arhynchobdellida | Erpobdellidae | *Erpobdella octoculata* |
| | | | Rhynchobdellida | Glossiphoniidae | *Glossiphonia complanata* |
| | | Oligochaeta | Crassiclitellata | Lumbricidae | *Eiseniella tetraedra* |
| | | | Haplotaxida | Naididae | Naididae Gen. sp. |
| | | | | Tubificidae | Tubificidae Gen. sp. |
| Arthropoda | Arachnida | Acari | | | Acari Gen. sp. |
| | Crustacea | Malacostrata | Isopoda | Asellidae | *Asellus aquaticus* |
| | | | Amphipoda | Gammaridae | *Gammarus pulex* |
| | Insecta | Pterygota | Coleoptera | | Coleoptera Gen. sp. |
| | | | Diptera | Chaoboridae | *Chaoborus* sp. |
| | | | | Chironomidae | Chironomidae Gen. sp. |
| | | | | Culicidae | *Culex* sp. |
| | | | | Ceratopogidae | *Bezzia* sp. |
| | | | Ephemeroptera | Baetidae | *Cloeon dipterum* |
| | | | | Ephemeridae | *Ephemera danica* |
| | | | Megaloptera | Sialidae | *Sialis lutaria* |
| | | | Odonata | n.b. | Anisoptera Gen. sp. |
| | | | | n.b. | Zygoptera Gen. sp. |
| | | | Plecoptera | n.b. | Plecoptera Gen. sp. |
| | | | Trichoptera | Leptoceridae | Leptoceridae Gen. sp. |
| | | | | Limnephilidae | Limnephilidae Gen. sp. |
| | | | | Polycentropodidae | *Polycentropus flavomaculatus* |
| Mollusca | Gastropoda | Heterobranchia | Hygrophila | Lymnaeidae | *Lymnea stagnalis* |
| | | | Hygrophila | Planorbidae | *Planorbis planorbis* |
| | | | | Lymnaeidae | *Radix* sp. |
| Plathel-minthes | Turbellaria | | Tricladida | Dugesiidae | *Dugesia* sp. |

# Attachment D

**A. 5: Daily weather data (min/max temperature, precipitation, sunshine duration) before and during case study I** (DWD (German Weather Station), 2019)

| Date | air temperature min/max [°C] | | precipitation [l/m²] | sunshine duration [h] |
|---|---|---|---|---|
| 01.02.2015 | -3.3 | 0.3 | 0.6 | 1.1 |
| 02.02.2015 | -2.8 | 0.7 | 0.1 | 0.9 |
| 03.02.2015 | -3.8 | 0.4 | 0.1 | 1.4 |
| 04.02.2015 | -5.4 | 0.4 | 0.1 | 2.7 |
| 05.02.2015 | -5.1 | -2.0 | 0.1 | 1.2 |
| 06.02.2015 | -6.5 | -1.4 | 0 | 6.7 |
| 07.02.2015 | -8.7 | 1.9 | 0.2 | 9.0 |
| 08.02.2015 | -2.3 | 2.5 | 0.1 | 5.8 |
| 09.02.2015 | -0.6 | 3.9 | 0.1 | 0 |
| 10.02.2015 | 1.9 | 4.2 | 0.1 | 0 |
| 11.02.2015 | -1.5 | 2.7 | 0 | 0 |
| 12.02.2015 | -2.0 | -1.0 | 0.1 | 0 |
| 13.02.2015 | -2.1 | 2.8 | 0.1 | 3.4 |
| 14.02.2015 | -3.1 | 6.2 | 0 | 6.6 |
| 15.02.2015 | -3.5 | 4.9 | 0 | 9.1 |
| 16.02.2015 | -3.4 | 2.3 | 0 | 2.7 |
| 17.02.2015 | -0.7 | 2.0 | 0 | 0.1 |
| 18.02.2015 | -0.7 | 3.7 | 0 | 0 |
| 19.02.2015 | -2.2 | -0.7 | 0 | 0 |
| 20.02.2015 | -1.2 | 7.9 | 1.6 | 1.9 |
| 21.02.2015 | 0.2 | 7.0 | 0.9 | 0 |
| 22.02.2015 | -0.8 | 5.1 | 0.3 | 5.5 |
| 23.02.2015 | -0.3 | 2.0 | 2.8 | 0 |
| 24.02.2015 | 0.5 | 4.3 | 0.2 | 3.6 |
| 25.02.2015 | -0.1 | 6.1 | 0.5 | 3.5 |
| 26.02.2015 | 1.4 | 6.1 | 0.1 | 1.2 |
| 27.02.2015 | 0.1 | 4.0 | 6.8 | 0 |
| 28.02.2015 | -2.0 | 5.2 | 1.8 | 5.6 |
| 01.03.2015 | 0.5 | 8.6 | 1.7 | 0.5 |
| 02.03.2015 | 0.2 | 6.8 | 0.6 | 4.8 |
| 03.03.2015 | -0.9 | 7.3 | 0.3 | 5.8 |
| 04.03.2015 | 0 | 4.6 | 0.1 | 1.6 |
| 05.03.2015 | 0.1 | 5.5 | 0 | 3.1 |
| 06.03.2015 | -0.8 | 5.4 | 0 | 0.7 |
| 07.03.2015 | 0.1 | 8.4 | 0 | 4.7 |
| 08.03.2015 | 1.8 | 12.9 | 0 | 7.3 |

| Date | air temperature min/max [°C] | | precipitation [l/m²] | sunshine duration [h] |
|---|---|---|---|---|
| 09.03.2015 | 1.3 | 13.5 | 0 | 5.6 |
| 10.03.2015 | 0.4 | 7.8 | 0.4 | 0.2 |
| 11.03.2015 | 0.2 | 8.4 | 0 | 9.0 |
| 12.03.2015 | -1.5 | 9.4 | 0 | 10.6 |
| 13.03.2015 | -0.1 | 4.5 | 0.1 | 0.3 |
| 14.03.2015 | -1.1 | 3.4 | 1.3 | 0 |
| 15.03.2015 | 1.2 | 4.6 | 0.1 | 0 |
| 16.03.2015 | 0.2 | 11.5 | 0 | 9.2 |
| 17.03.2015 | 2.6 | 14.9 | 0 | 10.2 |
| 18.03.2015 | 2.9 | 15.9 | 0 | 9.0 |
| 19.03.2015 | 3.2 | 12.7 | 0 | 6.5 |
| 20.03.2015 | 1.0 | 11.9 | 0.1 | 10.5 |
| 21.03.2015 | 0 | 5.8 | 0.7 | 0.1 |
| 22.03.2015 | -2.4 | 2.5 | 0 | 0.8 |
| 23.03.2015 | -3.6 | 9.1 | 0 | 5.7 |
| 24.03.2015 | 0.3 | 9.3 | 0 | 0.6 |
| 25.03.2015 | 2.5 | 16.4 | 0.1 | 8.5 |
| 26.03.2015 | 3.0 | 8.1 | 0.7 | 0.5 |
| 27.03.2015 | 3.0 | 6.6 | 0.1 | 0.6 |
| 28.03.2015 | 2.1 | 11.2 | 1.1 | 6.2 |
| 29.03.2015 | 5.8 | 9.8 | 18.7 | 0 |
| 30.03.2015 | 2.8 | 7.8 | 5.3 | 7.1 |
| 31.03.2015 | 1.0 | 10.7 | 4.7 | 4.2 |
| 01.04.2015 | -0.2 | 5.3 | 4.3 | 2.8 |
| 02.04.2015 | 0.4 | 5.6 | 0.4 | 4.3 |
| 03.04.2015 | -0.7 | 9.0 | 0 | 7.6 |
| 04.04.2015 | 0.5 | 6.5 | 0.1 | 5.7 |
| 05.04.2015 | -1.1 | 8.2 | 0 | 9.0 |
| 06.04.2015 | 0 | 8.5 | 0 | 4.1 |
| 07.04.2015 | -1.1 | 11.5 | 0 | 9.0 |
| 08.04.2015 | 1.0 | 13.5 | 0 | 8.0 |
| 09.04.2015 | 3.3 | 17.4 | 0 | 12.1 |
| 10.04.2015 | 6.4 | 19.7 | 0 | 12.0 |
| 11.04.2015 | 6.3 | 14.6 | 0.4 | 2.7 |
| 12.04.2015 | 3.3 | 15.5 | 0.1 | 11.6 |
| 13.04.2015 | 4.6 | 12.9 | 0.1 | 7.7 |

| Date | air temperature min/max [°C] | | precipitation [l/m²] | sunshine duration [h] |
|---|---|---|---|---|
| 14.04.2015 | 2.1 | 19.5 | 0 | 11.3 |
| 15.04.2015 | 9.5 | 22.8 | 0 | 12.8 |
| 16.04.2015 | 7.4 | 19.4 | 0.1 | 9.4 |
| 17.04.2015 | 3.0 | 11.4 | 1.5 | 9.0 |
| 18.04.2015 | 1.6 | 11.4 | 0 | 11.7 |
| 19.04.2015 | 0.7 | 15.0 | 0 | 13.2 |
| 20.04.2015 | 3.3 | 17.2 | 0 | 13.2 |
| 21.04.2015 | 5.7 | 21.5 | 0 | 13.7 |
| 22.04.2015 | 2.1 | 10.7 | 0 | 6.6 |
| 23.04.2015 | 1.6 | 18.0 | 0 | 13.1 |
| 24.04.2015 | 5.7 | 19.1 | 0 | 12.7 |
| 25.04.2015 | 9.6 | 14.3 | 2.2 | 0.2 |
| 26.04.2015 | 9.6 | 17.4 | 6.5 | 4.6 |
| 27.04.2015 | 5.2 | 11.4 | 10.0 | 0 |
| 28.04.2015 | 2.5 | 11.6 | 0 | 9.5 |
| 29.04.2015 | 0.8 | 13.3 | 0 | 9.9 |
| 30.04.2015 | 4.1 | 11.8 | 3.5 | 2.4 |
| 01.05.2015 | 0.6 | 11.9 | 0 | 3.5 |
| 02.05.2015 | 2.9 | 14.3 | 0.1 | 9.2 |
| 03.05.2015 | 7.7 | 14.7 | 4.8 | 0 |
| 04.05.2015 | 11.2 | 19.5 | 0.5 | 5.9 |
| 05.05.2015 | 9.8 | 22.7 | 0.2 | 4.3 |
| 06.05.2015 | 7.3 | 16.8 | 0.2 | 9.5 |
| 07.05.2015 | 6.3 | 16.1 | 0 | 8.6 |
| 08.05.2015 | 6.2 | 20.4 | 0.1 | 7.0 |
| 09.05.2015 | 9.5 | 19.6 | 2.2 | 3.5 |
| 10.05.2015 | 8.2 | 18.1 | 0 | 11.8 |
| 11.05.2015 | 7.2 | 22.4 | 0 | 9.8 |
| 12.05.2015 | 9.1 | 26.4 | 0.1 | 8.4 |
| 13.05.2015 | 6.2 | 19.3 | 0 | 12.9 |
| 14.05.2015 | 5.4 | 16.0 | 0.1 | 10.5 |
| 15.05.2015 | 7.8 | 19.1 | 0 | 13.1 |
| 16.05.2015 | 4.3 | 16.1 | 0.1 | 5.2 |
| 17.05.2015 | 4.4 | 16.0 | 0 | 9.0 |
| 18.05.2015 | 6.8 | 20.3 | 0.1 | 12.5 |
| 19.05.2015 | 8.2 | 15.5 | 0.4 | 8.6 |
| 20.05.2015 | 4.7 | 14.3 | 0.1 | 4.3 |
| 21.05.2015 | 2.2 | 15.4 | 0 | 8.4 |
| 22.05.2015 | 5.8 | 18.9 | 0 | 10.1 |

| Date | air temperature min/max [°C] | | precipitation [l/m²] | sunshine duration [h] |
|---|---|---|---|---|
| 23.05.2015 | 10.9 | 17.7 | 0.1 | 0.1 |
| 24.05.2015 | 9.4 | 19.2 | 0.9 | 1.7 |
| 25.05.2015 | 7.8 | 16.7 | 0.1 | 2.6 |
| 26.05.2015 | 6.0 | 13.5 | 0.1 | 1.1 |
| 27.05.2015 | 4.8 | 13.0 | 0 | 0.3 |
| 28.05.2015 | 6.9 | 17.9 | 0.1 | 3.5 |
| 29.05.2015 | 7.6 | 15.9 | 3.2 | 6.8 |
| 30.05.2015 | 6.2 | 14.5 | 0.6 | 5.0 |
| 31.05.2015 | 4.5 | 19.2 | 2.5 | 5.6 |
| 01.06.2015 | 8.7 | 14.4 | 0.6 | 3.6 |
| 02.06.2015 | 8.3 | 22.8 | 0.1 | 4.0 |
| 03.06.2015 | 11.1 | 20.1 | 0 | 6.1 |
| 04.06.2015 | 10.0 | 22.9 | 0.1 | 14.8 |
| 05.06.2015 | 11.0 | 29.9 | 2.2 | 15.3 |
| 06.06.2015 | 13.7 | 23.9 | 0 | 7.6 |
| 07.06.2015 | 10.7 | 22.6 | 0 | 12.9 |
| 08.06.2015 | 9.2 | 16.4 | 0 | 4.1 |
| 09.06.2015 | 7.8 | 15.5 | 0 | 5.0 |
| 10.06.2015 | 9.0 | 21.0 | 0 | 8.6 |
| 11.06.2015 | 12.1 | 21.9 | 0 | 5.3 |
| 12.06.2015 | 10.2 | 28.4 | 0.7 | 12.4 |
| 13.06.2015 | 14.0 | 22.9 | 0.1 | 4.0 |
| 14.06.2015 | 11.4 | 25.0 | 0 | 15.2 |
| 15.06.2015 | 9.1 | 18.5 | 0 | 5.2 |
| 16.06.2015 | 6.3 | 17.3 | 0 | 7.5 |
| 17.06.2015 | 6.1 | 19.9 | 3.0 | 10.4 |
| 18.06.2015 | 10.7 | 20.4 | 2.5 | 3.4 |
| 19.06.2015 | 9.1 | 12.9 | 2.2 | 0.3 |
| 20.06.2015 | 8.3 | 15.0 | 0.1 | 1.0 |
| 21.06.2015 | 9.5 | 16.6 | 1.3 | 0.8 |
| 22.06.2015 | 8.9 | 13.1 | 18.9 | 0.1 |
| 23.06.2015 | 7.8 | 15.5 | 0.4 | 2.9 |
| 24.06.2015 | 9.5 | 16.0 | 0 | 2.4 |
| 25.06.2015 | 8.5 | 22.4 | 0 | 7.2 |
| 26.06.2015 | 14.0 | 25.3 | 1.3 | 4.4 |
| 27.06.2015 | 11.8 | 23.6 | 1.5 | 3.7 |
| 28.06.2015 | 10.2 | 22.0 | 0.1 | 10.5 |
| 29.06.2015 | 14.3 | 26.8 | 0 | 10.2 |
| 30.06.2015 | 12.7 | 26.8 | 0 | 15.6 |

| Date | air temperature min/max [°C] | | precipitation [l/m²] | sunshine duration [h] |
|---|---|---|---|---|
| 01.07.2015 | 14.6 | 30.8 | 0 | 15.6 |
| 02.07.2015 | 17.6 | 33.2 | 0.1 | 15.1 |
| 03.07.2015 | 19.4 | 33.4 | 1.4 | 11.7 |
| 04.07.2015 | 21.1 | 35.3 | 0 | 13.7 |
| 05.07.2015 | 16.7 | 35.8 | 6.0 | 10.5 |
| 06.07.2015 | 14.1 | 25.2 | 0 | 14.4 |
| 07.07.2015 | 13.4 | 31.5 | 1.1 | 11.1 |
| 08.07.2015 | 12.4 | 20.0 | 1.1 | 3.7 |
| 09.07.2015 | 8.9 | 18.3 | 0.1 | 7.9 |
| 10.07.2015 | 6.2 | 21.7 | 0 | 15.5 |
| 11.07.2015 | 10.4 | 28.2 | 0 | 11.6 |
| 12.07.2015 | 13.2 | 24.2 | 3.5 | 6.6 |
| 13.07.2015 | 13.3 | 16.2 | 2.5 | 0 |
| 14.07.2015 | 15.3 | 21.0 | 0.1 | 0.2 |
| 15.07.2015 | 15.2 | 25.2 | 0.1 | 4.0 |
| 16.07.2015 | 16.1 | 29.0 | 0.1 | 11.5 |
| 17.07.2015 | 17.3 | 33.8 | 0.1 | 9.3 |
| 18.07.2015 | 17.5 | 29.4 | 19.5 | 9.3 |
| 19.07.2015 | 13.5 | 23.6 | 7.4 | 2.4 |
| 20.07.2015 | 14.9 | 22.5 | 0.1 | 0.7 |
| 21.07.2015 | 18.4 | 28.7 | 0 | 7.7 |
| 22.07.2015 | 17.7 | 29.4 | 0.1 | 8.8 |
| 23.07.2015 | 14.7 | 23.4 | 0 | 7.3 |
| 24.07.2015 | 12.6 | 28.6 | 5.2 | 9.0 |
| 25.07.2015 | 10.8 | 20.6 | 1.1 | 4.2 |
| 26.07.2015 | 8.7 | 19.6 | 4.4 | 8.4 |
| 27.07.2015 | 11.8 | 18.5 | 22.8 | 3.5 |
| 28.07.2015 | 10.9 | 18.9 | 0 | 6.1 |
| 29.07.2015 | 10.7 | 19.1 | 0.1 | 8.8 |
| 30.07.2015 | 8.3 | 17.8 | 1.2 | 6.7 |
| 31.07.2015 | 8.7 | 19.1 | 0 | 14.2 |
| 01.08.2015 | 9.9 | 25.2 | 0 | 9.5 |
| 02.08.2015 | 13.1 | 27.0 | 0 | 12.6 |
| 03.08.2015 | 14.0 | 29.9 | 0 | 13.8 |
| 04.08.2015 | 12.5 | 28.4 | 5.8 | 6.4 |
| 05.08.2015 | 9.4 | 24.9 | 0 | 12.2 |
| 06.08.2015 | 13.7 | 31.7 | 0 | 12.4 |
| 07.08.2015 | 17.3 | 34.6 | 0.2 | 13.8 |
| 08.08.2015 | 17.3 | 28.2 | 1.3 | 8.5 |
| 09.08.2015 | 16.4 | 27.3 | 0.1 | 4.1 |

| Date | air temperature min/max [°C] | | precipitation [l/m²] | sunshine duration [h] |
|---|---|---|---|---|
| 10.08.2015 | 17.4 | 31.1 | 0.1 | 10.2 |
| 11.08.2015 | 15.8 | 26.6 | 0 | 10.1 |
| 12.08.2015 | 17.1 | 27.6 | 0 | 3.5 |
| 13.08.2015 | 16.0 | 31.1 | 0.3 | 12.5 |
| 14.08.2015 | 18.4 | 26.6 | 0.1 | 4.8 |
| 15.08.2015 | 16.1 | 24.2 | 0.3 | 6.2 |
| 16.08.2015 | 14.1 | 16.3 | 52.7 | 0 |
| 17.08.2015 | 12.6 | 14.2 | 5.2 | 0 |
| 18.08.2015 | 12.0 | 17.2 | 0 | 0 |
| 19.08.2015 | 10.5 | 19.5 | 0 | 5.9 |
| 20.08.2015 | 11.4 | 21.6 | 0 | 2.2 |
| 21.08.2015 | 10.7 | 23.7 | 0 | 6.7 |
| 22.08.2015 | 11.7 | 24.4 | 0 | 12.0 |
| 23.08.2015 | 13.5 | 24.9 | 2.7 | 10.1 |
| 24.08.2015 | 13.7 | 22.3 | 4.8 | 2.3 |
| 25.08.2015 | 12.7 | 18.0 | 0.1 | 2.0 |
| 26.08.2015 | 13.4 | 24.3 | 0 | 11.2 |
| 27.08.2015 | 14.2 | 22.8 | 14.4 | 1.0 |
| 28.08.2015 | 13.0 | 19.1 | 0.1 | 0.5 |
| 29.08.2015 | 9.1 | 26.5 | 0 | 10.1 |
| 30.08.2015 | 16.5 | 29.8 | 0 | 10.8 |
| 31.08.2015 | 17.7 | 29.7 | 0.1 | 12.2 |
| 01.09.2015 | 11.1 | 22.2 | 22.1 | 1.1 |
| 02.09.2015 | 9.0 | 18.4 | 0.1 | 4.9 |
| 03.09.2015 | 9.3 | 17.9 | 0 | 7.4 |
| 04.09.2015 | 8.8 | 15.6 | 0.1 | 3.7 |
| 05.09.2015 | 7.9 | 17.3 | 1.2 | 0 |
| 06.09.2015 | 7.2 | 13.9 | 0.2 | 0 |
| 07.09.2015 | 9.5 | 15.3 | 2.2 | 0 |
| 08.09.2015 | 9.9 | 16.6 | 0 | 0 |
| 09.09.2015 | 9.3 | 16.7 | 0 | n. d. |
| 10.09.2015 | 7.3 | 16.6 | 0 | n. d. |
| 11.09.2015 | 7.1 | 18.4 | 0 | n. d. |
| 12.09.2015 | 8.1 | 21.7 | 0.6 | n. d. |
| 13.09.2015 | 12.5 | 18.6 | 1.9 | n. d. |
| 14.09.2015 | 10.3 | 19.0 | 8.7 | n. d. |
| 15.09.2015 | 8.2 | 15.5 | 3.4 | n. d. |
| 16.09.2015 | 9.9 | 16.8 | 12.7 | n. d. |
| 17.09.2015 | 9.8 | 14.6 | 4.2 | n. d. |
| 18.09.2015 | 9.4 | 17.4 | 0.6 | n. d. |

| Date | air temperature min/max [°C] | | precipi-tation [l/m²] | sunshine duration [h] |
|---|---|---|---|---|
| 19.09.2015 | 10.4 | 16.3 | 0.1 | n. d. |
| 20.09.2015 | 8.7 | 15.1 | 0.1 | n. d. |
| 21.09.2015 | 8.4 | 16.8 | 0.1 | n. d. |
| 22.09.2015 | 7.9 | 12.8 | 5.9 | n. d. |
| 23.09.2015 | 7.6 | 13.8 | 1.0 | n. d. |
| 24.09.2015 | 9.3 | 15.4 | 0 | n. d. |
| 25.09.2015 | 9.8 | 16.0 | 0 | n. d. |

**A. 6: Daily weather data (min/max temperature, precipitation, sunshine duration) before and during case study II** (DWD (German Weather Station), 2019)

| Date | air temperature min/max [°C] | | precipitation [l/m²] | sunshine duration [h] |
|---|---|---|---|---|
| 01.02.2017 | -0.6 | 1.7 | 0.3 | 0.3 |
| 02.02.2017 | 0.5 | 6.7 | 0.6 | 1.2 |
| 03.02.2017 | 1.2 | 7.0 | 0.6 | 0 |
| 04.02.2017 | 2.4 | 6.3 | 2.2 | 1.9 |
| 05.02.2017 | 0.1 | 5.8 | 0 | 2.6 |
| 06.02.2017 | -1.1 | 3.4 | 0 | 0 |
| 07.02.2017 | -1.7 | 0.6 | 0 | 0 |
| 08.02.2017 | -4.3 | 0.7 | 0 | 2.4 |
| 09.02.2017 | -4.9 | 0.8 | 0 | 3.9 |
| 10.02.2017 | -3.7 | -1.7 | 0 | 0 |
| 11.02.2017 | -4.2 | 5.0 | 0 | 3.7 |
| 12.02.2017 | -1.6 | 2.8 | 0 | 3.3 |
| 13.02.2017 | -1.4 | 6.5 | 0 | 8.7 |
| 14.02.2017 | -3.0 | 7.9 | 0 | 9.2 |
| 15.02.2017 | -2.5 | 7.8 | 0 | 8.9 |
| 16.02.2017 | 0 | 9.7 | 3.2 | 5.4 |
| 17.02.2017 | 1.4 | 5.7 | 1.2 | 0 |
| 18.02.2017 | 0.9 | 5.7 | 0 | 0 |
| 19.02.2017 | -0.8 | 4.4 | 0.3 | 0.4 |
| 20.02.2017 | 3.7 | 7.8 | 1.6 | 0 |
| 21.02.2017 | 5.8 | 10.3 | 0 | 0.8 |
| 22.02.2017 | 7.7 | 9.0 | 4.2 | 0 |
| 23.02.2017 | 3.9 | 8.1 | 1.0 | 0 |
| 24.02.2017 | -0.6 | 5.7 | 0.4 | 4.3 |
| 25.02.2017 | -1.6 | 6.5 | 0 | 5.3 |
| 26.02.2017 | 2.7 | 10.4 | 0 | 5.0 |
| 27.02.2017 | 5.2 | 11.6 | 4.9 | 1.8 |
| 28.02.2017 | 1.3 | 6.3 | 6.4 | 2.8 |
| 01.03.2017 | 0.6 | 7.6 | 2.9 | 2.2 |
| 02.03.2017 | 3.7 | 7.6 | 0 | 1.4 |
| 03.03.2017 | 0.7 | 9.1 | 0 | 1.7 |
| 04.03.2017 | 4.8 | 14.4 | 1.9 | 2.0 |
| 05.03.2017 | 2.9 | 7.7 | 2.6 | 1.4 |
| 06.03.2017 | 2.6 | 6.3 | 3.8 | 0.3 |
| 07.03.2017 | 2.7 | 7.8 | 1.3 | 2.4 |
| 08.03.2017 | 1.1 | 5.7 | 4.0 | 0 |
| 09.03.2017 | 3.4 | 11.1 | 0.9 | 0.9 |
| 10.03.2017 | 2.1 | 9.1 | 0 | 3.6 |

| Date | air temperature min/max [°C] | | precipitation [l/m²] | sunshine duration [h] |
|---|---|---|---|---|
| 11.03.2017 | 0.1 | 11.1 | 0 | 7.3 |
| 12.03.2017 | -0.5 | 10.7 | 0 | 10.3 |
| 13.03.2017 | -0.9 | 11.3 | 0 | 7.3 |
| 14.03.2017 | 2.4 | 13.1 | 0 | 7.5 |
| 15.03.2017 | 5.9 | 13.2 | 0 | 4.9 |
| 16.03.2017 | 1.4 | 15.1 | 0 | 10.7 |
| 17.03.2017 | 5.1 | 10.2 | 0.1 | 0.3 |
| 18.03.2017 | 3.7 | 10.3 | 7.3 | 0 |
| 19.03.2017 | 6.7 | 13.2 | 0 | 0.1 |
| 20.03.2017 | 7.6 | 12.1 | 0 | 0.1 |
| 21.03.2017 | 4.0 | 10.1 | 2.1 | 0 |
| 10.05.2017 | 1.5 | 16.2 | 0 | 12.9 |
| 11.05.2017 | 4.0 | 19.7 | 0.6 | 7.4 |
| 12.05.2017 | 10.2 | 17.9 | 2.5 | 2.7 |
| 13.05.2017 | 10.2 | 20.7 | 1.3 | 8.3 |
| 14.05.2017 | 10.9 | 19.2 | 3.7 | 3.5 |
| 15.05.2017 | 8.7 | 19.4 | 0 | 8.8 |
| 16.05.2017 | 9.2 | 22.6 | 0 | 8.5 |
| 17.05.2017 | 13.5 | 24.8 | 0 | 12.0 |
| 18.05.2017 | 14.4 | 23.7 | 7.0 | 5.1 |
| 19.05.2017 | 9.2 | 16.3 | 21.0 | 0 |
| 20.05.2017 | 7.8 | 15.4 | 0.5 | 6.5 |
| 21.05.2017 | 5.4 | 19.1 | 0 | 9.6 |
| 22.05.2017 | 9.2 | 21.4 | 0 | 6.2 |
| 23.05.2017 | 10.9 | 22.6 | 0 | 8.4 |
| 24.05.2017 | 9.0 | 17.4 | 0 | 2.4 |
| 25.05.2017 | 9.2 | 20.6 | 0 | 13.1 |
| 26.05.2017 | 10.5 | 23.1 | 0 | 11.4 |
| 27.05.2017 | 11.8 | 25.6 | 0 | 15.2 |
| 28.05.2017 | 14.6 | 27.7 | 0 | 11.1 |
| 29.05.2017 | 16.6 | 28.9 | 0 | 11.2 |
| 30.05.2017 | 13.6 | 26.2 | 13.4 | 5.5 |
| 31.05.2017 | 11.8 | 21.9 | 0 | 11.4 |
| 01.06.2017 | 11.4 | 23.4 | 0 | 12.1 |
| 02.06.2017 | 12.4 | 25.0 | 0.2 | 4.1 |
| 03.06.2017 | 15.1 | 24.6 | 12.3 | 5.3 |
| 04.06.2017 | 10.6 | 19.7 | 0.1 | 6.4 |
| 05.06.2017 | 9.2 | 19.7 | 0 | 8.0 |

| Date | air temperature min/max [°C] | | precipi-tation [l/m²] | sunshine duration [h] |
|---|---|---|---|---|
| 06.06.2017 | 8.5 | 16.5 | 3.7 | 4.3 |
| 07.06.2017 | 7.5 | 15.1 | 0.7 | 5.3 |
| 08.06.2017 | 9.8 | 19.1 | 0.1 | 5.9 |
| 09.06.2017 | 10.6 | 20.2 | 2.9 | 6.7 |
| 10.06.2017 | 9.6 | 21.9 | 0 | 13.2 |
| 11.06.2017 | 13.5 | 25.9 | 0.1 | 11.5 |
| 12.06.2017 | 11.1 | 20.4 | 0 | 12.6 |
| 13.06.2017 | 11.2 | 19.0 | 0 | 1.1 |
| 14.06.2017 | 9.3 | 23.2 | 0 | 14.8 |
| 15.06.2017 | 12.0 | 26.2 | 0.3 | 9.0 |
| 16.06.2017 | 11.3 | 19.3 | 0 | 6.2 |
| 17.06.2017 | 8.6 | 16.1 | 0 | 1.8 |
| 18.06.2017 | 14.1 | 23.9 | 0 | 6.5 |
| 19.06.2017 | 13.3 | 28.5 | 0 | 15.4 |
| 20.06.2017 | 15.0 | 30.2 | 0 | 14.2 |
| 21.06.2017 | 14.6 | 28.3 | 0 | 15.1 |
| 22.06.2017 | 15.6 | 30.8 | 70.8 | 8.8 |
| 23.06.2017 | 14.6 | 24.3 | 0 | 7.7 |
| 24.06.2017 | 12.7 | 22.8 | 0 | 8.4 |
| 25.06.2017 | 13.4 | 21.8 | 0 | 4.7 |
| 26.06.2017 | 13.6 | 21.6 | 0 | 7.5 |
| 27.06.2017 | 12.4 | 20.0 | 0.2 | 0.1 |
| 28.06.2017 | 14.4 | 21.5 | 2.6 | 1.8 |
| 29.06.2017 | 13.2 | 19.1 | 3.6 | 1.3 |
| 20.08.2017 | 10.3 | 16.9 | 0 | 6.1 |
| 21.08.2017 | 10.3 | 19.1 | 0 | 9.4 |
| 22.08.2017 | 9.9 | 21.5 | 0 | 9.2 |
| 23.08.2017 | 9.9 | 23.5 | 0 | 12.5 |
| 24.08.2017 | 14.4 | 20.9 | 0 | 9.0 |
| 25.08.2017 | 12.1 | 24.0 | 18.5 | 9.7 |
| 26.08.2017 | 15.8 | 23.9 | 0 | 5.4 |
| 27.08.2017 | 14.1 | 23.3 | 0 | 8.4 |
| 28.08.2017 | 12.0 | 24.3 | 0 | 12.7 |
| 29.08.2017 | 12.8 | 26.1 | 0 | 12.5 |
| 30.08.2017 | 15.2 | 26.6 | 0 | 8.0 |
| 31.08.2017 | 11.1 | 19.5 | 2.5 | 0.9 |
| 01.09.2017 | 7.6 | 18.2 | 0 | 8.0 |
| 02.09.2017 | 8.3 | 16.3 | 0 | 5.2 |
| 03.09.2017 | 6.1 | 18.1 | 0 | 8.2 |

| Date | air temperature min/max [°C] | | precipi-tation [l/m²] | sunshine duration [h] |
|---|---|---|---|---|
| 04.09.2017 | 5.8 | 19.6 | 0 | 11.1 |
| 05.09.2017 | 12.2 | 22.2 | 0 | 3.9 |
| 06.09.2017 | 11.0 | 18.1 | 0.4 | 1.6 |
| 07.09.2017 | 10.3 | 17.6 | 0 | 3.3 |
| 08.09.2017 | 11.8 | 16.8 | 1.9 | 0.8 |
| 09.09.2017 | 10.0 | 17.3 | 2.4 | 2.5 |
| 10.09.2017 | 9.2 | 15.8 | 0 | 2.4 |
| 11.09.2017 | 9.9 | 16.5 | 3.3 | 3.6 |
| 12.09.2017 | 9.2 | 15.0 | 6.3 | 3.5 |
| 13.09.2017 | 9.5 | 16.7 | 6.0 | 2.8 |
| 14.09.2017 | 8.3 | 13.5 | 17.1 | 3.5 |
| 15.09.2017 | 6.8 | 12.5 | 1.9 | 3.3 |
| 16.09.2017 | 6.6 | 13.7 | 0 | 6.2 |
| 17.09.2017 | 6.1 | 14.2 | 0 | 4.6 |
| 18.09.2017 | 5.0 | 13.3 | 0 | 2.6 |
| 19.09.2017 | 7.4 | 14.8 | 3.5 | 3.3 |
| 20.09.2017 | 6.0 | 13.1 | 0.3 | 1.5 |
| 21.09.2017 | 8.4 | 16.2 | 0 | 5.7 |
| 22.09.2017 | 5.7 | 18.2 | 0 | 7.2 |
| 23.09.2017 | 9.3 | 17.5 | 0 | 3.7 |
| 24.09.2017 | 10.4 | 16.3 | 0 | 0.6 |
| 25.09.2017 | 9.2 | 15.4 | 0 | 1.2 |
| 26.09.2017 | 8.8 | 17.6 | 0 | 4.4 |
| 27.09.2017 | 8.7 | 17.8 | 0.9 | 5.8 |
| 28.09.2017 | 8.7 | 19.2 | 0 | 2.1 |
| 29.09.2017 | 13.0 | 19.5 | 0.2 | 2.9 |
| 30.09.2017 | 10.2 | 20.4 | 3.6 | 3.1 |
| 01.10.2017 | 6.0 | 14.2 | 0.3 | 4.6 |
| 02.10.2017 | 9.2 | 14.0 | 11.4 | 0.1 |
| 03.10.2017 | 8.2 | 14.0 | 0.1 | 3.5 |
| 04.10.2017 | 6.9 | 12.1 | 0.3 | 1.5 |
| 05.10.2017 | 7.6 | 13.2 | 1.4 | 2.2 |
| 06.10.2017 | 7.0 | 10.9 | 0.1 | 2.2 |
| 07.10.2017 | 7.6 | 9.6 | 2.7 | 0.5 |
| 08.10.2017 | 6.5 | 12.5 | 3.9 | 0.7 |
| 09.10.2017 | 3.5 | 10.4 | 1.8 | 2.4 |
| 30.11.2017 | -1.8 | 0.3 | 3.2 | 0.2 |
| 22.03.2017 | 0.7 | 8.5 | 0 | 1.2 |
| 23.03.2017 | 2.7 | 12.4 | 0 | 6.9 |

| Date | air temperature min/max [°C] | | precipitation [l/m²] | sunshine duration [h] |
|---|---|---|---|---|
| 24.03.2017 | 4.3 | 10.7 | 0 | 6.8 |
| 25.03.2017 | 1.3 | 13.8 | 0 | 10.6 |
| 26.03.2017 | 2.1 | 13.1 | 0 | 11.2 |
| 27.03.2017 | 1.5 | 17.1 | 0 | 12.3 |
| 28.03.2017 | 4.2 | 19.3 | 0 | 11.7 |
| 29.03.2017 | 7.4 | 15.3 | 0 | 4.5 |
| 30.03.2017 | 10.2 | 18.8 | 0 | 7.3 |
| 31.03.2017 | 8.5 | 21.1 | 0 | 8.5 |
| 01.04.2017 | 7.3 | 18.2 | 0 | 4.1 |
| 02.04.2017 | 7.7 | 16.4 | 0 | 2.7 |
| 03.04.2017 | 4.5 | 13.2 | 0 | 11.7 |
| 04.04.2017 | 1.2 | 13.7 | 0 | 9.9 |
| 05.04.2017 | 2.2 | 14.2 | 0.1 | 6.7 |
| 06.04.2017 | 3.4 | 11.1 | 0.4 | 6.0 |
| 07.04.2017 | 1.9 | 11.1 | 0 | 0.2 |
| 08.04.2017 | 7.7 | 14.8 | 0 | 3.4 |
| 09.04.2017 | 4.0 | 20.8 | 0 | 12.5 |
| 10.04.2017 | 5.4 | 17.2 | 0 | 7.5 |
| 11.04.2017 | 3.9 | 9.7 | 0 | 2.9 |
| 12.04.2017 | 5.5 | 14.1 | 0 | 0.3 |
| 13.04.2017 | 5.0 | 10.7 | 0 | 1.4 |
| 14.04.2017 | 2.2 | 11.3 | 0 | 7.5 |
| 15.04.2017 | 3.1 | 9.7 | 0.4 | 0 |
| 16.04.2017 | 2.8 | 9.0 | 5.5 | 1.8 |
| 17.04.2017 | -0.1 | 7.7 | 13.3 | 2.0 |
| 18.04.2017 | -1.0 | 6.8 | 1.1 | 2.4 |
| 19.04.2017 | -1.3 | 5.9 | 0 | 5.4 |
| 20.04.2017 | -3.0 | 9.9 | 0 | 12.6 |
| 21.04.2017 | 2.0 | 10.8 | 0 | 3.2 |
| 22.04.2017 | 1.9 | 8.8 | 0.9 | 1.3 |
| 23.04.2017 | 0.5 | 9.5 | 0 | 1.8 |
| 24.04.2017 | -0.3 | 13.8 | 0 | 11.8 |
| 25.04.2017 | 2.6 | 9.0 | 0.1 | 2.5 |
| 26.04.2017 | 0.8 | 10.0 | 0 | 7.9 |
| 27.04.2017 | -0.5 | 10.4 | 0 | 8.2 |
| 28.04.2017 | -1.4 | 10.3 | 0 | 6.6 |
| 29.04.2017 | 2.6 | 11.7 | 0 | 9.9 |
| 30.04.2017 | 1.1 | 15.9 | 0 | 12.0 |
| 01.05.2017 | 5.8 | 11.7 | 9.6 | 0 |
| 02.05.2017 | 2.7 | 8.9 | 22.5 | 0 |

| Date | air temperature min/max [°C] | | precipitation [l/m²] | sunshine duration [h] |
|---|---|---|---|---|
| 03.05.2017 | 1.3 | 12.9 | 1.6 | 0 |
| 04.05.2017 | 6.5 | 10.2 | 0 | 0 |
| 05.05.2017 | 6.2 | 11.2 | 0 | 0 |
| 06.05.2017 | 4.6 | 18.0 | 0 | 8.6 |
| 07.05.2017 | 8.8 | 17.2 | 2.3 | 0.6 |
| 08.05.2017 | 5.2 | 10.4 | 0.2 | 0 |
| 09.05.2017 | 2.4 | 11.3 | 0 | 9.1 |
| 30.06.2017 | 12.4 | 19.6 | 1.8 | 4.7 |
| 01.07.2017 | 12.6 | 16.3 | 5.4 | 0.1 |
| 02.07.2017 | 11.9 | 17.4 | 0.2 | 1.8 |
| 03.07.2017 | 9.7 | 22.3 | 0 | 10.5 |
| 04.07.2017 | 12.4 | 24.0 | 0 | 12.1 |
| 05.07.2017 | 11.9 | 26.5 | 0 | 11.8 |
| 06.07.2017 | 14.5 | 28.6 | 3.3 | 10.9 |
| 07.07.2017 | 14.5 | 29.4 | 0 | 11.8 |
| 08.07.2017 | 15.6 | 27.4 | 0 | 11.8 |
| 09.07.2017 | 15.8 | 27.9 | 5.3 | 10.0 |
| 10.07.2017 | 15.8 | 22.6 | 20.0 | 1.8 |
| 11.07.2017 | 14.7 | 22.6 | 0.7 | 5.6 |
| 12.07.2017 | 9.3 | 17.4 | 12.0 | 0.7 |
| 13.07.2017 | 7.5 | 19.0 | 0 | 9.3 |
| 14.07.2017 | 9.0 | 21.3 | 1.6 | 4.6 |
| 15.07.2017 | 9.1 | 19.5 | 0 | 3.9 |
| 16.07.2017 | 11.9 | 21.5 | 0 | 1.6 |
| 17.07.2017 | 14.1 | 22.4 | 0 | 4.7 |
| 18.07.2017 | 12.3 | 26.6 | 0 | 13.4 |
| 19.07.2017 | 15.9 | 30.6 | 16.7 | 10.6 |
| 20.07.2017 | 15.3 | 22.6 | 3.8 | 2.5 |
| 21.07.2017 | 13.9 | 24.9 | 8.3 | 10.7 |
| 22.07.2017 | 14.8 | 24.4 | 5.6 | 4.4 |
| 23.07.2017 | 13.4 | 21.5 | 1.6 | 7.0 |
| 24.07.2017 | 11.1 | 16.5 | 29.3 | 1.3 |
| 25.07.2017 | 11.3 | 13.6 | 36.3 | 0 |
| 26.07.2017 | 11.9 | 19.9 | 0.7 | 2.7 |
| 27.07.2017 | 13.2 | 20.5 | 7.1 | 1.2 |
| 28.07.2017 | 11.8 | 20.9 | 0 | 7.0 |
| 29.07.2017 | 14.4 | 24.8 | 0.1 | 4.6 |
| 30.07.2017 | 17.4 | 27.3 | 0 | 11.3 |
| 31.07.2017 | 16.0 | 26.2 | 19.6 | 7.9 |
| 01.08.2017 | 16.2 | 23.4 | 1.9 | 3.6 |

| Date | air temperature min/max [°C] | | precipitation [l/m²] | sunshine duration [h] |
|---|---|---|---|---|
| 02.08.2017 | 15.7 | 26.2 | 0 | 6.3 |
| 03.08.2017 | 17.1 | 26.7 | 0 | 5.3 |
| 04.08.2017 | 15.6 | 24.2 | 0 | 6.7 |
| 05.08.2017 | 13.0 | 23.5 | 6.0 | 5.1 |
| 06.08.2017 | 10.7 | 21.0 | 0 | 11.9 |
| 07.08.2017 | 9.2 | 24.1 | 0 | 12.6 |
| 08.08.2017 | 12.0 | 21.3 | 0 | 2.6 |
| 09.08.2017 | 12.0 | 21.9 | 2.0 | 11.6 |
| 10.08.2017 | 11.6 | 15.8 | 14.7 | 0 |
| 11.08.2017 | 11.8 | 13.9 | 48.3 | 0 |
| 12.08.2017 | 10.2 | 14.8 | 3.4 | 0 |
| 13.08.2017 | 12.4 | 21.1 | 0 | 3.9 |
| 14.08.2017 | 10.7 | 24.4 | 0 | 9.4 |
| 15.08.2017 | 14.3 | 25.4 | 2.0 | 7.3 |
| 16.08.2017 | 15.1 | 23.1 | 0.1 | 7.7 |
| 17.08.2017 | 14.7 | 24.8 | 0 | 6.4 |
| 18.08.2017 | 14.3 | 22.1 | 35.5 | 0.7 |
| 19.08.2017 | 10.8 | 18.0 | 0.6 | 7.4 |
| 10.10.2017 | 9.3 | 13.0 | 0.2 | 0 |
| 11.10.2017 | 10.7 | 13.3 | 0 | 0.3 |
| 12.10.2017 | 9.4 | 14.8 | 0 | 5.2 |
| 13.10.2017 | 9.2 | 16.2 | 0 | 0.7 |
| 14.10.2017 | 12.1 | 19.0 | 0 | 7.8 |
| 15.10.2017 | 7.0 | 18.8 | 0 | 10.2 |
| 16.10.2017 | 11.2 | 18.4 | 0 | 5.5 |
| 17.10.2017 | 9.2 | 19.5 | 0 | 9.5 |
| 18.10.2017 | 6.2 | 17.0 | 0 | 7.6 |
| 19.10.2017 | 5.2 | 18.4 | 0 | 8.4 |
| 20.10.2017 | 7.6 | 15.3 | 0.9 | 3.7 |
| 21.10.2017 | 7.6 | 13.4 | 1.4 | 1.1 |
| 22.10.2017 | 7.1 | 9.3 | 2.5 | 0.1 |
| 23.10.2017 | 6.6 | 9.4 | 0.1 | 0.1 |
| 24.10.2017 | 7.3 | 11.9 | 0 | 0 |
| 25.10.2017 | 10.9 | 16.5 | 0 | 3.7 |
| 26.10.2017 | 9.6 | 14.3 | 2.9 | 1.7 |
| 27.10.2017 | 7.8 | 11.5 | 0 | 3.2 |
| 28.10.2017 | 6.6 | 9.3 | 2.2 | 0 |
| 29.10.2017 | 4.5 | 12.1 | 0 | 3.1 |
| 30.10.2017 | 4.1 | 8.3 | 1.1 | 2.8 |

| Date | air temperature min/max [°C] | | precipitation [l/m²] | sunshine duration [h] |
|---|---|---|---|---|
| 31.10.2017 | 3.1 | 8.0 | 1.8 | 0.8 |
| 01.11.2017 | 5.0 | 10.7 | 0 | 2.6 |
| 02.11.2017 | 5.2 | 10.3 | 0 | 4.8 |
| 03.11.2017 | 5.2 | 11.1 | 0 | 3.3 |
| 04.11.2017 | 1.2 | 11.1 | 5.0 | 0.7 |
| 05.11.2017 | 3.4 | 10.4 | 10.1 | 0 |
| 06.11.2017 | 0.6 | 6.5 | 0 | 2.5 |
| 07.11.2017 | 0.5 | 7.0 | 0 | 1.3 |
| 08.11.2017 | 4.8 | 7.3 | 0 | 0 |
| 09.11.2017 | 4.4 | 6.8 | 0.1 | 0 |
| 10.11.2017 | 3.8 | 6.9 | 1.9 | 0 |
| 11.11.2017 | 2.9 | 4.9 | 1.3 | 0 |
| 12.11.2017 | 1.2 | 3.4 | 8.0 | 0 |
| 13.11.2017 | 0.5 | 6.4 | 0.1 | 6.1 |
| 14.11.2017 | -0.9 | 4.8 | 0 | 0.8 |
| 15.11.2017 | 2.8 | 5.6 | 0 | 0 |
| 16.11.2017 | 1.3 | 5.0 | 0 | 0 |
| 17.11.2017 | 1.2 | 4.3 | 0.5 | 0 |
| 18.11.2017 | 1.4 | 3.0 | 4.3 | 0.1 |
| 19.11.2017 | 0.5 | 5.4 | 0 | 0.9 |
| 20.11.2017 | 0.8 | 4.5 | 2.7 | 0 |
| 21.11.2017 | 4.5 | 8.4 | 0.7 | 0 |
| 22.11.2017 | 5.8 | 8.4 | 0 | 3.8 |
| 23.11.2017 | 5.8 | 12.0 | 2.2 | 1.4 |
| 24.11.2017 | 5.7 | 10.6 | 3.7 | 0 |
| 25.11.2017 | 0.5 | 6.3 | 6.4 | 0 |
| 26.11.2017 | 0.4 | 2.6 | 0.2 | 0.3 |
| 27.11.2017 | 0.5 | 3.8 | 13.6 | 0 |
| 28.11.2017 | 0.1 | 4.2 | 0.7 | 2.4 |
| 29.11.2017 | -0.9 | 2.0 | 0.2 | 0 |

# 8 Acknowledgements

First, I would like to thank all friends, colleagues, and family members, who played an important role during this part of my life. You all were and of course still are awesome!

Particularly, I would like to thank…

…Prof. Dr. Klaus Peter Ebke. Many thanks for the opportunity to conduct the doctoral thesis at your facility Mesocosm GmbH in Homberg (Ohm). You always supported me and discussed several difficulties regarding experimental setups. Your supervision was excellent!

…Prof. Dr. Rolf-Alexander Düring and Prof. Dr. Hans-Werner Koyro for taking over the official supervision of the doctoral thesis.

…the IME Fraunhofer Institute in Schmallenberg for the analytical verification of the right dosing of the two model substances used in my thesis. I would particularly like to thank Dr. Udo Hommen for the guidance for the writing of the carbaryl manuscript. Although the opinions were sometime strongly divided, the discussions were as much more fruitful.

…Dr. Jochen P. Zubrod for getting the opportunity to perform experiments at the University of Koblenz-Landau and for supporting the progress within my thesis.

…Prof. Dr. László Dören for supporting me indispensable during the time at Mesocosm and afterwards.

…the former team from Mesocosm GmbH including Rabea, Kathrin, Ute, Heike, Betti, Petra, Siggi, Steffi, Marlene, Marga, Sabine, Thomas, Ilja, Benny, Ralf and Martin. My special thanks belong to Ralf and Martin for their vocational skills and for their assistance for building the experimental setup. I also would like to thank the bachelor and master students, which supported me during my thesis. Many thanks belong to Christian and Nicole.

…my family, especially my mother for giving me the opportunity and the financial support to study biology and giving me the strength and enthusiasm to perform this thesis.

…last but not least to my partner Florian. I am quite sure living with me at this time was sometimes not easy, but you always supported me. THANK YOU!

# 9 Declaration of academic honesty

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and cited all text passages that are derived verbatim from or are based on the content of published work of others, and all information relating to verbal communications. I consent to the use of an anti-plagiarism software to check my thesis. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University Giessen „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ in carrying out the investigations described in the dissertation:

_____ _____

Date Name