EFFECTS OF FUNGICIDES ON DECOMPOSER COMMUNITIES AND LITTER DECOMPOSITION IN STREAMS

by

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Summary

Agriculture covers one third of the world land area and has become a major source of water pollution due to its heavy reliance on chemical inputs, namely fertilisers and pesticides. Several thousands of tonnes of these chemicals are applied worldwide annually and partly reach freshwaters. Despite their widespread use and relatively unspecific modes of action, fungicides are the least studied group of pesticides. It remains unclear whether the taxonomic groups used in pesticide risk assessment are protective for non-target freshwater fungi. Fungi and bacteria are the main microbial decomposers converting allochthonous organic matter (litter) into a more nutritious food resource for leaf-shredding macroinvertebrates. This process of litter decomposition (LD) is central for aquatic ecosystem because it fuels local and downstream food webs with energy and nutrients. Effects of fungicides on decomposer communities and LD have been mainly analysed under laboratory conditions with limited representation of the multiple factors that may moderate effects in the field.

In this thesis a field study was conducted in a German vineyard area to characterise recurrent episodic exposure to fungicides in agricultural streams (chapter 2) and its effects on decomposer communities and LD (chapter 3). Additionally, potential interaction effects of nutrient enrichment and fungicides on decomposer communities and LD were analysed in a mesocosm experiment (chapter 4).

In the field study event-driven water sampling (EDS) and passive sampling with Empore^{TM} styrene-divinylbenzene reverse phase sulfonated disks (SDB disks) were used to assess exposure to 15 fungicides and 4 insecticides. A total of 17 streams were monitored during 4 rainfall events within the local application period of fungicides in 2012. EDS exceeded the time-weighted average concentrations provided by the SDB disks by a factor of 3, though high variability among compounds was observed. Most compounds were detected in more than half of the sites and mean and maximum peak (EDS) concentrations were under 1 and 3 µg/l, respectively. Besides, SDB disk-sampling rates and a free-software solution to derive sampling rates under time-variable exposure were provided.

Several biotic endpoints related to decomposers and LD were measured in the same sampling sites as the fungicide monitoring, coinciding with the major litter input period. Our results suggest that polar organic fungicides in streams change the structure of the fungal community. Causality of this finding was supported by a subsequent microcosm experiment. Whether other effects observed in the field study, such as reduced fungal biomass, increased bacterial density or reduced microbial LD can be attributed to fungicides remains speculative and requires further investigation. By contrast, neither the invertebrate LD nor in-situ measured gammarid feeding rates correlated with water-borne fungicide toxicity, but both were negatively associated with sediment copper concentrations. The mesocosm experiment showed that fungicides and nutrients affect microbial decomposers differently and that they can alter community structure, though longer experiments are needed to determine whether these changes may propagate to invertebrate communities and LD. Overall, further studies should include representative field surveys in terms of fungicide pollution and physical, chemical and biological conditions. This should be combined with experiments under controlled conditions to test for the causality of field observations.

CONTENTS

1. Introduction and objectives

1.1 Freshwater ecosystems and litter decomposition

Despite rivers and streams make up less than 0.01% of global freshwater resources (Shiklomanov, 1993), they hold outstanding socioeconomic and ecological values. Human health and well-being directly depend on freshwater resources, which are irreplaceable to satisfy the global demands of drinking water and irrigation of agricultural land. Besides, they support other socioeconomic values such as waste disposal, navigation, energy production, fishing or recreation (MEA, 2005). From the ecological point of view, lotic freshwaters host at least 100,000 species (almost 6% of the known biodiversity on Earth; Dudgeon et al., 2006) and perform services of central importance, such as water purification, nutrient recycling and organic matter processing.

Protecting stream biodiversity and functionality implies the maintenance of aquatic foodwebs, which are mainly supported by two processes: primary production and the decomposition of allochthonous organic matter (Vannote et al., 1980). This latter process is defined as the catabolism of organic matter into its inorganic constituents (e.g. CO_2, N, P) from leaching of soluble compounds, physical fractionation, microbial conditioning and invertebrate feeding (Tank et al., 2010). Allochthonous organic matter in streams, hereafter litter, refers to organic material with a terrestrial origin and it mainly includes logs, branches and leaves from the riparian vegetation. Litter decomposition in streams fuels local and downstream foodwebs with energy and nutrients (Wallace et al., 1997) and is particularly relevant in reaches with limited primary production (Fisher and Likens, 1973), mainly low-order streams due to light interception by the riparian vegetation. Considering that 50% of the approximately 1.2 million kilometres of the stream network (scale 1:250,000) in Europe are small rivers and streams (Globevnik, 2007) and that dominance of litter decomposition over primary production can extend up to tens of kilometres from headwaters (Webster, 2007), litter inputs are of central importance.

Bacteria and fungi are the main microbial litter decomposers. Fungi are generally considered as dominating this process (Duarte et al., 2010; Fischer et al., 2006; Pascoal and Cássio, 2004), though similar importance of fungi and bacteria has been reported occasionally (Hieber and Gessner, 2002). When entering the streams, litter is already colonised by terrestrial fungi, but their activity is supposed to be severely depressed in the water (Graça and Rodrigues, 1997). Bacteria and aquatic fungi rapidly colonise litter and convert it into a more palatable food resource for macroinvertebrate shredders, which generally lack the enzymatic capability to use the structural components of leaves (Graça and Canhoto, 2006). Besides, microbial activity turn leaf material into (i) fine particulate organic matter, which will be mainly processed by bacteria (Fischer et al., 2006) and invertebrate collectors, and (ii) microbial biomass, which apart from increasing litter palatability is also directly consumed by some shredders (Chung and Suberkropp, 2009; Graça et al., 2000). The structure of fungal and bacterial communities has been suggested to change throughout the decomposition process (Duarte et al., 2010). Whereas the number of fungal species at a given time remains usually under 10-15 species (Dimitrov et al., 2014; Duarte et al., 2010; Jabiol et al., 2013), bacterial diversity can greatly exceed that of fungi (Dimitrov et al., 2014).

Stream invertebrates constitute the other major group of decomposers in freshwater ecosystems. The main invertebrate decomposers are shredders and collectors by feeding on coarse and fine particulate organic matter, respectively. Although invertebrate

decomposer activity can greatly exceed that of microorganisms (Hieber and Gessner, 2002), contrasting results have been reported and differences may originate from different invertebrate densities (Graça, 2001).

1.2 Freshwater threats in agricultural areas

Freshwaters are among the most threatened ecosystems in the world. Human population growth and increasing economic development are the primary drivers of this situation (MEA, 2005). The pressure that human activities exert on freshwaters encompasses a wide range of stressors, from which hydromorphological alterations and water pollution are of central importance, because of their large contribution to the degradation of freshwater ecosystems at a global scale (Gleick, 2003; Schwarzenbach et al., 2010; Vörösmarty et al., 2010). Hydromorphological alterations (such as dams, stream channelization and water diversion) have caused severe disruptions in the magnitude and timing of natural river flows and sediment transport, loss of aquatic and riparian habitats, fragmentation of biological corridors and the spread of non-indigenous species, among other impacts (Poff et al., 2007; Rosenberg et al., 2000). Water pollution, which is a major threat for aquatic life and human health, originates mainly from agriculture, industrial and urban wastewater, mining activities and landfills (Schwarzenbach et al., 2010).

Agriculture covers already a third of the world land area (UNEP, 2014) and has become a major source of water pollution due to its heavy reliance on chemical inputs, namely fertilisers and pesticides. Fertilizer application, either as manure or mineral fertilisers, provide agricultural soil with the nutrient level (mainly of nitrogen, phosphorus and potassium) necessary to support current crop yields (Roberts, 2009). Pesticides, chemicals or biological agents used to control crop pests, are also considered essential to secure the global food supply (Strange and Scott, 2005). Over the second half of the 20th century, production of pesticides and fertilizers have experienced a rapid increase (>750% for pesticides and N fertilizers, 300% for P fertilizers; Tilman et al., 2001). The subsequent increase in pesticide and fertilizer applications has lead to a significant improvement in crop yields (3-fold for cereals, Trewavas, 2002) and allowed human population to reach 6 billion people at the end of the 20th century (Cohen, 1996). In the last 15 years the consumption of pesticides and fertilisers has stopped its increasing trend and has become relatively steady (European Environment Agency, 2014, 2013; Grube et al., 2011). Nonetheless, agriculture remains a major threat for water quality and freshwater biodiversity worldwide (Vörösmarty et al., 2010).

The large amount of pesticides applied worldwide annually (200,000 tonnes only in Europe; EUROSTAT, 2007) partly reach surface and groundwaters (Schwarzenbach et al., 2010; Stehle and Schulz, 2015), where they pose significant risks of acute and chronic toxicity (Malaj et al., 2014; Stehle and Schulz, 2015). Pesticides enter streams mainly via diffuse sources such as wind drift, surface runoff, tile drains or groundwater (Schulz, 2004). Runoff-related pesticide input triggered by precipitation events has been identified as a major pathway (Bereswill et al., 2012; Leu et al., 2004; Rabiet et al., 2010) and positive relationships between pesticide concentrations and stream discharge have been reported (Rabiet et al., 2010; Taghavi et al., 2010). The episodic nature of pesticide pollution is reflected in a recent study on insecticide pollution at global scale (Stehle and Schulz, 2015) showing that that no insecticides were detected in more than 97% of the samples, whereas more than the half of the samples containing insecticides at detectable

levels exceeded regulatory threshold values for water or sediments. Once they reach streams, pesticides can affect decomposer communities (Beketov et al., 2013) and reduce litter decomposition rates (Rasmussen et al., 2012b; Schäfer et al., 2012b).

Several stressors are likely to co-occur with pesticides in agricultural streams and influence litter decomposition. Firstly, the use of fertilizers in agriculture is linked to elevated phosphorous and nitrogen concentrations in stream water. Nutrient enrichment can accelerate litter decomposition (e.g. Pascoal and Cássio, 2004; Robinson and Gessner, 2000), whereas eutrophic and hypertrophic states can dramatically reduce this process (Woodward et al., 2012). Besides, channelization and dredging of agricultural streams lead to homogenisation of physical and hydraulic conditions, which in turn has been suggested to also reduce litter decomposition (Rasmussen et al., 2012b). Finally, changes in water physico-chemical parameters potentially driven by agriculture, such as increased temperature, increased conductivity or reduced dissolve oxygen, can also affect decomposition (Bruder et al., 2015; Piggott et al., 2012; Schäfer et al., 2012a).

1.3 Fungicide effects on decomposers and litter decomposition

Fungicides are chemicals used to control or prevent fungal pests on a wide range of crops, including soybean, vine and potatoes. Whereas inorganic fungicides (mainly copper and sulphur) persist in the environment, synthetic organic fungicides break down throughout different abiotic and biotic mechanisms, such as hydrolysis, photolysis and microbial degradation. Thus, inorganic and organic fungicides are associated with chronic and recurrent acute toxicity in freshwaters, respectively. Despite fungicides are the most heavily used group of pest control agents in regions such as the European Union, they are the least studied (Köhler and Triebskorn, 2013). Some of them present relatively unspecific modes of action, affecting one or several cell components or functions, such as amino acid synthesis, cell division, energy production, membrane integrity or sterol biosynthesis (Maltby et al., 2009; Van den Brink et al., 2007). Thus, it is still not clear whether the taxonomic groups used in pesticide risk assessment (vertebrates, invertebrates and primary producers) are protective for non-target freshwater fungi. In this regard, Dijksterhuis et al. (2011) reported that this was not the case for epoxiconazole and tebuconazole (two fungicides from the group triazoles, which inhibit an enzyme involved in sterol production).

Field studies on the effects of pesticides on litter decomposition and decomposers are scarce and typically target not only fungicides. These complex pesticide mixtures have been reported to decrease microbial (e.g. Rasmussen et al., 2012b) and invertebrate-mediated decomposition (e.g. Schäfer et al., 2012b). Under these conditions, it is difficult to attribute effects to a single individual group (e.g. fungicides). To date, fungicide effects have been mainly reported under laboratory conditions and include changes in fungal community structure (Bundschuh et al., 2011; Zubrod et al., 2015) and decreased fungal biomass (Bundschuh et al., 2011), which may lead to a decrease in microbial decomposition (Artigas et al., 2012; Rasmussen et al., 2012a). In addition, fungicides impaired leaf palatability to shredders (Bundschuh et al., 2011; Zubrod et al., 2015), which can also be directly affected at environmentally relevant concentrations (Flores et al., 2014; Zubrod et al., 2014). Nonetheless, laboratory tests usually lack of some processes typically occurring in the field, such as recurrent episodic exposure to fungicides, biotic interactions or recolonisation. Therefore, it remains unknown whether these results are representative of the field situation.

1.4 Fungicide regulation, monitoring and evaluation

In Europe, risk assessment of pesticides to aquatic ecosystems follows a tiered approach (EFSA PPR Panel, 2013). In the first tier, acute laboratory toxicity tests are carried out with a limited number of standard test species (a fish, a crustacean and an alga) to establish dose-response relationships. The assessed toxicity/exposure ratio, which is the toxic effect value (e.g. EC50) divided by the predicted environmental concentration (assessed with FOCUS simulations; FOCUS, 2001), will be used as criteria to authorize or refuse the registration of single pesticides (EEC, 1991). If this is the only toxicity data available, safety factors (e.g. 10-100; European Commission, 2011) are applied to the lowest median lethal concentration of the test species in order to account for uncertainties including chronic effects and differences with field conditions. In the second tier, toxicity data for a greater number of test species is generated to establish species sensitivity distributions (SSD), which are used to assess the concentration affecting a specific proportion of the species. In higher tiers, more complex toxicity tests (e.g. mesocosms) are performed. Despite the existing framework for performing complex ecotoxicological tests, bottom-up approaches currently dominate ecotoxicology and the available data come mainly from single-compound laboratory studies (Van den Brink et al., 2007). The current body of knowledge should be complemented with top-down approaches (e.g. field studies) to properly determine effects on large-scale ecological systems (Beketov and Liess, 2012), which remain largely unknown.

Reporting data obligations in the European Union with respect to freshwaters are collected into the WISE (Water Information System for Europe) – SoE (State of the Environment) Rivers database (European Environment Agency, 2012a). Apart from some deficiencies in the data provided by some Member States in terms of number of monitored priority substances and water bodies (European Environment Agency, 2012b), sampling strategies are often inappropriate to catch episodic peaks of pesticide pollution. Monitoring of pollutants within the Water Framework Directive (WFD) frame, for example, suggest a grab water sample each month for priority pollutants and every three months for other pollutants. This strategy provides accurate information at a specific point time, but is likely to underestimate peak concentrations. Time-proportional and flow-proportional sampling are required to properly cover the temporal variability of pollution and the magnitude of peak contamination episodes, respectively (Bundschuh et al., 2014).

Mixtures of polar organic fungicides are common in streams flowing across fungicide-treated agricultural landscapes (e.g. Battaglin et al., 2011; Herrero-Hernández et al., 2013). A common approach to deal with chemical mixtures is the assessment of Toxic Units (TU; Ohe and Zwart, 2013), in which the predicted or measured concentration of each pollutant is divided by its effective concentration 50 (EC_{50}). The sum of TU aggregates the toxicity of the different compounds and assumes a similar mode of action.

1.5 Objectives and structure of the thesis

This thesis has three main objectives:

- Characterise the current exposure to fungicides in agricultural streams (chapter 2).
- Determine whether the resulting fungicide toxicity affects aquatic decomposers and litter decomposition, and in which extent (chapter 3).
- Determine whether nutrient enrichment compensates for effects of fungicides on leaf decomposition (chapter 4).

Each objective has been analysed in a different chapter of this thesis and implied one or more experiments (Figure 1.1).

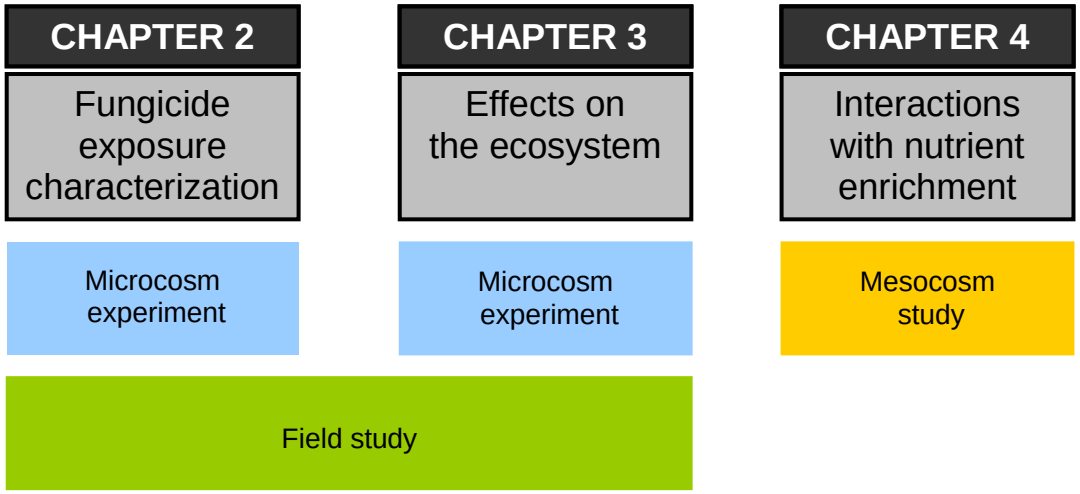

Figure 1.1: structure of this thesis.

Recurrent episodic exposure to fungicides in agricultural streams and its effects on decomposer communities and leaf decomposition were analysed in the same field study (chapter 2 and 3). A German vineyard area where fungicides are the main pest control agent was selected as study area, and 17 stream sites were selected covering a presumed gradient in fungicide exposure. Fungicides were monitored in association with rainfall events within the application period, to determine the magnitude of peak concentrations occurring during the recurrent episodic pollution events. Decomposition and several other biotic endpoints were measured in the same sampling sites at the time of major litter input.

Event triggered samples and passive samplers were used to measure fungicide concentrations in stream water (chapter 2). A microcosm experiment was conducted to determine the calibration information required to assess the concentration of each analyte in the stream water from the mass accumulated in the passive sampler. Additionally, a second microcosm experiment was conducted to identify whether the found correlations between microbial community change and fungicide toxicity were causal (chapter 3).

Finally, potential interaction effects of nutrient enrichment and fungicides on decomposer communities and litter decomposition were analysed in a mesocosm experiment simulating a single pollution event (chapter 4).

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2. Fungicide exposure characterization

Calibration and field application of passive sampling for episodic exposure to polar organic pesticides in streams

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2.1 Abstract

Rainfall-triggered runoff is a major driver of pesticide input in streams. Only few studies have examined the suitability of passive sampling to quantify such episodic exposures. In this study, we used Empore^{TM} styrene-divenylbenzene reverse phase sulfonated disks (SDB disks) and event-driven water samples (EDS) to assess exposure to 15 fungicides and 4 insecticides in 17 streams in a German vineyard area during 4 rainfall events. We also conducted a microcosm experiment to determine the SDB-disk sampling rates and provide a free-software solution to derive sampling rates under time-variable exposure. Sampling rates ranged from 0.26 to 0.77 L d^{-1} and time-weighted average (TWA) concentrations from 0.05 to 2.11 µg/L. The 2 sampling systems were in good agreement and EDS exceeded TWA concentrations on average by a factor of 3. Our study demonstrates that passive sampling is suitable to quantify episodic exposures from polar organic pesticides.

2.2 Introduction

Large amounts of pesticides including hundreds of different active ingredients are applied worldwide annually and may partly reach surface and groundwaters (Schwarzenbach et al., 2010). Pesticide pollution is consequently of great concern and may result in ecological effects on non-target organisms (Beketov et al., 2013). Beside the diversity of compounds, a major challenge for pesticide monitoring in lotic ecosystems is the variability of concentrations due to the dynamic nature of pesticide input. Runoff-related pesticide input triggered by precipitation events has been identified as a major driver of pesticide input in streams (Bereswill et al., 2012; Leu et al., 2004; Rabiet et al., 2010), and positive relationships between pesticide concentrations and stream discharge have been reported (Rabiet et al., 2010; Taghavi et al., 2010). Moreover, maximum exposure concentrations of pesticides in streams following precipitation have been linked to adverse effects on freshwater communities as well as on essential ecosystem functions (Schäfer et al., 2012). Hence, capturing peak pesticide concentrations during episodic inputs is pivotal for an ecologically relevant characterisation of exposure.

Grab water sampling at individual time points is very likely to miss relevant exposure events unless event-triggered or flow proportional samples are taken (Stehle et al., 2013). While automatic sampling equipment is expensive and requires technical maintenance, event-driven water samplers (EDS) sensu Liess et al. (1996) represent an economic alternative. Nevertheless, EDS require immediate retrieval and sample processing shortly after the rainfall events, to prevent degradation of compounds, which would result in underestimation of the exposure. This renders larger scale applications of this technique laborious.

Passive sampling constitutes an alternative to water sampling (Kot et al., 2000; Vrana et al., 2005) and through concentration of compounds may allow for lower quantification limits compared to extracted water samples. Besides, passive samplers provide an integrated measure of the pesticide concentration during the deployment period and are less logistically constraining for the monitoring of pesticides than repeated grab sampling. Thus, they are becoming popular for characterising field exposure. Semi-permeable membrane devices (SPMD; Huckins et al., 2006), Chemcatcher (Sánchez-Bayo et al., 2013; Schäfer et al., 2008b) and Polar Organic Chemical Integrative Samplers (POCIS; Bartelt-Hunt et al., 2011; Thomatou et al., 2011) are among the most used passive samplers for this purpose, although other samplers have also provided with satisfactory results (Assoumani et al., 2013; Hyne et al., 2004). However, only few studies examined the suitability of passive sampling for pesticide episodic exposure characterisation (Schäfer et al., 2008b; Shaw and Mueller, 2009). Furthermore, to determine field concentrations after field exposure requires so-called substance-specific sampling rates (i.e. volume of water sampled per unit of time), which allow users to compute time-weighted average (TWA) concentrations from the compound mass in the receiving phase (Gunold et al., 2008). While several calibration studies have been conducted for pharmaceuticals and polar herbicides and insecticides, there is a scarcity of fungicide calibration data.

In this study, we used Empore^{TM} styrene-divenylbenzene reverse phase sulfonated disks (hereafter SDB disks) to assess the exposure to 15 and 4 polar organic fungicides and insecticides, respectively, in 17 streams in a vineyard area in the south-west of Germany. SDB disks were deployed shortly before 4 presumed rainfall events, in concert with EDS. In addition, a 6-day microcosm calibration study was conducted to determine sampling rates of the 19 target pesticides under close-to-natural conditions. Sampling rates were subsequently used to estimate TWA concentrations, which were in turn compared to concentrations from the EDS to evaluate the suitability of SDB disks for capturing episodic exposure. Moreover, we provide a free open source software solution to derive sampling rates under variable exposure in calibration experiments.

2.3 Material and methods

2.3.1 Study area and survey design

The study was conducted in 17 streams in the South of the federal state of Rhineland-Palatinate (southwest Germany), which is the largest German vine-growing region characterised by 23,000 ha of vineyards (Statistisches Landesamt RLP, 2011). Fungicides are applied every 10-14 days from end of April to middle August (Bereswill et al., 2012)

and are the most used pesticides for grapes (96% of all applications), whereas herbicides (1.5%) and insecticides and acaricides (2.5%) play a minor role (Roßberg, 2010).

A natural conserve, the Palatinate forest, is located upstream of the vineyards and is the source of all streams in the region so that other than vinicultural pesticide input can largely be excluded. The selected streams covered a presumed gradient of fungicide exposure including 4 reference sites without exposure, located in the Palatinate Forest (Appendix A, Figure A.1). This presumed gradient of fungicide exposure was estimated from the proportion of vineyards with respect to the total catchment area, using Corine Land Cover (Büttner and Kosztra, 2007) maps. The sites were monitored for 15 fungicides and 4 insecticides (environmental properties in Appendix A, Table A.1) in 2012 from July to September, and physico-chemical variables at the sampling location were also measured (Appendix A, Table A.2). Pesticides were selected based on: (i) information on exposure from a previous pesticide study in the region (Bereswill et al., 2012) and (ii) spraying recommendations from local authorities. The monitoring was based on precipitation information and both passive samplers and EDS were deployed 1-2 days preceding forecasted precipitation events (>10 mm/day; Appendix A, Table A.3). Samplers were retrieved within 2 days after precipitation events (except for the fourth precipitation event, were samplers were retrieved after 5 days due to logistic constraints).

2.3.2 Passive samplers

All solvents used were HPLC grade (Carl Roth, Karlsruhe, Germany). Before deployment, SDB disks (47 mm diameter; Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) were conditioned for each 30 min in methanol and in ultrapure water under gentle rotation (100 rpm) in a shaker. Subsequently, they were placed between 2 stainless steel sheets (2 mm thickness), one of them presenting a 40 mm diameter circular opening (Vermeirssen et al., 2012; Appendix A, Figure A.2) and kept submerged in ultrapure water until field deployment. The whole device was fixed in duplicate (2 disks) with a single metal stake in the stream bed with the disks facing the riverbanks, to protect the disks from damage from water-transported materials. Upon retrieval, the disks were rolled up and stored in 7 mL acetone at -21°C. Each disk was extracted for 30 min under gentle rotation in a shaker (100 rpm). Acetone was quantitatively moved to a new vial and concentrated to 0.5 mL in a nitrogen stream, while the disk was extracted a second time in the vial with 7 mL of methanol. These 2 extractions were deemed sufficient as a third extraction with methanol yielded only negligible concentrations (Appendix A, Table A.4). The 2 extracts were combined and passed through a 0.45 µm PTFE membrane in a polypropylene housing (Altmann Analytik Gmbh & Co. KG, Munich, Germany). Then the solvent was evaporated to dryness under a gentle stream of nitrogen and the analytes were retrieved in 1 mL methanol LC-MS grade. The extracts were analysed using liquid chromatography-high resolution mass spectrometry (LC-HRMS; see section 2.3.5). The concentrations reported for the passive samplers (both calibration and field data) were adjusted for matrix effects and to the recovery for each pesticide (Appendix A, Table A.4, mean recovery: 77% ± 8 RSD). Recovery was determined by testing the loss of the analyte in an extraction procedure without disks: reduction of acetone containing a known concentration of the pesticides, methanol addition, mixture filtration and make up in 1 mL methanol.

2.3.3 Calibration of SDB disks, modelling of sampling rates and calculation of TWA concentrations

To assess the sampling rates of the disks for the target analytes, a microcosm experiment was performed using 4 artificial channels (50 L each) made from stainless steel (Appendix A, Figure A.3) and were run with stream water at 0.15 – 0.2 m/s in a circular flow (velocities adjusted to median of sampled streams). The microcosms were situated on a field station in a distance of 5 m to a stream to mimic field conditions. The water temperatures ranged from 10 to 16°C due to daily variation and were representative of the temperature in the streams during deployment. Each channel was spiked with 30 mL of a pesticide mixture in methanol containing 66.7 mg/L of each target analyte (99% purity; Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) to obtain an initial concentration of approximately 40 µg/L per microcosm. Although this concentration was higher than field concentrations, we chose this calibration concentration to: (*i*) facilitate detection in SDB disk extracts for short calibration periods and (ii) overlay any possible effects from the river water used in the calibration experiment. Besides, the sampling rate gives the extracted volume of water per unit of exposure time (L/d) and is generally independent from the water concentration level (Booij et al., 2007; Vrana et al., 2005). Hence, the higher calibration concentrations in our experiment did not affect the relevance of the derived sampling rates for field conditions. At the start of the experiment 6 disks were submerged in each channel. On each day after exposure, 1 disk was retrieved from each microcosm in concert with a water sample. Disks were extracted and analysed as described above, while water samples were centrifuged 1 minute at 10,000 rpm and directly analysed into LC-HRMS (see section 2.3.5). At this step matrix effects were also considered for both SDB disk and water samples (Appendix A, Table A.4). The uptake in the samplers was modelled by optimising a one-compartment first-order kinetic model with respect to the measured mass of pesticide accumulated in the disk msorb and the concentration of the respective pesticide in the water C_W:

$$\frac{dm_{sorb}}{dt} = k_{WS} \cdot C_W - k_{SW} \cdot m_{sorb} \quad (1)$$

where the rate constants for the transfer from water to sampler (k_{WS}) and from sampler to water (k_{SW}) were used from the best fit model. Briefly, the optimisation relied on the Flexible Modelling Environment package to create a function calculating the model residuals, which are then minimised using the Levenberg-Marquardt algorithm (Ranke et al., 2013; for details see computer code in Supplementary Data). Once the parameters were obtained, equation 1 was solved using (i) C_W = 1 µg/L, as this value is a realistic field concentration during episodic exposures and it does not affect the calculation of sampling rates, (ii) the obtained parameters and (iii) the exposure time for the respective sampler *t*. Then, C_W (here C_{TWA}), *t* and m_{sorb} were used to estimate the sampling rate R_S (L/day; Gunold et al., 2008; Kingston et al., 2000):

$$R_S = m_{sorb} / C_{TWA} t \quad (2)$$

Finally, the sampling rate and the mass absorbed in the disks during field deployment were used to calculate the C_{TWA} for each individual compound, using t = 2 days as an approximation, as pesticide concentration is assumed to rapidly decrease after the peak of flow (Leu et al., 2004; Taghavi et al., 2010; Wittmer et al., 2010). The modelling was done in R (R Development Core Team, 2012) with the additional packages "mkin" (Ranke et al., 2013) and "kinfit" (Ranke and Lindenberger, 2012) and deSolve (Soetaert

et al., 2010). The algorithm, which allows the assessment of sampling rates under time-variable exposure and is available in the Supplementary Data, can be adapted for different compounds and calibration conditions by modifying the input parameters.

2.3.4 Event-driven water sampling

The sampling system consisted of 2 1-liter brown glass bottles that were fixed to a steel bar and placed in the stream with the bottle opening approximately 10 and 20 cm above the normal water level (Appendix A, Figure A.4; Liess et al., 1996; Schulz, 2001). Bottle lids were fixed 1 cm above the opening to prevent rainfall to enter the bottle and dilute the sample. Stream water samples were retrieved after the 4 monitored rain events, stored in a fridge and solid-phase-extracted within 24 h after retrieval. When the 2 bottles were filled, the lowest one was discarded as the peak concentration of pesticides occur simultaneously to the increase in water level (Rabiet et al., 2010; Taghavi et al., 2010). Due to loss of samplers and fixing the bottles too high above the water level, the number of EDS was 23. Prior to extraction, water samples were centrifuged to remove large particles (20 min at 4000 rpm; Heraeus Multifuge 4KR, Thermo) and then acidified with HCl (2 M) to pH 2. Oasis® HLB 6 cc 500 mg extraction cartridges (Waters, Milford, USA) were used for extraction. Cartridges were conditioned with 6 mL methanol (HPLC grade; Carl Roth, Karlsruhe, Germany) and 12 mL ultra pure water, and between 850 to 980 mL of a water sample were loaded. Finally, the cartridges were dried for 30 min in a nitrogen stream and stored at -21°C. Before elution, the cartridges were washed with distilled water (pH 2), dried for 30 min in a nitrogen stream and then eluted with 4 mL of methanol. The eluate was evaporated to dryness in a nitrogen stream and the residues reconstituted in 1 mL methanol. Finally, the extract was 1:20 diluted in methanol LC-MS grade and measured using LC-HRMS. Due to the high dilution factor (matrix content of 5%), matrix effects were not considered. Results were adjusted to the cartridge recovery determined for each pesticide (Appendix A, Table A.4, mean recovery = 34% ± 8 RSD). The low recovery presumably resulted from an erroneous washing step with distilled water prior to elution with methanol and the recoveries should be higher if omitting this step. However, due to acceptable RSDs (<15% for all compounds except azoxystrobin and dimethoate) and the good correlation with TWA concentrations, EDS results are deemed reliable.

2.3.5 Target analysis of the pesticides

Samples and standards were analysed using a LC-HRMS Exactive system from Thermo® (Thermo Fisher Scientific System; Dreiech, Germany) conformed by a quaternary Accela® pump and an Exactive® Orbitrap MS detector (Thermo Fisher Scientific, Waltham, USA). Separation of the pesticides was achieved on a 50×2.1 mm Thermo Hypersil GOLD™ column (1.9 µm particle size) and the flow rate was 200 µL/min (retention times shown in Appendix A Table A.4). The mobile phase consisted of 0.1% formic acid and 4 mM NH_4 formate in methanol (A) and 0.1% formic acid and 4 mM NH_4 formate in water (B) at flow rate 0.2 mL/min. The gradient started with 3 min 5% A – 95% B, followed by 7 min of 100% phase A. The column was re-equilibrated by 2 min of 5% A – 95% B. The Orbitrap mass analyser was operated in the full scan mode and all ions generated in the ion source were detected. The ions were trapped in the Orbitrap around a central electrode. The oscillation of the ions was used for determination of specific transition masses (m/z; Appendix A, Table A.4). All pesticides showed a linear range from 0.2 – 200 µg/L. Matrix effects were estimated by a post-

extraction spike method at 7 different concentrations within the working range. Strong ion enhancement or suppression was observed for all pesticide when SDB disks were used. Thus, quantification of samples was corrected to compensate the effect of the matrix. Since the matrix effect was stronger at very low concentration (0.2 µg/L), extracts from passive samplers with a concentration under 2 µg/L (less than 10 ng/L in terms of concentration in water) were not considered for further calculations. Remaining extracts were corrected using the mean value of the matrix effects obtained at the other 6 concentrations (see Appendix A, Table A.4), as most of the effects were homogeneous in the range 2-200 µg/L (<15% RSD; Appendix A, Table A.5). A few data were excluded for the mean calculations at the lowest concentrations due to a signal close to the non-spiked extract, resulting in unrealistically high matrix effects. The matrix effect of the stream water used in the calibration experiment were negligible for most of the compounds (values around ± 15 % for most compounds) and correction was only made for compounds with effects over 20%. The extracts from EDS were also prone to strong matrix effects (Supplementary Data Table S4), although quantification of samples was not corrected as the high dilution factor of the extract was supposed to effectively reduce matrix effects (Gosetti et al., 2010; Kruve et al., 2009). The limits of detection and quantification (LOD and LOQ, respectively; Appendix A, Table A.4) were determined based on the lowest quantity of analyte that can be clearly distinguished from the background (LOD; signal-to-noise ratio = 3) or quantified (LOQ; signal-to-noise ratio = 6).

2.3.6 Data analysis

Linear regression was performed to analyse the relationship between sampling rates and log K_{OW}. Linear regression models were also used to determine the association between the 2 sampling methods (for the 23 paired SDB disk- EDS samples), as a linear relationship was expected. This was done individually and together for all compounds. All analyses were conducted in R. Computer code and raw data were provided as Supplementary Data in Fernández et al. (2014) to allow for reproducibility of our analysis and can be found at http://dx.doi.org/10.1016/j.envpol.2014.08.001.

2.4 Results

2.4.1 Uptake kinetics in SDB disks

The duration of the linear uptake phase during the calibration experiment was approximately 5 days for most compounds (Appendix A, Figure A.5). The sampling rates ranged from 0.26 (fenhexamid) to 0.77 (quinoxyfen), and were weakly related to the log K_{OW} (linear regression equation: $Rs = 0.29 + 0.06*\log K_{OW}$; $r^2 = 0.26$, $p = 0.03$; Appendix A, Figure A.6). The sampling rate for tolyfluanid could not be determined and was estimated based on the regression with log K_{OW} (see above).

2.4.2 Comparison of the 2 sampling methods

The pesticide concentrations obtained with the 2 sampling methods were significantly correlated (Figure 2.1; model parameters for log-transformed concentration without

intercept: r^2 = 0.87, p < 0.01, n = 175). Peak concentrations from the EDS exceeded TWA concentrations on an average by a factor of 2.6, although there was high variability among compounds (0.58 for iprovalicarb to 11.82 for cyprodinil). Linear regression models for each compound provided with significant relationships for 13 pesticides (Figure 2.2) and adjusted coefficients of determination ranged from 0.18 (fenhexamid) to 0.97 (cyprodinil).

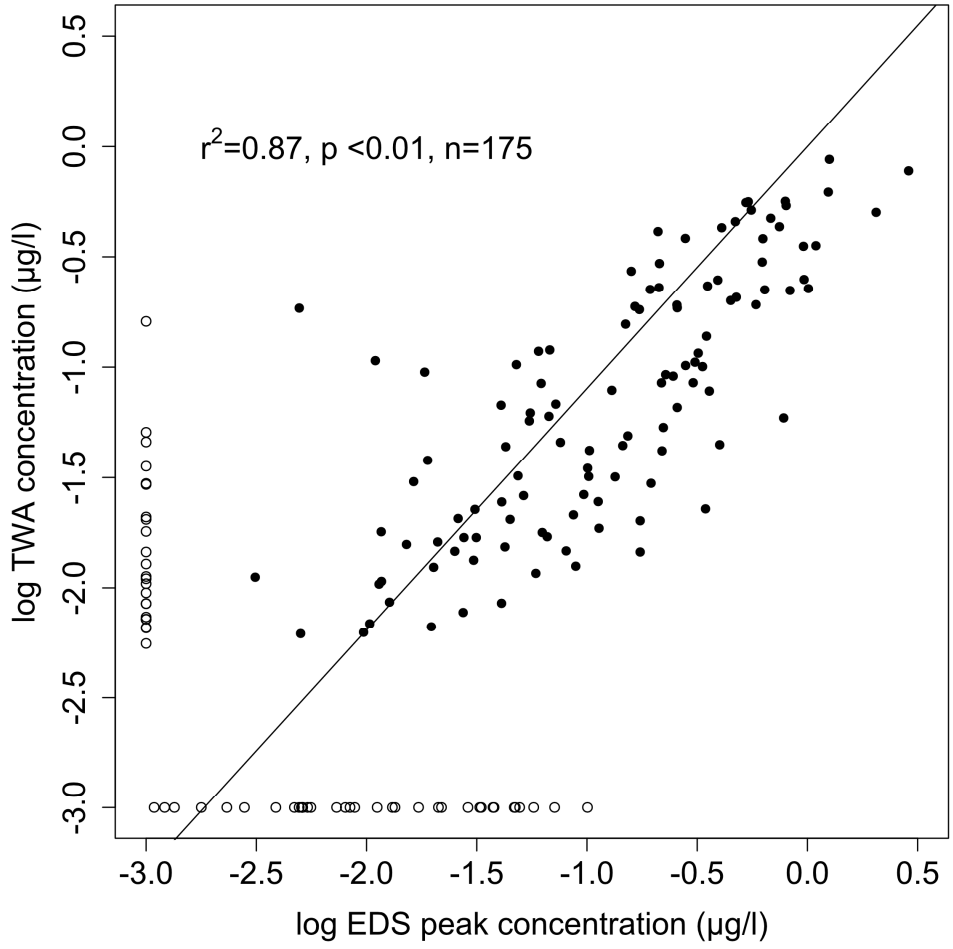

Figure 2.1: Relationship between the TWA concentrations obtained with the SDB disks and the EDS peak concentrations in 13 agricultural streams and 4 forested streams, on a double logarithmic scale. Regression line and coefficient of determination were assessed only with quantifiable concentrations in both sampling devices (full dots); concentrations which were quantifiable only with 1 device are also shown (empty dots).

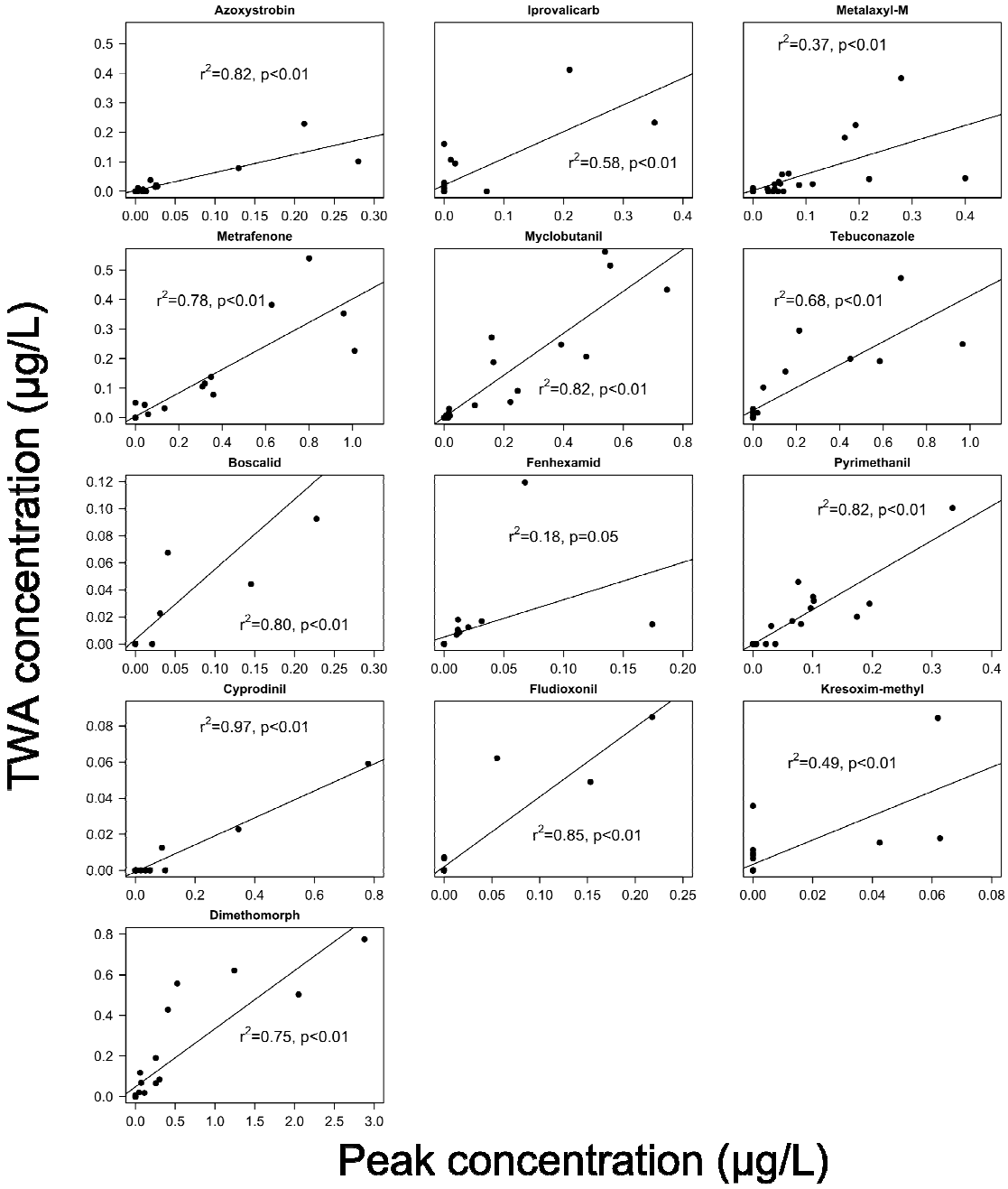

Figure 2.2: Relationships between the TWA concentrations obtained with the SDB disks and the EDS peak concentrations in 13 agricultural streams and 4 forested streams, for individual compounds. Only compounds with at least 3 pairs of concentrations over the limit of quantification are shown.

2.4.3 Fungicide exposure in the study area

We monitored 4 rainfall events during the field study. The first and the third rainfall events lead to 2 to 10 fold higher total inputs of pesticides in comparison to the second and the fourth events, respectively (Figure 2.3). Most of the pesticides were detected in more than 50% of the agricultural sites and only the insecticide tebufenpyrad and the fungicide tolyfluanid were not detected (Table 2.1). TWA mean and maximum concentrations for individual compounds ranged from 0.02 to 0.27 µg/L and from 0.05 to 2.11 µg/L, respectively (Table 1). Maximum TWA concentrations were found for the

fungicides cyprodinil, azoxystrobin and fenhexamid, and the highest mean TWA concentrations were found for cyprodinil, myclobutanil and boscalid. A couple of pesticides were detected in the forested sites above the LOQ, though the sum of the pesticide TWA concentrations in these sites was always lower than 0.1 µg/L (Figure 2.3).

Table 2.1: Sampling rates and TWA concentrations estimated for the field deployment.

Pesticide	Type	Sampling rate (L/day) [a]	TWA mean (µg/L) [b]	TWA max (µg/L) [b]	Detections in vineyard sites (%) [c]	Detections in forest sites (%) [c]
Azoxystrobin	F	0.59	0.107	1.481	62	0
Boscalid	F	0.42	0.187	0.874	77	0
Cyprodinil	F	0.55	0.268	2.112	31	0
Dimethoate	I	0.29	0.091	0.184	23	0
Dimethomorph	F	0.44	0.158	0.775	77	0
Fenhexamid	F	0.26	0.143	1.469	69	0
Fludioxonil	F	0.61	0.153	1.327	46	0
Imidacloprid	I	0.28	0.088	0.324	23	0
Indoxacarb	I	0.34	0.018	0.047	53	0
Iprovalicarb	F	0.36	0.077	0.412	69	0
Kresoxim-methyl	F	0.52	0.020	0.084	62	0
Metalaxyl-M	F	0.45	0.061	0.383	85	50
Metrafenone	F	0.54	0.151	1.237	69	0
Myclobutanil	F	0.46	0.199	1.457	100	0
Pyrimethanil	F	0.64	0.033	0.113	70	0
Quinoxyfen	F	0.77	0.015	0.062	38	0
Tebuconazole	F	0.44	0.081	0.473	76	25
Tebufenpyrad	F	0.54	0.000	0.000	0	0
Tolyfluanid	I	0.51*	0.000	0.000	0	0

(F=fungicide; I= Insecticide).

[a] Sampling rates obtained in the calibration experiment. * = Value derived from the regression (see above)

[b] TWA mean and maximum concentration in the vineyard sites obtain with the SDB disks. Only positive samples were considered in the assessment.

[c] Proportion of vineyard (n=13) or forest (n=4) sites presenting detections above the LOQ at least in one rainfall event.

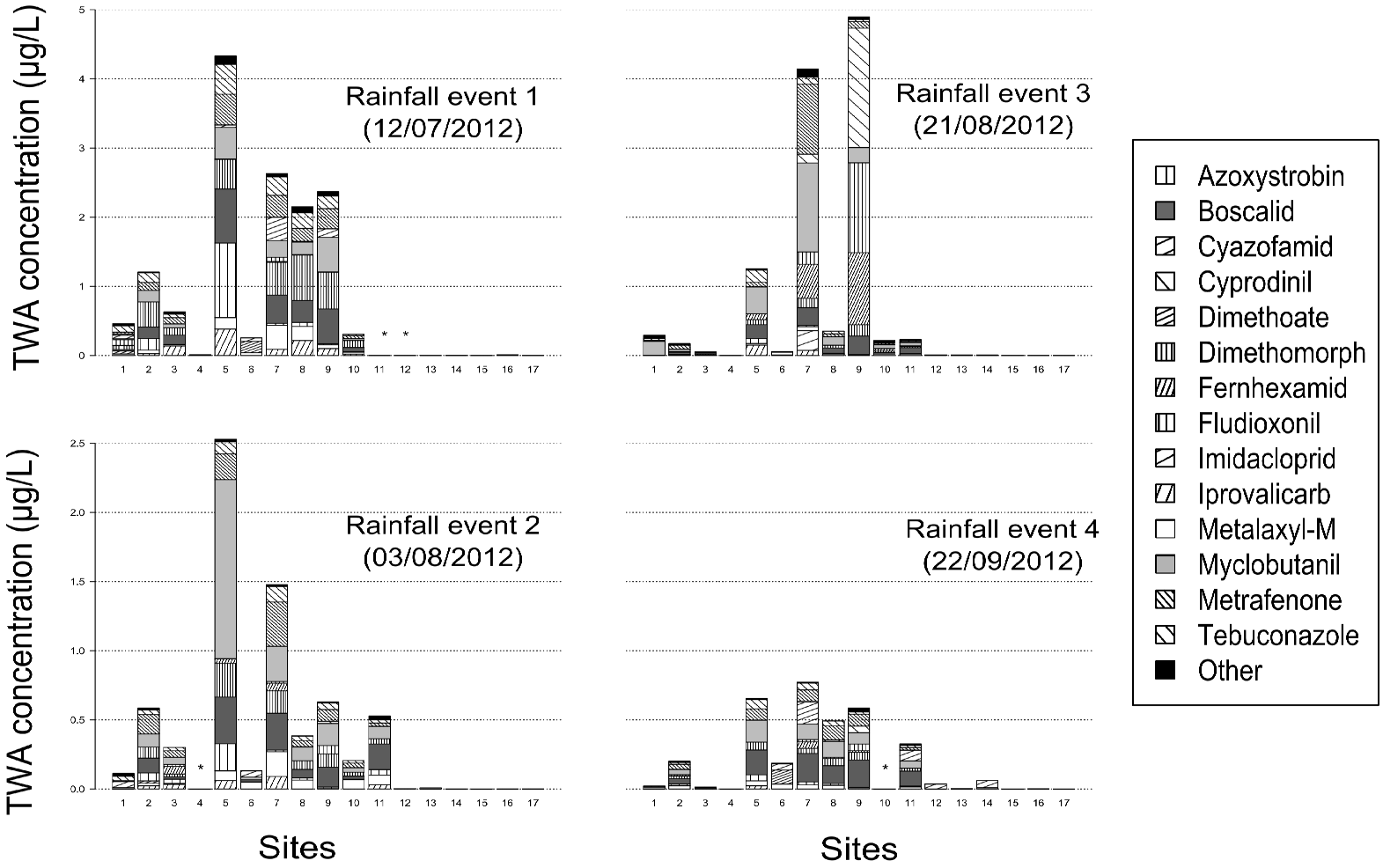

Figure 2.3: TWA pesticide concentrations at the impacted (1-13) and reference (14-17) sampling sites during the 4 monitored rainfall events in summer 2012 (*: no data available).

2.5 Discussion

2.5.1 Calibration of passive samplers

The range of sampling rates and the 5-day duration of the linear uptake phase for most compounds is very similar to those reported by Shaw et al. (2009) during a calibration study for polar compounds in our range of K_{OW} using the same sorbent. No sampling rates have been previously reported for our set of compounds except for azoxystrobin (0.15; Ibrahim et al., 2013), tebuconazole (0.19; Gunold et al., 2008) and pyrimethanil (0.24; Thomatou et al., 2011), but the sorbents, passive sampling devices, calibration systems and environmental conditions of these studies differed, presumably resulting in different sampling rates.

2.5.2 Relationship between passive sampling and event-driven sampling

The significant correlation between the concentrations obtained with the SDB disks and those obtained with the EDS suggest a good agreement, despite that sampling rates were not corrected by flow. Most flow rates in our sampling sites were above 0.1 ms^{-1} under normal conditions, and presumably increased markedly during rain events. Though flow effects decrease with increasing velocity, they are still expected to occur for SDB disks under our flow conditions (Vermeirssen et al., 2009). However, the pesticide concentration range, which varied over 3 logarithmic units, might have overruled potential variability in the TWA concentrations originating from flow effects. The 3-fold difference between EDS and TWA concentrations is close to the factor of 4-5 reported by Schäfer et al. (2008) for 10 pesticides using SDB-XC Empore disks. This difference is within the expected range (3 to 12-fold higher concentrations for EDS, *sensu* Schäfer et al. 2008), as EDS were assumed to represent peak concentrations for a few hours, while our passive samplers provide TWA concentration for 2 days, and in this period the concentration may drop to less than 10% of the peak concentrations (Leu et al., 2004). Finally, the EDS/SDB disk concentration ratio also depends on the physicochemical properties of the target pesticides, and therefore we obtained different ratios for each compound (Figure 2.2, Appendix A, Table A.4). The highest value for this ratio was obtained for the fungicide cyprodinil, which might suffer a rapid release from the passive sampler.

2.5.3 Fungicide concentrations in the field

The catchment area and land-use composition upstream of the most polluted vineyard sites (5,7,9) and of less polluted vineyard sites (4, 10, 11) was similar (Appendix A, Figure A.1). However, these high-polluted sites were subject to lower flow velocities than low-polluted sites (0.09±0.04 versus 0.23±0.03 m/s on average), thus potentially reflecting the influence of hydraulic conditions on the pollution state of a site. The discharge point of the erosion rills, which has been shown to govern the entry of pesticides in vineyards (Bereswill et al., 2012), may also partly explain site-specific pollution profile. Sampling locations downstream of an erosion-rill discharge most likely exhibit higher pesticide concentrations than those located upstream. In the reference sites, the detections over the LOQ may originate from very small vineyards and crops upstream

the sites or from spray drift or wet deposition (Ohliger and Schulz, 2010) for those close to the vineyard area (Appendix A, Figure A.1).

Several studies reported pesticide concentrations in streams including for those compounds analysed in our study (Battaglin et al., 2011; Bereswill et al., 2012; Herrero-Hernández et al., 2013; Reilly et al., 2012; Wightwick et al., 2012). These studies covered a wide range of regions from the USA, Europe and Australia and different crops, which are associated with the use of fungicides (soybean, vine, potatoes). The sampling relied on grab water samples, which were solid-phase extracted and subsequently analysed using gas or liquid chromatography. Only one study sampled event-driven after rainfall events that lead to high-flow conditions (Bereswill et al., 2012). Our mean and maximum peak (EDS) concentrations were in the same range as these studies (less than 1 and 3 µg/L for mean and maximum concentrations, respectively; Appendix A, Figure A.7). However, pesticide concentrations highly depend on the temporal and spatial distribution of the rainfall (Rabiet et al., 2010), and rain after long dry periods can lead to an order of magnitude higher concentrations with respect to events after short dry periods (Bereswill et al., 2012). During our sampling period the temporal distribution of the rainfall was relatively even and events were similar regarding amount and intensity (Appendix A, Table A.3), though there was spatial variability in the distribution of rainfall. The fourth event lead to the lowest concentrations in the sites, which may be the consequence of lower or no pesticide applications between the third and fourth event, because the local peak application period of fungicides is between July and August (Roßberg, 2010). In addition, there may have been losses for some unstable pesticides, as the SDB disks were submerged for 5 days after the fourth event. Thus, beside hydrological conditions, sampling schemes should be tailored to the pesticide application schedule, which in turn depends on climatic conditions and previous infections because they determine the respective disease pressure (Bereswill et al., 2012).

2.5.4 Implications for future studies

We demonstrate that SDB disks can be used for the quantification of episodic exposure to fungicides and polar insecticides. As previous studies have shown, SDB disks can also be employed to monitor other polar organic pollutants such as herbicides, pharmaceuticals or industrial chemicals (Shaw and Mueller, 2009; Vermeirssen et al., 2013). Moreover, for hydrophobic compounds C-18 disks have been successfully applied (Vrana et al., 2007). The disks can be used with or without a diffusion-limiting membrane. A diffusion-limiting membrane decreases the uptake of analytes and in turn the sampling rate, but increases the time where the sampler operates in the linear uptake regime that is required to estimate TWA concentrations (Schäfer et al., 2008a). Using the disks without diffusion-limiting membrane, as in our study, is most suitable to achieve high sampling rates during short episodic exposures, i.e. to capture peak exposures of pollutants with strong concentration oscillations (e.g. pesticides). However, disks without membrane usually biofoul after a few days of deployment, which depresses the sampling rates (Schäfer et al., 2008a). Hence, a diffusion-limiting membrane should be used when the aim is to monitor for longer time periods or when the exposure peaks cannot be anticipated and the sampler might be deployed for a few weeks. The algorithm to derive sampling rates under variable exposure could be adapted to this situation by including the diffusion-limiting membrane as a third compartment in the first-order kinetic model (see Vermeirssen et al., 2012 for details).

To conclude, this study provides SDB-disk sampling rates for 19 polar organic pesticides, assessed under climatic and hydraulic conditions typical for small low-slope streams flowing across temperate vinicultural areas. Moreover, we present a free-software solution to derive sampling rates under time-variable exposure. Finally, we successfully captured and quantified episodic exposures of organic pesticides using passive sampling.

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3. Effects on the ecosystem

Effects of fungicides on decomposer communities and litter decomposition in vineyard streams

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3.1 Abstract

Large amounts of fungicides are applied globally and partly enter freshwater ecosystems. A few laboratory studies examined their effects on decomposer communities and the ecosystem process of litter decomposition (LD), whereas the field situation remains largely unknown. We conducted a field study with 17 stream sites in a German vineyard area where fungicides represent the dominant pest control agent. Passive samplers were used to monitor 15 fungicides and 4 insecticides in streams and their toxicity was described using the toxic unit approach, whereas sediment samples were taken to characterise total copper concentrations. Microbial and leaf-shredding invertebrate community composition and related LD rates were assessed at each site. The structure of microbial and shredder communities as well as fungal biomass changed along the fungicide toxicity gradient. The changes in microbial endpoints were associated with a reduction of microbial LD rate of up to 40% in polluted streams. By contrast, neither the invertebrate LD rate nor *in-situ* measured gammarid feeding rates correlated with fungicide toxicity, but both were negatively associated with sediment copper concentrations. A subsequent laboratory experiment employing field fungicide concentrations suggested that the microbial community changes are causal. Overall, our results suggest that fungicides can affect LD under field conditions.

3.2 Introduction

Human activities exert high pressure on freshwater ecosystems. Among the different stressors, pollution has been identified as a major driver of freshwater biodiversity loss (MEA, 2005; Vörösmarty et al., 2010) consequently threatening ecosystem health and the provisioning of ecosystem processes and services (Dudgeon et al., 2006). In this context, pesticides contribute substantially to freshwater pollution because large amounts are applied worldwide and partly enter surface waters (Köhler and Triebskorn, 2013; Schwarzenbach et al., 2010) with rainfall-triggered runoff as a major input path (Leu et al., 2004a). When entering a stream, they may affect the different groups of organisms

involved in allochthonous coarse litter decomposition (LD) which in turn might propagate to a reduction of this ecosystem process (Peters et al., 2013; Schäfer et al., 2007). LD plays a key role in stream ecosystems, because it represents the dominant energy and nutrient source for the heterotrophic food web (Wallace et al., 1997), particularly in headwaters (Fisher and Likens, 1973). In addition, dominance of allochthonous inputs over primary production can extend up to tens of kilometres from headwaters (Webster, 2007). Considering that 50% of the approximately 1.2 million kilometres of the stream network (scale 1:250,000) in Europe are small rivers and streams (Globevnik, 2007) and that heterotrophic metabolism dominates in low-order streams (Vannote et al., 1980), allochthonous inputs are of central importance. In this context, fungi and bacteria are the main microbial decomposers converting organic matter into a more nutritious food resource for leaf-shredding macroinvertebrates (Gessner et al., 2007). Despite the fact that fungicides are the most heavily used group of pest control agents in regions such as the European Union and that they can affect non-target freshwater fungi (Dijksterhuis et al., 2011), they represent the least studied group of pesticides (Köhler and Triebskorn, 2013). Under laboratory conditions, fungicides change fungal community structure and decrease fungal biomass (Bundschuh et al., 2011b), which resulted in a decrease in microbial LD (Artigas et al., 2012; Rasmussen et al., 2012a). Moreover, shredders showed preference for non-exposed leaves in comparison to leaves that have been exposed to fungicides (Bundschuh et al., 2011b) and were directly affected by fungicides at environmentally relevant concentrations (Flores et al., 2014; Zubrod et al., 2014). However, it remains open whether and to which extent these studies can be extrapolated to the field situation, where multiple factors such as biotic interactions, abiotic factors and recolonisation may moderate the effects of fungicides on LD.

In this study we assessed the effects of fungicides on LD and the associated decomposer communities in 17 streams within a vineyard area where fungicides are the dominant pest control agent. Microbial and leaf-shredding invertebrate communities were characterised together with microbial and invertebrate-mediated LD in autumn 2012 (Appendix B, Figure B.1), coinciding with the major leaf litter input into streams. Fungicide exposure in stream water was monitored the same year during four rainfall events in summer and early autumn, covering the main fungicide application period. At the same time, the ecotoxicological potential of the pesticide loads introduced during these run-off events was empirically estimated by means of in-situ bioassays using gammarid feeding as endpoint. Moreover, the toxicity of each pesticide for microorganisms and invertebrates at the different sampling sites was assessed using Toxic Units (Ohe and Zwart, 2013) and subsequently aggregated per site and rainfall event. Total copper in stream sediment was also assessed because of its use as fungicide in vineyards, where it tends to accumulate in the soil and reaches the stream mainly via physical erosion (Bereswill et al., 2012). Finally, a laboratory experiment was conducted to identify whether correlations between microbial community change and fungicide toxicity are causal. We hypothesised that fungicide toxicity would lead to a shift in the fungal community and a decrease in fungal biomass and that these changes would be associated with a reduction in microbial and invertebrate LD.

3.3 Methods

3.3.1 Study area

The study was conducted in the wine-growing area of Palatinate (southwest Germany; Appendix B, Figure B.2), which covers more than 23,000 ha of vineyards (Statistisches Landesamt RLP, 2011). Fungicides are applied every 10-14 days from end of April to mid August and are the most used pesticides for grapes (96% of all applications), whereas herbicides (1.5%) and insecticides/acaricides (2.5%) play a minor role (Roßberg, 2010), the latter ones owing to the use of pheromone traps. All streams originate in the Palatinate Forest Nature Park, a forested low-mountain range. After the forest, they discharge through an agricultural landscape that is dominated by vineyards in the first kilometres. We selected 17 sampling sites in different streams covering a presumed gradient of fungicide exposure based on the proportion of vineyards in the upstream catchment (Appendix B, Figure B.2). Four sites were located upstream from vineyards in the forest and served as reference sites without exposure. Sites were selected to exclude other major pressures upstream (such as large waste water treatment plants, large urban areas and industrial facilities) to allow for an identification of potential impacts from fungicides. Moreover, the sampling sites were located within a maximum distance of four km to the forest edge to allow for potential recovery of the invertebrate community from previous effects (Hatakeyama and Yokoyama, 1997; Liess and Ohe, 2005).

3.3.2 Environmental parameters

Physico-chemical data was collected in concert with leaf deployment at each site using the Rapid Bioassessment Methodology for Rivers and Streams (EPA, 2003; for parameters see Table 3.1). Nutrient concentrations were analysed on-site with Visocolor® test kits (Macherey-Nagel, Düren, Germany). Water temperature, pH, electrical conductivity and dissolved oxygen were measured using a multiparameter analyser Multi 340i (WTW, Weilheim, Germany) and flow velocity was measured with a flow meter (Höntzsch, Waiblingen, Germany).

3.3.3 Fungicide exposure and toxicity

Episodic fungicide exposure was assessed using passive sampling during four precipitation events (>10 mm/day) in summer 2012. Passive samplers were equipped with naked Empore^{TM} SDB-RPS discs and deployed preceding forecasted rainfall events, given that runoff represents a major input path in small agricultural streams (Leu et al., 2004b). They were retrieved within 2 days after the end of the precipitation event, extracted and analysed for 15 fungicides and 4 insecticides (Appendix B, Tables B.1 and B.2). The compounds were selected based on information from a previous study in this region (Bereswill et al., 2012) and spraying recommendations from local authorities (www.dlr.rlp.de). Subsequently, time-weighted average concentrations were determined. For further details see Fernández et al. (2014) The toxicity of the pesticide exposure for microorganisms and invertebrates was assessed using Toxic Units ($TU = C_i/EC50_i$, where C_i is the concentration of the pesticide *i* and $EC50_i$ is the median acute effect concentration for a standard test species for this substance). The algae *Pseudokirchneriella subcapitata* (72-h EC50) and the invertebrate *Daphnia magna* (48-h EC50) were used as proxies for the mixture toxicity for aquatic fungi and macroinvertebrates, respectively. The selection of an algal species as a surrogate for fungi is motivated by previous studies showing that in the absence of toxicity data for fungal

Table 3.1: Environmental variables characterising the 17 sampling sites included in this study.

Variable	Minimum	Maximum	Median	Mean	SD
Stream width (m)	0.80	7.30	1.67	2.21	1.61
Stream depth (m)	0.07	0.43	0.15	0.19	0.10
Current velocity (m/s)	0.01	0.67	0.23	0.26	0.17
Temperature (°C)	11.21	13.77	12.62	12.50	0.81
pH	7.51	8.26	7.87	7.85	0.24
Oxygen (mg/L)	5.30	10.61	9.60	9.10	1.30
Conductivity (µS/cm)	110	1290	332	481	340
Nitrite (mg/L)	0.00	0.80	0.04	0.09	0.19
Nitrate (mg/L)	2	60	5	9	14
Phosphate (mg/L)	0.10	0.60	0.20	0.25	0.13
Ammonium (mg/L)	0.00	0.20	0.00	0.01	0.05
Riffle sections (%)	0	100	80	70	36
Pool sections (%)	0	100	20	30	36
Leaves and wood (< 10 cm diam.)[a]	1	3	1	-	-
Wood (> 10 cm diameter)[a]	1	2	1	-	-
Filamentous algae[b]	0	2	0	-	-
Macrophytes[b]	0	2	0	-	-
Shading[b]	2	5	4	-	-
WRF (width of the riparian forest; m)[c, d]	1	7.5	2.25	-	-
Tree cover along the bank[b, d]	0.5	5	2.5	-	-
Large substrates (boulder + cobble; %)	0	100	20	31	31
Medium substrates (pebble + gravel; %)	0	60	5	19	23
Fine substrates (sand + silt; %)	0	100	35	50	39
sum $TU_{\textit{P. subcapitata}}$	-4.87	-2.02	-3.73	-3.66	1.07
sum $TU_{\textit{D. magna}}$	-5.34	-1.87	-3.86	-3.91	1.25

[a] Measured using an ordinal scale indicating the coverage, ranging from 1 (<5%) to 3 (>20%)
[b] Measured using an ordinal scale indicating the coverage, ranging from 0 (absent) to 5 (very high)
[c] Width of the riparian forest. When riparian forest was connected with the hillside forest, a maximum of 10 m was assigned.
[d] Mean from the left and right bank.

species TU based on *P. subcapitata* allowed to establish a relationship with functional microbial community composition as well as LD (Peters et al., 2013; Schäfer et al., 2012a). Toxicity data were compiled from the Pesticide Properties Database (Agriculture & Environment Research Unit of the University of Hertfordshire, 2013). To aggregate the toxicity from different pesticides the logarithmic sum of TUs (sumTU) was used (see Appendix B Table B.3 for rationale):

$$sumTU = \log\left(\sum_{i=1}^{n} \frac{c_i}{EC50_i}\right) \quad (1)$$

Sites where no pesticide was detected were assigned a sumTU corresponding to 1/10 of the minimum TU observed in the sites with measured concentrations (-4.9 for $sumTU_{P. subcapitata}$ and -5.3 for $sumTU_{D. Magna}$). The pesticide toxicity from different rainfall events in each site was aggregated using the maximum sumTU (for rationale see Schäfer et al., 2011). The aggregated toxicity exhibited stronger associations with the biotic endpoints than that related to individual events, suggesting that it is most indicative of the pollution state of a site.

To assess total copper, three sediment samples were taken from the stream bed at each site using a sediment sampler (260 ml volume; Kersting GmbH, Brilon, Germany). Subsequently the three samples from one site were mixed, resulting in a single composite sample. Only one composite sample was taken at each site during the passive sampling period, as copper sediment concentrations can be assumed to be relatively stable compared to water concentrations of polar organic pesticides, due to its accumulation over time periods that substantially exceed discrete rainfall events. In the laboratory, samples were dried at 105°C, sieved (2-mm mesh size), ground using agate grinding balls and extracted using microwave-induced (MARS Xpress, CEM GmbH) reversed aqua regia (3 HNO3 + HCl) digestion. The extracts were measured with inductively coupled plasma mass spectrometry (ICP-MS, Thermo Fisher Scientific Q-ICP-MS XSeries2).

3.3.4 Leaf decomposition

Five replicates of coarse-mesh and fine-mesh leaf bags (8 mm and 250 µm mesh size, respectively) were filled with 3 (± 0.07 SD; n=180) grams of dried (at 60°C for 24 h) alder leaves (*Alnus glutinosa*) collected in October 2011 at the time of abscission from a location close to Landau in der Pfalz, Germany (49° 12' 7" N; 8° 8' 37" E) and stored at −21 ◦C. Leaf bags were submerged at each sampling site for 21 days starting in mid September to assess for the microorganism and invertebrate-mediated LD, respectively. In addition, temperature loggers were deployed (HOBO Pendant, Synotech, Hückelhoven) to allow for temperature-standardisation of decomposition rates among sites. After retrieval, the remaining leaf material was gently rinsed to remove mineral particles, oven-dried at 60°C for 24 h and weighed to the nearest 0.001 g. At one of the sampling sites additional replicates of each bag type (n=5) were returned to the laboratory immediately after immersion and also after 24h of immersion to determine handling and leaching losses, respectively. The leaf decomposition rate *k* per sum of degree days ($ddays^{-1}$) was estimated for each site *i* using the following formula (Benfield, 2007):

$$k = \frac{-\ln\left(\frac{S_i(t)}{S_i(0)}\right)}{\Sigma_{j=1}^{t} \overline{T}_i(j)} \quad (2)$$

where S is the leaf mass as a function of deployment time t and T is the mean temperature for a day j. $S_i(t)$ was corrected for handling and leaching losses. S was assessed from fine-mesh bags when calculating $k_{microbial}$, whereas for $k_{invertebrates}$ $S_i(0)$ was the initial mass in the coarse-mesh ba*gs* and $S_i(t)$ was the sum of remaining leaf mass in the coarse-mesh bag and the mean mass loss in the fine-mesh bags at site i.

3.3.5 Microbial and macroinvertebrate community characterisation

Three additional coarse-mesh leaf bags were deployed at each site to sample the decomposer communities (shredders and microorganisms). These bags were retrieved one week before those used for the assessment of leaf decomposition, namely two weeks after deployment, when more leaf material was remaining to analyse microbial endpoints. The latter include: fungal community structure, fungal biomass and bacterial density. In case of insufficient leaf material, fungal biomass and diversity were prioritised over bacterial density. The bags were gently retrieved using a net (250 µm) to prevent the loss of invertebrates feeding on the leaves. The invertebrates were preserved in 70% ethanol and identified to genus or species level (Bährmann, 2011; Brohmer et al., 2000).

Fungal community structure was determined via conidia identification as described by Bärlocher (1982). Three leaf discs were cut randomly from the remaining leaf material and placed individually in a six-well-plate containing 4 mL of distilled water per well. After incubation for 96 h in darkness on an orbital shaker at 55 rounds per minute, 0.5 mL of lactophenol cotton blue were added to fix samples and to stain the spores. Subsequently, conidia were identified (Ingold, 1975) and counted under a light microscope at 100-fold magnification. For highly abundant spores, only the first 500 were counted. For each sampling site, the mean abundance from the three replicates for each species was used in analysis. Leaf-associated fungal biomass was estimated from ergosterol content, which is a cell-membrane component of eumycotic fungi, following the method of Gessner and Schmitt (1996). Briefly, ergosterol was extracted in alkaline methanol, purified by solid-phase extraction (SPE) and high-performance liquid chromatography and quantified by measuring absorbance at 282 nm. Bacterial density was determined by epifluorescence microscopy as described by Buesing (2005) Briefly, formalin-preserved cells were detached from leaf discs by ultrasonication, filtered on Anodisc membrane filters (Whatman, UK), stained with SYBRGreen II, and automatically counted on digital photographs using an image-analysis system (AxioVision 4.8, Carl Zeiss).

3.3.6 Feeding rate of gammarids

To assess the ecotoxicological potential of the pesticide loads introduced during run-off events in-situ bioassays using gammarid (*Gammarus fossarum*) feeding were used as measure. Test individuals were collected in a natural stream (Hainbach) near Landau, Germany (49°14' N; 8°03' E) and adults of both sexes with a cephalothorax length between 1.2 and 1.6 mm were selected using a passive underwater separation technique (Franke, 1977). These in situ bioassays were involved during two of the four precipitation events for which fungicide toxicity was assessed. Twenty cages (length=5.0 cm, diameter=3.0 cm, covered by a 1.0 mm mesh screen) containing one specimen of *G. fossarum* together with two preconditioned (Bundschuh et al., 2011a) leaf discs of known dry weight were placed at each site preceding the rainfall events. Five additional cages that contained only leaf discs were deployed at each site to account for microbial

decomposition and abiotic losses. These bioassays can display direct implications of water quality on feeding behaviour. Within 2 days after the end of the rain event, the amphipods together with the remaining leaf material were recovered, dried and weighed. The feeding rate was expressed as consumed leaf mass per mg animal and day and was calculated as described in Maltby et al. (2000).

3.3.7 Laboratory experiment

A laboratory experiment was performed after the field study to disentangle the influence of two variables (fungicide toxicity and pH) that showed a high association with microbial endpoints in the field. The experiment followed a two-factorial design with minimum and maximum field levels: fungicide exposure (two levels: no fungicides and a mixture of 8 frequently found fungicides at a concentration corresponding to a $logTU_{\textit{P. subcapitata}}$ of -2 each, resulting in a sumTU of -1.1) and pH (7.5 and 8.3). The mixture included the following fungicides: azoxystrobin, boscalid, cyprodinil, fludioxonil, kresoxim-methyl, metrafenone, pyrimethanil and tebuconazole. A toxicity level of -2 TU was selected because it was frequently found for several fungicides such as azoxystrobin and cyprodinil in the field. We selected a toxicity level in the range of the maximum field toxicity to improve statistical power to detect potential adverse effects, which is usually rather low for experiments such as ours with low replication (see Szöcs and Schäfer, 2015). This toxicity level was used for all fungicides to include potential mixture effects and avoid that responses would only be driven by one compound. Briefly, triplicate 1-L beakers were filled with 800 mL of stream water from a pristine site. Approximately 5 grams of alder leaves (wet weight), which were deployed for 10 days at the same pristine site to allow for microbial colonisation, were put in each beaker. The beakers were placed on magnetic stirrers (150 rpm), aerated and kept at room temperature (16°C) and darkness for 6 days. The temperature was slightly higher than that of field conditions, which should, however, be irrelevant for the main results of our study because it would have affected all treatments in a similar way. The pH was measured daily and adjusted if necessary using sodium hydroxide (2N) and hydrochloric acid (2N). After the experiment, the leaf-associated fungal community structure was determined as described above.

3.3.8 Data analysis

The relationship between fungicide toxicity and both LD and univariate biotic endpoints (fungal biomass and richness, bacterial density and gammarid feeding) was tested using Pearson correlation. Subsequently, linear models were used to identify the environmental variables (including fungicide toxicity) exhibiting the strongest explanatory power for the decomposition rates and the biotic endpoints. Temperature was omitted in the analysis of LD, because LD was temperature-standardised. Fungicide toxicity was included as $sumTU_{\textit{P. subcapitata}}$ for $k_{\text{microbial}}$ and as $sumTU_{\textit{D. magna}}$ for $k_{\text{invertebrates}}$. To avoid collinearity, predictors were checked for pairwise correlations > 0.7 before performing the linear models and the ecologically less relevant variable was removed, which was determined based on expert judgement. Automatic forward model building was performed with the Bayesian Information Criterion (BIC) as stepwise model selection criterion (Schwarz, 1978). The models were checked for error assumptions (normality and homoscedasticity) and unusual observations (leverage, outliers). Finally, the relative importance of retained predictors was assessed using hierarchical partitioning (function lmg in the R package "relaimpo"; Grömping, 2006).

Redundancy analysis (RDA) was used to determine the effects of environmental variables and fungicide toxicity on the community structure of fungi (field study and laboratory experiment) and invertebrates (field study). Collinear variables (Pearson's r > 0.7), variables showing minor variation across the study area and rare species occurring in less than 20% of sites and samples from the field and laboratory, respectively, were removed before analysis. Species data were Hellinger transformed to achieve standardisation and circumvent the problems associated with using the Euclidean distance for ecological data (Legendre and Gallagher, 2001). Then automatic model building with forward stepwise model selection was performed (function ordiR2step in the R package "vegan; Oksanen et al., 2010). Given inflation of the p value in stepwise model building based on hypothesis testing, the p value was set to 0.01 for variable entry. The individual effects from pH and fungicide toxicity in the laboratory experiment were determined using RDA after partialling out the other factor (Peres-Neto et al., 2006). All statistical analyses were conducted in R version 3.1.2. (R Core Team, 2014). Computer code and raw data were provided as Supplementary Data in Fernández et al. (2015) to allow for reproducibility of our analysis and can be found at http://dx.doi.org/10.1016/j.scitotenv.2015.06.090).

3.4 Results

3.4.1 Effects of fungicide toxicity on LD and biotic endpoints

On average, fungicides accounted for 99.2% of the pesticide toxicity in terms of $sumTU_{P.\ subcapitata}$ in polluted sites, except for one site where the insecticide dimethoate occurred in ecotoxicologically relevant concentrations. Still, fungicides accounted for the majority (62.2 %) of toxicity in this site. $SumTU_{P.\ subcapitata}$ exhibited statistically significant negative correlations with $k_{microbial}$, fungal biomass (expressed as ergosterol) and fungal richness, whereas the correlation with bacterial density was positive, albeit not statistically significant (Figure 3.1). Fungicide toxicity in terms of $sumTU_{D.\ magna}$ did neither correlate with $k_{invertebrates}$ nor the gammarid feeding rate (Appendix B, Figure B.3), but both endpoints correlated close to statistical significance with total copper concentrations in the sediment (Figure 3.1). $SumTU_{P.\ subcapitata}$ explained the largest proportion of variance of ergosterol content and fungal richness, whereas physicochemical and habitat variables were better predictors of $k_{microbial}$ and $k_{invertebrates}$, respectively (Table 3.2).

Copper showed high collinearity with several environmental variables such as conductivity (r=0.76; p<0.01), nitrite (r=0.73; p<0.01) and dissolved oxygen (r= -0.77; p<0.01), and moderate collinearity with $sumTU_{D.\ magna}$ (r=0.47, p=0.05). As these chemical variables were relevant for LD in other studies (Pascoal and Cássio, 2004; Schäfer et al., 2012a) they were retained in the linear models whereas copper was omitted. Additional models were performed retaining copper and excluding collinear chemical variables. Water chemistry still presented the highest explanatory power for $k_{microbial}$, whereas for $k_{invertebrates}$ copper explained 32% of the relative explained variance (Appendix B, Figure B.4).

3.4.2 Effects of environmental variables on the community structure of decomposers

For fungi, only sumTU$_{P.\ subcapitata}$ was a statistically significant predictor for community structure in RDA (Figure 3.2a) and explained 12% and 100% of the total and constrained variance, respectively. Conidia abundance of several species increased along the toxicity gradient (e.g. *Anguillospora spp.* and *Lunuluspora curvula*), whereas others showed an inverse trend (e.g. *Tetrachaetum elegans* and *Clavatospora longibrachiata*). Regarding the shredder community, gammarids represented the only shredders in 9 sites and in a further 4 sites they constituted 95% of the shredder community (Appendix B, Figure B.5). Again, only pesticide toxicity in terms of sumTU$_{D.\ magna}$ was included as explanatory variable of leaf-shredding macroinvertebrate community composition. *Gammarus roeseli* was more abundant in fungicide-polluted streams, whereas *G. fossarum* presented an opposite trend and *G. pulex* showed no response to the fungicide toxicity gradient (Figure 3.2b).

Table 3.2: Environmental variables (see Table 1 for full names) with highest explanatory power for LD and microbial endpoints. Percentage contribution in hierarchical partitioning, r^2, Bayesian information Criteria (BIC) for the best-fit model and sample size *n* are provided.

Response variable	Explained variance										r^2	BIC	n
	Tree cover	Stream depth	NO_2	PO_4	sumTU *P. subcapitata*	pH	Fine substr.	Med. substr.	Others				
$k_{microbial}$ [a]			47 (25)			53 (75)					0.71 (0.63)	-267 (-277)	15 (17)
$k_{invertebrates}$	52						48				0.42	-224	17
Ergosterol					100						0.25	122	17
Fungal richness		35			65						0.44	41	17
Bacterial density				48	18		9	8	7	9	0.86	-91	13

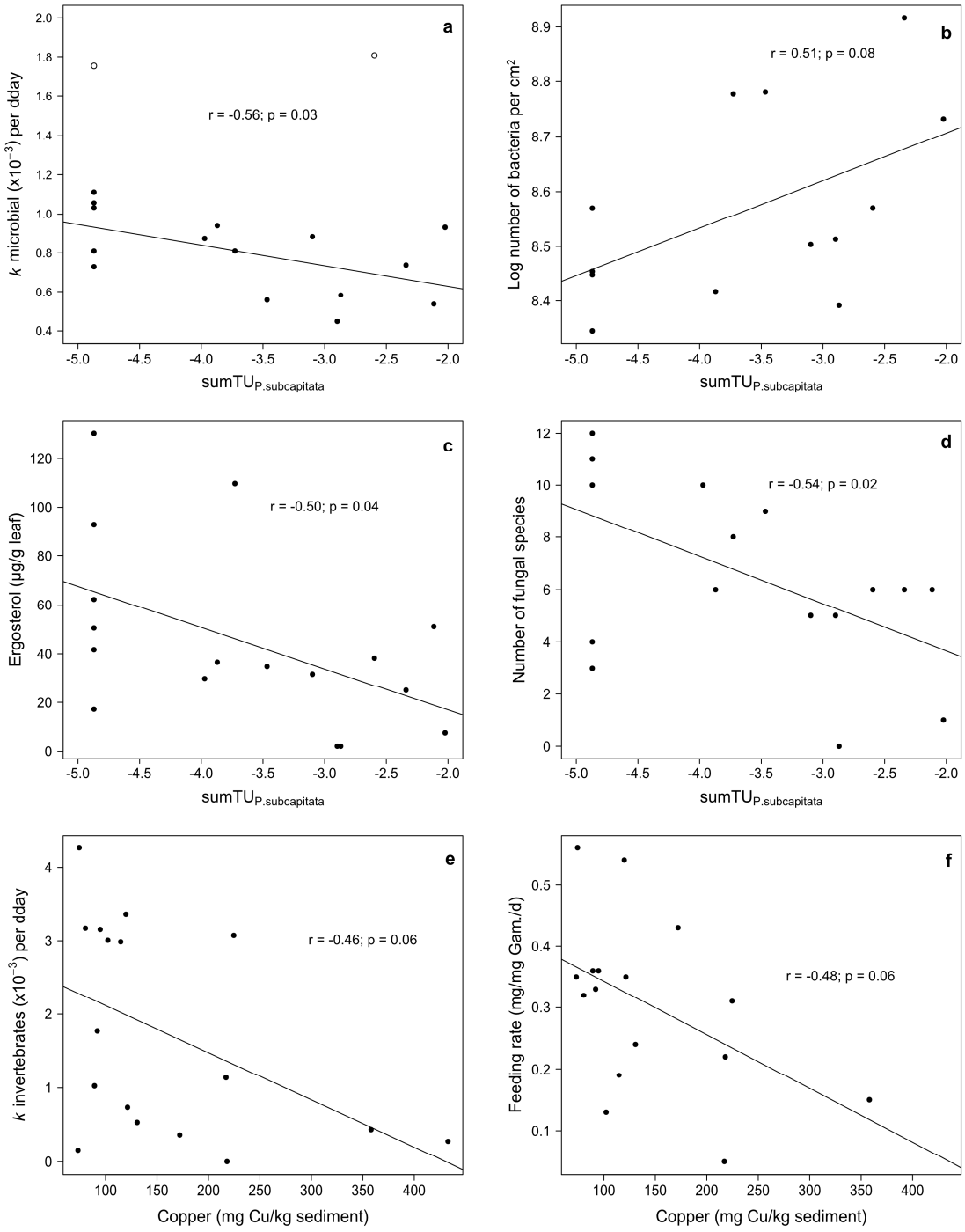

Figure 3.1: Correlation between fungicide toxicity and several biotic endpoints. Regression lines are added to visualize the positive or negative correlation. **a)** Correlation between $k_{\text{microbial}}$ and fungicide toxicity in terms of the sum of toxic units for *P. subcapitata* (sumTU) in 17 German streams using time-weighted average concentrations. Empty points in the upper graph (sites 1 and 16) were excluded from the analysis because of undue influence according to Cook's distance. **b)** Correlation between bacterial density and sum $TU_{P.\ subcapitata}$. Four sites not shown because of insufficient leaf material to determine bacterial density. **c)** Correlation between fungal biomass expressed as ergosterol and sum $TU_{P.\ subcapitata}$. **d)** Relationship between the number of fungal species and sum $TU_{P.\ subcapitata}$. **e)** Correlation between invertebrate leaf decomposition rate and total inorganic copper in stream sediment. **f)** Relationship between the feeding rate of *G. fossarum* during the third monitored rainfall event and total inorganic copper in stream sediment.

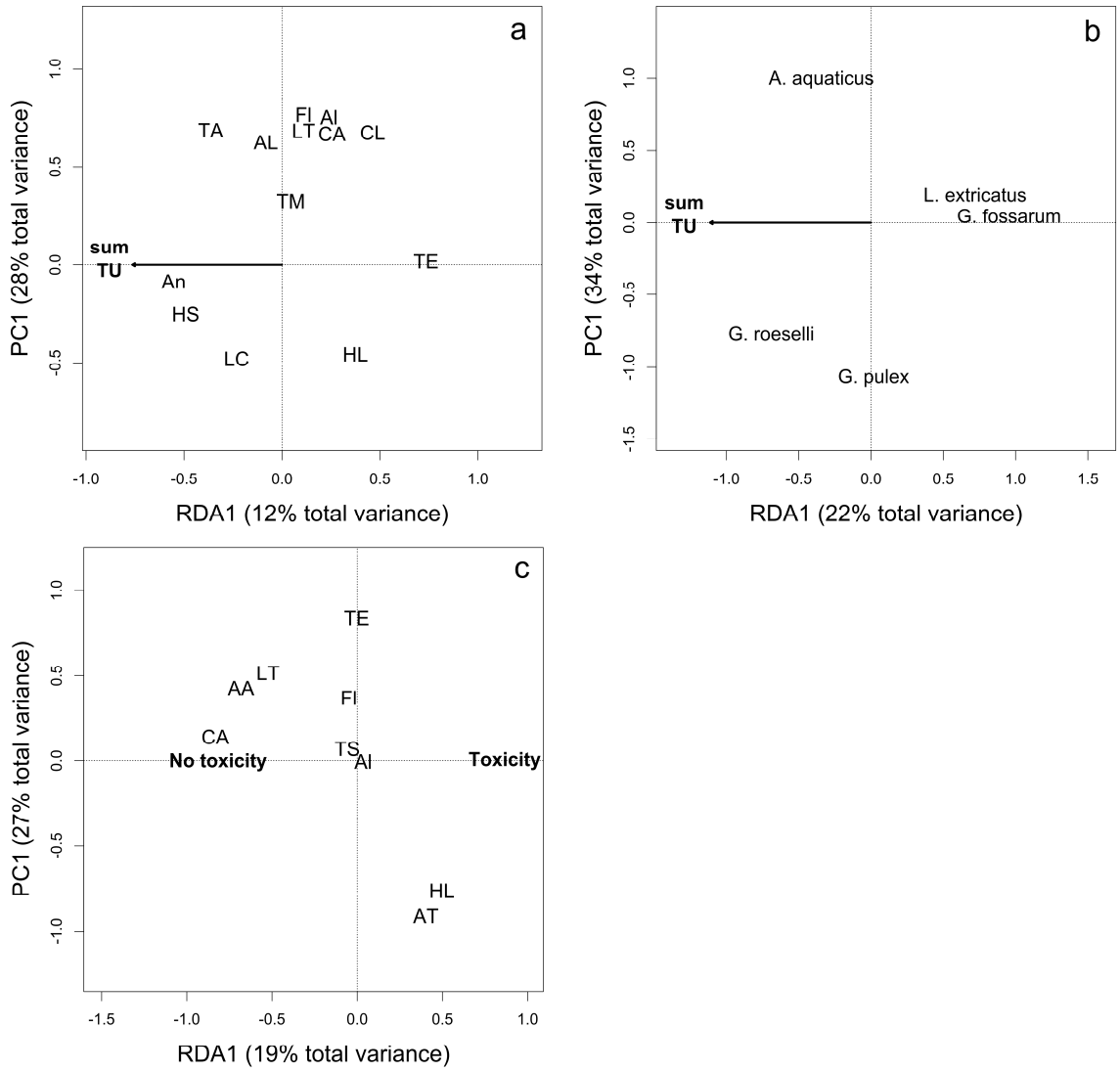

Figure 3.2: **a)** Triplot from the redundancy analysis using fungal species (field experiment) as response variables and the variables selected by the automatic model building as explanatory variables. Species: *Alatospora sp*. (Al), *Anguilospora longissima* (AL), *Anguillospora sp.* (An), *C. aquatica* (CA), *C. longibrachiata* (CL), *Flagelospora sp.* (Fl), *H. lugdunensis* (HL), *Heliscella stellata* (HS), *L. curvula* (LC), *Lemonniera terrestris* (LT), *Tricladium angulatum* (TA), *T. marchalianum* (TM) and *T. elegans* (TE). **b)** Triplot using invertebrate species (field experiment) as response variables and the variables selected by the automatic model building as explanatory variables. **c)** Triplot from the redundancy analysis using fungal species (laboratory experiment) as response variables and fungicide toxicity after partialling out pH effect as explanatory variable. Species not present in Fig. 2a: *Articulospora atra* (AA), *Articulospora tetracladia* (AT) and *Unknown sp.* (Un).

For the laboratory experiment, fungicides caused a statistically significant effect on the community structure of leaf-associated fungi ($p = 0.03$), whereas this was not the case for pH after partialling out the fungicide induced effect ($p=0.27$). The species specific response to toxicity partly deviated from the pattern found in the field (Figure 3.2a, c).

3.5 Discussion

3.5.1 Effects of fungicides on microbial community and microbial LD

Differences in conidia abundance were found for the sampling sites, which suggests differences in the fungal community composition (Bärlocher, 2005). Based on RDA results, pesticide toxicity in terms of $sumTU_{P.\ subcapitata}$ was the major driver of these differences. The toxicity can be mainly attributed to fungicides because insecticides exhibited a minor contribution to the sumTU. The results of the laboratory experiment support that fungicide toxicity causally contributed to the fungal community change observed under field conditions. For example, *Clavariopsis aquatica* decreased with increasing toxicity during both the field and the laboratory experiment. Besides, our field results (Figure 3.2a) are in general agreement with a study (Bundschuh et al., 2011b) reporting that the leaf-borne fungal species *Alatospora acuminata*, *C. aquatica* and *Flagellospora curvula* were reduced by the fungicide tebuconazole, whereas *Tetracladium marchalianum* exhibited no response to this toxicant. Nevertheless, some patterns found in the field deviated from the response in our laboratory experiment. For example, *Heliscus lugdunensis* showed opposite responses in the field study (Figure 3.2a) and laboratory experiment (Figure 3.2c). The positive relationship between conidia abundance of *H. lugdunensis* and fungicide toxicity found in the laboratory was also reported in an experiment with the fungicide cyprodinil (Zubrod et al., 2015), which was also included in our experiment. Generally, differences between the field situation and laboratory experiments are not limited to fungicides and have been reported for several stressors (Schäfer, 2014). Explanations include different exposure patterns and recovery occurring in the field compared to our short-term experiment and field communities are shaped by the environmental context, i.e. a wide range of abiotic and biotic variables (Liess and Beketov, 2011).

Beside changes in fungal community composition, fungicide toxicity was associated with a decrease in fungal biomass and an increase in the density of bacteria. In linear modelling that included additional explanatory variables, fungicide toxicity was the only predictor of fungal biomass, whereas multiple physico-chemical and habitat variables, dominated by phosphate, explained the increase in bacteria. These results suggest that indeed fungicide toxicity affected fungal communities including their biomass, whereas the increase in bacteria may be related to elevated nutrient levels. Moreover, the decrease in fungal biomass along the fungicide toxicity gradient may have contributed to the decrease in $k_{\text{microbial}}$ (Gessner and Chauvet, 1994), which reached up to 40% reduction compared to sites with no or minor fungicide exposure. However, this explanation relies on the assumption that fungi are more important than bacteria for LD, which has been shown in several studies (Duarte et al., 2010; Pascoal and Cássio, 2004), though similar importance of fungi and bacteria has been reported occasionally (Hieber and Gessner, 2002). It remains speculative whether the increase in bacterial density of up to a half logarithmic unit in sites with high fungicide toxicity partially buffered the decrease in $k_{\text{microbial}}$.

Despite the fact that fungicide toxicity exhibited the highest correlation with several endpoints related to the microbial decomposer community, pH and nitrite exhibited the strongest explanatory power for $k_{\text{microbial}}$ (Table 3.2), displaying a negative relationship. The potential effect of pH on freshwater fungi, and subsequently on $k_{\text{microbial}}$, has been suggested to be indirect (by affecting for example the solubility of metals; Bärlocher, 2005). Nitrite was significantly correlated with conductivity and the percentage of

dissolved oxygen (r = 0.61 and -0.60, respectively). Both increasing conductivity and decreasing dissolved oxygen have been reported to negatively affect $k_{microbial}$ (Pascoal and Cássio, 2004; Schäfer et al., 2012a). Overall, the results suggest that fungicides were the main driver of the effects on microbial-community related endpoints, whereas shifts in the microbial decomposition rate were largely driven by physico-chemical variables. This mismatch, i.e. that the changes in the ecosystem structure and processes were related to different stressors, can be explained by (1) the integrative nature of ecosystem processes that are influenced by a magnitude of environmental variables (Tank et al., 2010) and by (2) the fact that the response of ecosystem processes depends rather on changes in the functional composition of communities than on the taxonomic composition (Vandewalle et al., 2010). Moreover, complex fungicide mixtures occur in the field and their effect on ecosystem processes may not be captured with the very simplified toxic unit approach. For example, the sumTU assumes additive mixture effects (concentration addition), whereas non-additive effects of fungicide mixtures can occur (Zubrod et al., 2015). In addition, the sample size in our study was relatively low and the results from models with multiple variables should be interpreted with caution and against the results from previous studies (Rasmussen et al., 2012; Schäfer et al., 2012a). Future studies should try to unravel the effect mechanisms using a combination of experiments under controlled conditions and field surveys covering a larger set of sites.

3.5.2 Effect of fungicides on shredder community and invertebrate LD

Fungicide toxicity in stream water was not related to $k_{invertebrates}$, lending no support to the hypothesis that fungicide toxicity lead to a reduction in this ecosystem process. This contrasts with our expectations of finding an indirect effect on $k_{invertebrates}$, as fungal species preferred by gammarids were more abundant in non or low-polluted sites, which in turn indicates that food intake may not be affected by the fungal community composition if no alternative food is present (e.g. *Alatospora sp.* or *C. aquatica;* Arsuffi and Suberkropp, 1989; Jabiol and Chauvet, 2012). The absence of detectable effects may be explained by the fact that measured fungicide concentrations were below effect thresholds for gammarids (Zubrod et al., 2015, 2014), together with the temporal mismatch between pesticide sampling and LD assessment. Nonetheless, gammarid feeding measured *in situ* was similarly unrelated to fungicide toxicity either, despite being measured in concert with pesticide sampling. Besides, no relationship between gammarid feeding and $k_{invertebrates}$ was found (Appendix B, Figure B.6), which may also be attributed to the temporal mismatch.

Our results contrast with previous field studies reporting a decrease in $k_{invertebrates}$ because of the loss of pesticide-sensitive species (Schäfer et al., 2012a, 2012b). However, these studies reported data from agricultural areas with a more diverse shredder community, whereas a high dominance of gammarids was found in our study area. In addition, the present study is in agreement with Rasmussen et al. (Rasmussen et al., 2012b) where a *G. pulex*-dominated shredder community in Danish agricultural streams was unrelated to a pesticide gradient of a comparable TU range. Rasmussen et al. (2012b) found a positive correlation of $k_{invertebrates}$ with the density of gammarids, whereas no significant correlation was found in our study between $k_{invertebrates}$ and total gammarid abundance, though $k_{invertebrates}$ tended to be higher at higher gammarid abundances (Appendix B, Figure B.7). Nonetheless, the fact that in our study LD and decomposers were sampled in autumn implies that (i) insect shredders may have emerged and that the shredder community can be more heterogeneous and potentially more sensitive during other seasons, and (ii) the exposure to fungicides was lower than in summer (Fernández et al.,

2014). Therefore, effects on $k_{invertebrates}$ from recurrent fungicide exposure could occur earlier in the year as has been shown in Schäfer et al. (2012b). Indeed, the distribution of the three dominant gammarid species was associated with water-borne fungicide toxicity, although the latter correlated with several other environment variables. Moreover, both $k_{invertebrates}$ and the gammarid feeding rate were negatively associated with sediment-borne copper concentrations. Decreasing abundance and diversity of crustaceans and other macroinvertebrates have been reported in other studies at sediment copper concentrations detected also during the present study (Kraft and Sypniewski, 1981; Mebane, C.A., 2002). Nonetheless, the association should be interpreted with caution because sediment copper toxicity for aquatic organisms is governed by the availability of free copper ions in the water column (Kramer et al., 2004), which in turn depends and dissolved organic carbon and pH (De Schamphelaere and Janssen, 2004).

In conclusion, our results suggest that recurrent exposure to fungicides in stream water affects microbial communities. However, whether these changes result in a decrease in $k_{microbial}$ remains speculative and requires further investigation. Both field studies and experiments under controlled conditions are required to disentangle the effects of environmental variables and fungicide toxicity on microbial decomposers and LD. $k_{invertebrates}$ was negatively associated to sediment copper, whereas no response to fungicide toxicity in water was observed.

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4. Interaction with nutrient enrichment

Does nutrient enrichment compensate fungicide effects on litter decomposition and decomposer communities in streams?

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4.1 Abstract

Nutrient and pesticide pollution are widespread agricultural stressors. Fungicides may affect freshwater fungi, which play an important role in litter decomposition (LD), whereas moderate nutrient enrichment can stimulate LD. We examined potential interaction effects of nutrients and fungicides on decomposer communities and LD in a 14-day two-factorial (fungicide and nutrient treatments) mesocosm experiment. Fungicide exposure was limited to 4 days to simulate episodic contamination. Only the microbial community responded significantly to the experimental factors, though non-significant increases > 20% were found for invertebrate decomposer weight gain and LD under high-nutrient conditions. Fungal community structure responded stronger to fungicides than sporulation. Sporulation responded strongest to nutrients. Bacterial community structure was affected by both factors, although only nutrients influenced bacterial density. Our results suggest effects from fungicides at field-relevant levels on the microbial community. Whether these changes propagate to invertebrate communities and LD remains unclear and should be analysed under longer and recurrent fungicide exposure.

4.2 Introduction

The agricultural use of pesticides and fertilisers has increased crop yields (Strange and Scott, 2005; Trewavas, 2002), but also dominantly contributes to global freshwater pollution (Vörösmarty et al., 2010). In Europe, phosphate and nitrate concentrations, main drivers of eutrophication, have decreased in the last two decades. This is mainly due to improvements in the extent and quality of wastewater treatment, the reduction of phosphorus in detergents and the implementation of the Nitrates Directive (European Council, 1991). However, diffuse pollution from agriculture remains a significant pressure in more than 40% of European water bodies (European Environment Agency, 2015). Beside fertilisers, more than 200,000 tonnes of agricultural pesticides (active ingredients) are used annually in Europe (EUROSTAT, 2007) and can enter surface

waters, where they pose significant risks of acute and chronic toxicity (Malaj et al., 2014; Stehle and Schulz, 2015).

Both nutrient and pesticide pollution can affect the ecosystem process of litter decomposition (LD) in streams. Litter is a pivotal energy source for local and downstream food webs in the first tens of kilometres (Wallace et al., 1997; Webster, 2007). Fungi and bacteria are the main microbial decomposers and they convert leaves into a more nutritious food resource for invertebrate decomposers, also termed shredders (Gessner et al., 2007). Benthic algae, which are part of aquatic biofilms, may also play a role in LD even in systems dominated by heterotrophic processes. Algae can stimulate LD by the exudation of labile carbon, which can be used by decomposers and contribute to the mineralization of recalcitrant organic-matter (Guenet et al., 2010). This process, called “priming effect”, has been suggested to increase LD under low nutrient levels (Danger et al., 2013). Fungi typically contribute more than bacteria to microbial LD (Gulis and Suberkropp, 2003; Pascoal and Cássio, 2004) and invertebrate LD, in turn, can greatly exceed microbial LD (Hieber and Gessner, 2002). Nonetheless, the density of invertebrates is governed by several biotic and abiotic factors, which may lead to complex spatial and temporal patterns in their relative contribution to LD (Graça and Canhoto, 2006). Benthic algae can be expected to alleviate seasonal differences in the food supply for invertebrates because they represent an alternative food source for some opportunistic invertebrate detritivores (such as some caddis flies). For the same reason algae might contribute to the attenuation of fungicide effects in the invertebrate food web in streams by preventing steep population declines due to starvation or malnutrition of invertebrates following the reduction of fungal or bacterial LD.

Elevated nutrient concentrations, mainly phosphorous and nitrogen, can accelerate LD in streams (Pascoal and Cássio, 2004; Robinson and Gessner, 2000), whereas eutrophic and hypertrophic states can dramatically reduce LD (Baldy et al., 2007; Woodward et al., 2012). Both acceleration and reduction of LD can affect the availability of allochthonous organic carbon and as a consequence lead to changes in decomposer communities and the subsequent food web (Woodward et al., 2012). Higher LD under nutrient-enriched environments is typically linked to higher microbial biomass and production (Pascoal and Cássio, 2004), which subsequently can result in higher invertebrate biomass (France, 2011). Parallel, elevated nutrient concentrations increase the biomass of benthic algae in the biofilms (Elser et al., 2007; Smith, 2003), providing a better food supply for invertebrates. This positive effect on invertebrates can be suppressed at high levels of nitrate or ammonia due to direct toxicity (Camargo et al., 2005) or to oxygen depletion driven by algal blooms (Smith, 2003), albeit this rarely occurs in small streams. On the other hand, fungicides can adversely affect non-target freshwater fungi (Dijksterhuis et al., 2011) and they have been reported to alter fungal community structure and decrease fungal biomass both under laboratory and field conditions (Bundschuh et al., 2011; Fernández et al., 2015), which may lead to a decrease in microbial LD (Artigas et al., 2012; Rasmussen et al., 2012a). In addition, invertebrates have shown a feeding preference for leaves that have not been exposed to fungicides under laboratory conditions (Bundschuh et al., 2011) and can be directly affected by fungicides at concentrations in the order of tens of µg L-1 (Flores et al., 2014; Zubrod et al., 2014). These high fungicide concentrations in stream water only occur under extreme weather conditions in temperate agricultural regions (Bereswill et al., 2012), but can occur more frequently in regions such as the tropics (e.g. banana plantations in Costa Rica; Castillo et al., 2006). Fungicides may also affect biofilms and subsequently alter aquatic food webs

LD by changing the outcome of interactions between fungi and algae. Both groups may compete for inorganic nutrients and space within the biofilm, as suggested by bacteria-algae interactions (e.g. Daufresne and Loreau, 2001). Consequently, a selective impairment of fungi would positively affect algae by releasing them from competition.

Given that both fungicides and nutrients are of agricultural origin, they are likely to co-occur in agricultural streams. However, their potential interaction effects on decomposer communities have rarely been studied. For example, nutrient enrichment, which typically accelerates LD, could either compensate for a fungicide-induced reduction in LD or non-compensate because of the competitive advantages of algae in using the nutrients while suffering less from fungicides than fungal decomposers. In addition, ecological surprises, in terms of unforeseen synergistic effects, could occur (Segner et al., 2014). We studied potential interaction effects of nutrients and fungicides on benthic algae, decomposers and LD in a mesocosm experiment, where invertebrates, leaves colonised by microbes, and stones colonised by a more autotroph biofilm were exposed to minimum and maximum field levels of fungicides and to natural vs. elevated nutrient concentrations. We hypothesised that (1) fungicides alter the fungal community, decrease fungal biomass and increase algae biomass, (2) these effects would propagate to weight gain of invertebrate shredders and contribute to a reduced LD and (3) nutrients increase microbial biomass (bacteria, fungi, algae) and increase LD, which may partially compensate potential reductions from fungicide exposure.

4.3 Material and methods

4.3.1 Experimental design

The experiment followed a two-factorial design consisting of minimum and maximum field levels of nutrients and fungicide toxicity (Appendix C, Figure C.1), derived from a study in the wine-growing area of Rhineland-Palatinate (Fernández et al., 2014). The low-nutrient level corresponded to 3 and <0.01 mg L^{-1} of nitrate and phosphate, respectively, whereas 10 and 0.35 mg L^{-1} were used as high-nutrient level. The two fungicide levels were no fungicides and a mixture of eight polar organic fungicides at field-relevant concentrations (Table 4.1). This design resulted in four treatments: low nutrient and no fungicides (LN-NF), low nutrient and fungicides (LN-F), high nutrients and no fungicides (HN-NF) and high nutrients and fungicides (HN-F). The experiment was conducted in 24 stainless-steel artificial stream channels (hereafter called mesocosms; Appendix C, Figure C.2) in April and May 2014 at the Eußerthal Ecosystem Research Station, South West Germany (49°15' 14" N, 7° 57' 42" E), inside the Palatinate Forest Nature Park, which is an extensively-forested low-mountain range. The mesocosms had a volume of 72 litres (length: 1.2 m, width: 0.3 m, height: 0.2 m) and were placed outdoor adjacent to a pristine stream to mimic field conditions (day-night light cycle and air temperature variation). However, they were covered with an awning for protection against strong rainfall events and direct sunlight, which would otherwise be likely to overheat the mesocosms. Each mesocosm was filled with 40 litres of pre-filtered (0.2 mm µm sieve, to prevent unintended insertion of macroinvertebrates) stream water (pH=7.02, conductivity=124 µS/cm) and a paddle wheel created a circular flow of 0.08 m/s (0.03 m/s inside the enclosures, described below), which is similar to low flowing sections of the adjacent stream. These hydraulic conditions lead to a dissolved oxygen concentration of 11.46 mg L^{-1} ± 0.39 standard deviation. Six replicates of each of the four

treatments were randomly distributed across the 24 artificial streams. LN-NF was considered as control treatment where non-manipulated stream water was used. The experiment was run twice for 14 days (run 1 and run 2), separated by 1 week, to increase replication. Mean water temperature was 9.93°C ± 0.25 and 10.80°C ± 0.19 during run 1 and 2, respectively.

Table 4.1: Fungicides and concentrations used in this study.

Fungicide	Concentration ($\mu g\ L^{-1}$)
Azoxystrobin	7.27
Boscalid	1.69
Cyprodinil	3.50
Fludioxonil	2.00
Kresoxim-methyl	0.19
Metrafenone	1.91
Myclobutanil	5.38
Tebuconazole	0.94

At the start of the experiment, a solution containing sodium nitrate and anhydrous disodium phosphate was added to the high-nutrient mesocosms to yield the above-mentioned nutrient concentrations. Two enclosures (length: 0.3 m, width: 0.15 m, height: 0.15 m) constructed from 250 µm stainless steel mesh were placed in each mesocosm. Each enclosure contained 10 gammarids (*Gammarus fossarum*) and three trichopterans (two individuals of *Potamophylax cingulatus* and one of *Sericostoma personatum*) collected in the adjacent stream (Appendix C, Figure C.1), together with the pre-conditioned leaf material from a leaf bag (see section "Leaf decomposition" for details). The selected invertebrates were typical representatives of shredders in the study region and were introduced in abundances relative to the abundances of the source stream. Invertebrates were collected, irrespective of sex, to yield a relatively homogeneous sample of individuals of the largest available size class. Given the lower invertebrate densities in the source stream, the variability in size classes was higher for the two trichopterans species, particularly during the first run. In addition, two stones from the adjacent stream were placed in each mesocosm outside the enclosures at the beginning of the first run to monitor algal biomass. Stones were selected to exhibit minimal differences in the initial biomass of biofilm. After 24 hours of invertebrate acclimatisation, 2 ml of a fungicide mixture (Table 4.1) in methanol was added to each fungicide mesocosm. No-fungicide mesocosms received 2 ml of methanol only to account for potential effects of the solvent. Water was renewed in all mesocosms after a 4-day exposure and spiked again with nutrients in high-nutrient mesocosms as outlined above. Due to technical difficulties, the fungicide boscalid was not included in run 1. After approximately two weeks from the start of the experiment the following endpoints were analysed: bacterial density, algal biomass, fungal biomass and sporulation, microbial community structure, invertebrate weight gain and LD.

4.3.2 Microbial endpoints

At the end of the experiment, the remaining leaf material of one randomly chosen enclosure in each mesocosm was used to determine the following microbial endpoints:

bacterial density, fungal biomass and microbial community structure. A cork borer was used to randomly cut 1-cm diameter leaf discs, which were treated as follows: 5 were preserved in formalin to determine bacterial density, 3 were immediately frozen for later analysis of microbial community structure via denaturing gradient gel electrophoresis (DGGE) and other 3 were submerged in distilled water to promote fungal sporulation and subsequently determine fungal community structure via conidia identification. The remaining leaf material was used to determine fungal biomass. Besides, in run 1, periphyton was brushed off from stones to analyse algal biomass. Sample processing for each endpoint is described below.

4.3.3 Bacterial density

Bacterial density was determined by epifluorescence microscopy as described by Buesing (2005). Briefly, formalin-preserved cells were detached from leaf discs by ultrasonication, filtered on Anodisc membrane filters (Whatman, UK), stained with SYBRGreen II, and automatically counted on digital photographs using an image-analysis system (AxioVision 4.8, Carl Zeiss).

4.3.4 Algal biomass

Chlorophyll a was quantified to determine benthic algal biomass. The upper part of each stone was brushed off separately with a plastic brush and subsequently a suspension was created with tap water. The volume of the suspension was measured and transferred to a beaker, where it was stirred to homogenise. An aliquot of 2 ml was transferred to an Eppendorf cap and centrifuged at 13,000 rpm for 3 min in a centrifuge (Mikro 200R, Hettich Zentrifugen, Tuttlingen, Germany). After discarding the supernatant, samples were shock-frozen in liquid nitrogen and stored at -80 °C until analysis. Before analysis, samples were homogenised in 500 µl buffered 96% ethanol (1 g $MgCO_3$ L^{-1}) using a dispersing device (IKA-ULTRA-TURRAX® T8, IKA®-Werke GmbH & Co. KG, Staufen, Germany). The disperser was rinsed with another 500 µl of buffered ethanol. Chlorophyll *a* concentration and turbidity were assessed in the resulting volume (1 ml of ethanol) using a photometer (Specord 200 plus, Analytik Jena, Jena, Germany) measuring absorbance at 665 nm (chlorophyll *a*) and 750 nm (turbidity). Concentrations were expressed per unit of area. The sampled area of each stone was determined by wrapping the brushed-off parts of the stone in aluminium foil, cutting off all protruding folds and edges, weighing the foil and comparing it with that of a reference foil with known dimensions.

4.3.5 Fungal biomass

Leaf-associated fungal biomass was estimated from ergosterol content, which is a cell-membrane component of eumycotic fungi, following the method of Gessner and Schmitt (1996). Briefly, ergosterol was extracted in alkaline methanol, purified by solid-phase extraction (Sep-Pak Vac RC tC18 500 mg sorbent, Waters) and quantified by high-performance liquid chromatography (1200 Series, Agilent Technologies).

4.3.6 Fungal sporulation

Sporulation of fungal species was determined via conidium identification as described by Bärlocher (1982). Three leaf discs were placed individually in a six-well-plate containing

4 ml of distilled water per well. After incubation for 96 h in darkness on an orbital shaker at 55 rpm, 0.5 ml of lactophenol cotton blue were added to fix samples and to stain the spores. Subsequently, one squared cm of the slide was analysed by identifying (e.g. Ingold, 1975) and counting conidia under a light microscope at 100-fold magnification. For each mesocosm and taxon, the mean abundance from the three replicates was used in analysis.

4.3.7 Microbial community structure

DGGE was used to assess microbial community structure (Duarte et al., 2012). First, 4 freeze-dried leaf disks from 2 different samples (2 disks per sample) of the same treatment and run were combined to a single composite sample. Microbial DNA was extracted from each composite sample using an UltraClean® Soil DNA Isolation Kit (MoBio, Carlsbad, CA, USA). Next, two polymerase chain reactions (PCR) were performed in a T100^{TM} Thermal Cycler (BioRad Laboratories, Hercules, CA, USA) to amplify fungal and subsequently bacterial DNA. The primer pair ITS3GC/ITS4 was used to amplify the ITS2 region of fungal rDNA (White et al., 1990), whereas the pair 338GC/518 was used to amplify the V3 region of bacterial 16S rDNA (Muyzer et al., 1993). DGGE analyses were performed using a DCode^{TM} Universal Mutation Detection System (BioRad Laboratories, Hercules, CA, USA). DNA from the amplification products (20 µl of each product, ca. 700 ng) were loaded on 8% (w/v) polyacrylamide gels in 1x Tris-acetate-EDTA (TAE) with denaturing gradients from 30% to 60% (fungi) and from 40% to 65% (bacteria), with 100% denaturant corresponding to 40% formamide and 7 M urea. Gels were run at 55V, 56 °C for16 h and stained for 10 min with 1x Midori Green in TAE 1x. Finally, images from the gel were taken under UV light in a ChemiDoc^{TM} XRS Molecular Imager® (BioRad Laboratories, Hercules, CA, USA).

4.3.8 Invertebrate weight gain

Length-dry mass relationships were used to evaluate initial invertebrate weight. Gammarid length was measured as the distance from the head to the end of the abdomen, excluding appendices. For trichopterans, the diameter of the case opening was used as predictor of dry mass as in previous studies (e.g. Campos and González, 2009; Canhoto, 1994; Martins et al., 2014) because it is a less invasive measure than body parameters such as head capsule width. For gammarids, the length at the beginning of the experiment was determined via image analysis with ImageJ using photos taken over a distance scale (Schneider et al., 2012). The length-dry mass relationship was established from additional individuals of the three invertebrate species (165 *G. fossarum*, 58 *P. cingulatus* and 23 *S. personatum*). A non-linear regression model was fitted to the data and subsequently used to predict the initial dry mass (Y) from the initial length or the initial case opening diameter (X) using the following power function:

$$Y = a \cdot X^{b} \quad (1)$$

where the parameters a and b were obtained via the non-linear least squares method, using the coefficients of a linear model with the log-transformed data as start values. The power-function parameters for the invertebrate initial-weight assessment are shown in Appendix C, Figure C.3. The weight gain was assessed for each species and enclosure by subtracting the mean value of the predicted initial dry weight from the mean value of the measured final dry weight, which was determined at the end of the experiment from all

organisms after drying at 60°C for 48 h. The weight gain was divided by the total experimental time per enclosure to account for differences in the termination of the experiment of up to 16 h. Finally, the weight gain was averaged per mesocosm from the two enclosures.

4.3.9 Leaf decomposition

Ten days before the start of each run, 53 leaf bags (2-mm mesh size) containing 3 (± 0.05 standard deviation; n=106) g of dried (at 60°C for 24 h) alder leaves (*Alnus glutinosa*), which were collected in October 2013 at the time of abscission and stored at -21°C, were submerged in the adjacent stream for ten days to allow for microbial colonisation. Subsequently, the remaining leaf material from 48 leaf bags was transferred to the 48 enclosures, whereas 5 bags were immediately returned to the laboratory to determine leaf mass loss during microbial colonisation. At the end of the experiment, the remaining leaf material from one randomly chosen enclosure from each mesocosm was gently rinsed to remove mineral particles, oven-dried at 60 °C for 24 h and weighed to the nearest 0.001 g. The leaf decomposition rate k per sum of degree days and unit of invertebrate biomass ($ddays^{-1}g^{-1}$) was estimated for each enclosure i as follows:

$$k_i = \frac{\left(\frac{-\ln\left(\frac{S_i(t)}{S_i(0)} \right)}{\Sigma_{j=1}^{t} \bar{T}_i(j)} \right)}{\sum_{z=1}^{3} (M_z \times N_z)^{0.75}} \quad (2)$$

where S is the leaf mass as a function of deployment time t, M_z is the mean average weight of individuals from the invertebrate species z in the enclosure i, N_z is the number of organisms of the species z in the enclosure i (see below) and $\bar{T}$ is the mean temperature for a day j. $S_i(0)$ is the leaf mass at the start of the experiment, which was obtained by subtracting losses during colonisation from the initial dry mass. The coefficient 0.75 to the power of M describes a relationship between body size and metabolic rate, which applies across most groups of organisms (Brown et al., 2004). We standardised leaf decomposition by metabolism because individuals escaped from the enclosures, presumably due to poor sealing. N was calculated as the number of remaining individuals at the end of the experiment plus half of the number of escaped individuals, assuming that they escaped at the middle of the experiment. Enclosures with less than six gammarids or less than two trichopterans were excluded from the analysis.

4.3.10 Fungicide analysis

Water samples for fungicide analysis were taken 24 (48 hours in run 1) and 72 hours after the addition of the fungicide mixture. Samples and standards were analysed using a LC-HRMS Exactive system from Thermo® (Thermo Fisher Scientific System; Dreiech, Germany) conformed by a quaternary Accela® pump and an Exactive® Orbitrap MS detector (Thermo Fisher Scientific, Waltham, USA). Separation of the pesticides was achieved on a 50×2.1 mm Thermo Hypersil GOLD^{TM} column (1.9 µm particle size); the

flow rate was 200 µl min^{-1}. The mobile phase consisted of 0.1% formic acid and 4 mM NH_4 formiate in methanol (A) and 0.1% formic acid and 4 mM NH_4 formiate in water (B) at flow rate 0.2 ml/min. The gradient started with 3 min 5% A – 95% B, followed by 7 min of 100% A. The column was re-equilibrated by 2 min of 5% A – 95% B. All solvents used were LC-MS grade (Carl Roth, Karlsruhe, Germany). The Orbitrap mass analyzer was operated in the full scan mode and all ions generated in the ion source were detected. The ions were trapped in the Orbitrap around a central electrode. The oscillation of the ions was used for determination of specific transition masses. All pesticides showed a linear range from 0.5 – 100 µg L^{-1}. Matrix effects of stream water were estimated using 3 different concentrations within the working range (1, 5 and 10 µg L^{-1}) and were found to be negligible (values around ± 10% for most compounds).

4.3.11 Physicochemical variables

Dissolved oxygen, conductivity and pH were measured using a multiparameter analyser Multi 340i (WTW, Weilheim, Germany). Flow velocity was measured with a flow meter (Höntzsch, Waiblingen, Germany). Temperature was recorded continuously (every 15 min) in each mesocosm using loggers (HOBO Pendant, Synotech, Hückelhoven). Nitrate and phosphate concentrations were measured from water samples taken approximately 24 and 120 hours after nutrient additions in run 1 and 2, respectively, using continuous flow analysis in an AutoAnalyzer 3 (Seal Analytical, Norderstedt, Germany). Nitrate and phosphate measurements were in agreement with DIN EN ISO 13395:1996 and DIN EN ISO 15681-2: 2003, respectively.

4.3.12 Data analysis

ANOVA analyses were used to determine the influence of experimental factors (nutrients and fungicides) on the following biotic endpoints: invertebrate weight gain, leaf decomposition, fungal biomass and bacterial density. For each endpoint, data from the two different runs were centred on the same mean and analysed jointly. Models were checked for unusual observations (leverage, outliers) and residuals were tested for normality and homogeneity of variance. Differences over 20% with respect to the control treatment were reported irrespective of statistical significance, together with the results from power analyses (the percentage of change required to achieve statistical significance at $\alpha = 0.05$ and ß = 0.8; R package “pwr”; Champely, 2015).

Microbial community response to the experimental factors was analysed using Redundancy Analysis (RDA), both for conidia and DGGE data. Regarding DGGE data, the relative intensity of the bands in the images was used as response variable for the multivariate analysis, as it is related to the relative abundance of the operational taxonomic units (OTUs; Duarte et al., 2009). Rare taxa occurring in less than 5% of the samples were removed before RDA and taxon data were Hellinger transformed to achieve standardisation and circumvent the problems associated with using the Euclidean distance for ecological data (Legendre and Gallagher, 2001). Nutrients and fungicides were included as categorical explanatory variables with two levels (high and low for nutrients, fungicides and no fungicides for fungicides). Automatic model building with stepwise model selection was performed to identify the best-fit model (R package “vegan”; Oksanen et al., 2010). DGGE data from each run was analysed separately because OTU numbers can slightly differ between runs. Conidium data from the two runs was analysed jointly after partialling out the effect of run (Peres-Neto et al., 2006).

Moreover, cluster analyses were performed with DGGE data to quantify the magnitude of community changes based on species abundances. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used and similarity matrices were computed using the Pearson coefficient. Clusters were performed using BioNumerics software (version 5.00 Applied Maths, Sint-Martens-Latem, Belgium). All other statistical analyses were conducted in R version 3.1.2 (R Core Team, 2014). Computer code and data are provided for reproducibility of the analysis (SD computer code).

4.4 Results

Measured fungicide concentrations (Appendix C, Figure C.4) indicated minor degradation for most pesticides during the 4-day exposure. Nutrient measurements, which were added on the first and fifth day, revealed that nitrate concentration remained stable, whereas phosphate declined by approximately 90% 5 days after addition (not shown).

4.4.1 Effects on microorganisms

ANOVA analysis showed a statistically significant effect of nutrients on bacterial density ($p < 0.001$). Nonetheless, the increment in the HN-NF and HN-F treatments (67% and 74%, respectively) was still insufficient to reach statistical significance in pairwise comparisons (> 78% needed, Figure 4.1a). *Chlorophyll a* concentration showed high variability within treatments and was relatively similar across treatments, with a slight (24%) increase under high nutrients (> 154% needed for statistical significance, Figure 4.1b). Fungal biomass was 40% higher than the control in the HN-F treatment (> 57% needed, Figure 4.1c).

In community analysis with RDA, nutrient level was the only significant predictor for fungal sporulation ($p = 0.001$, Figure 4.2a). Conidia from *Tetrachaetum elegans* and *Articulospora tetracladia* were more abundant in low nutrient treatments, whereas *Clavatospora longibrachiata* increased in high nutrient treatments. For the DGGE data, both runs produced similar results. Fungicides were the only significant predictor of fungal community composition based on the DGGE data ($p = 0.036$ in run 1 and $p = 0.025$ in run 2, Figure 4.2b, Appendix C Figure C.5). The bacterial community was significantly affected by fungicides ($p = 0.001$ in run 1 and $p = 0.010$ in run 2), nutrients ($p = 0.048$ in run 1 and $p = 0.024$ in run 2) and their interaction ($p = 0.063$ in run 1 and $p = 0.026$ in run 2, Figure 4.2c, Appendix C Figure C.5). Cluster analyses of the DGGE data supported the RDA findings and showed more pronounced differences in community structure for bacteria: the two most different groups of fungal samples exhibited 80% similarity, whereas the two most different groups of bacterial samples showed 50-70% similarity (Appendix C, Figures C.6 and C.7).

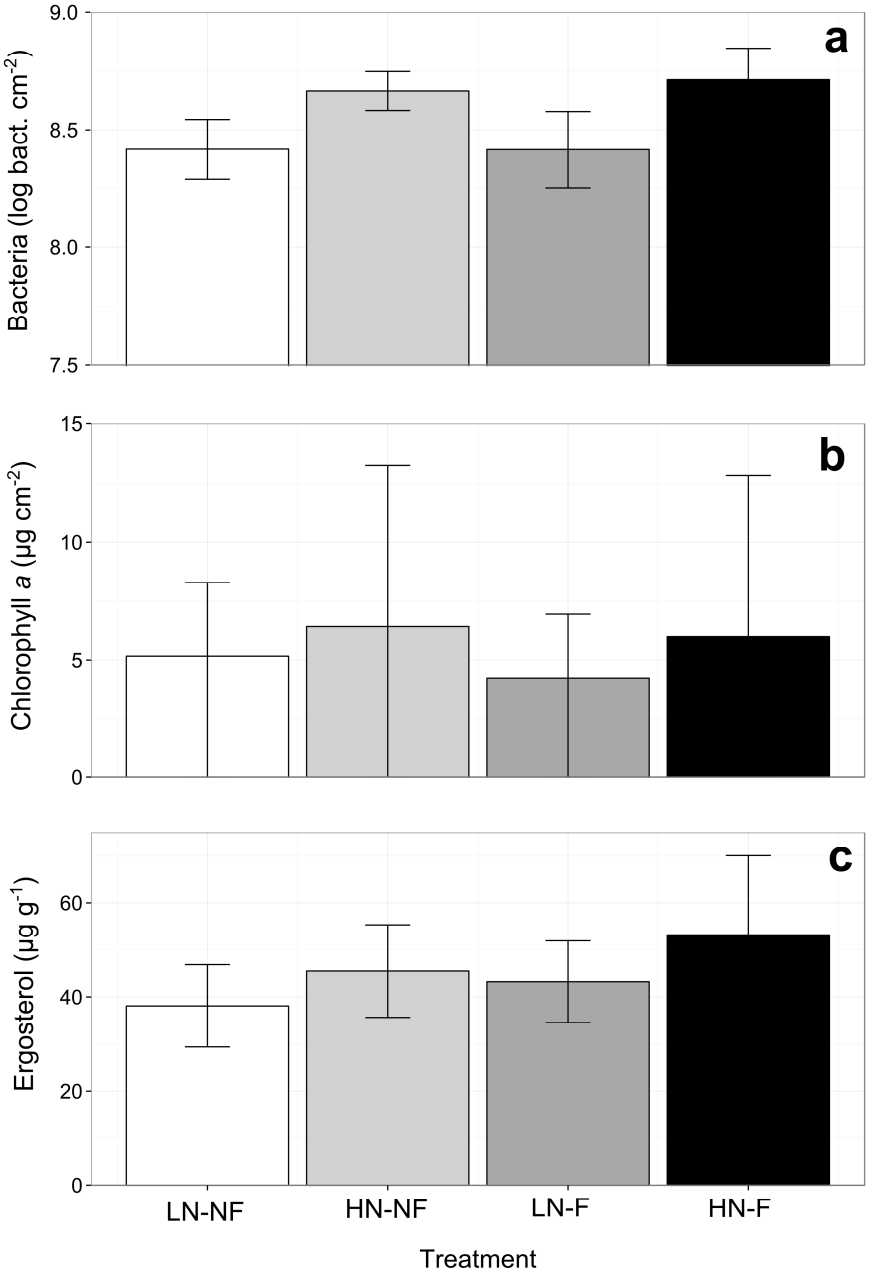

Figure 4.1: Mean value (with 95% CI) for bacterial density (a), chlorophyll *a* (b) and fungal biomass expressed as ergosterol content (c). Treatments: low nutrients and no fungicides (LN-NF, white), high nutrients and no fungicides (LN-F, light grey,), low nutrients and fungicides (HN-NF, dark grey) and high nutrients and fungicides (HN-F, black).

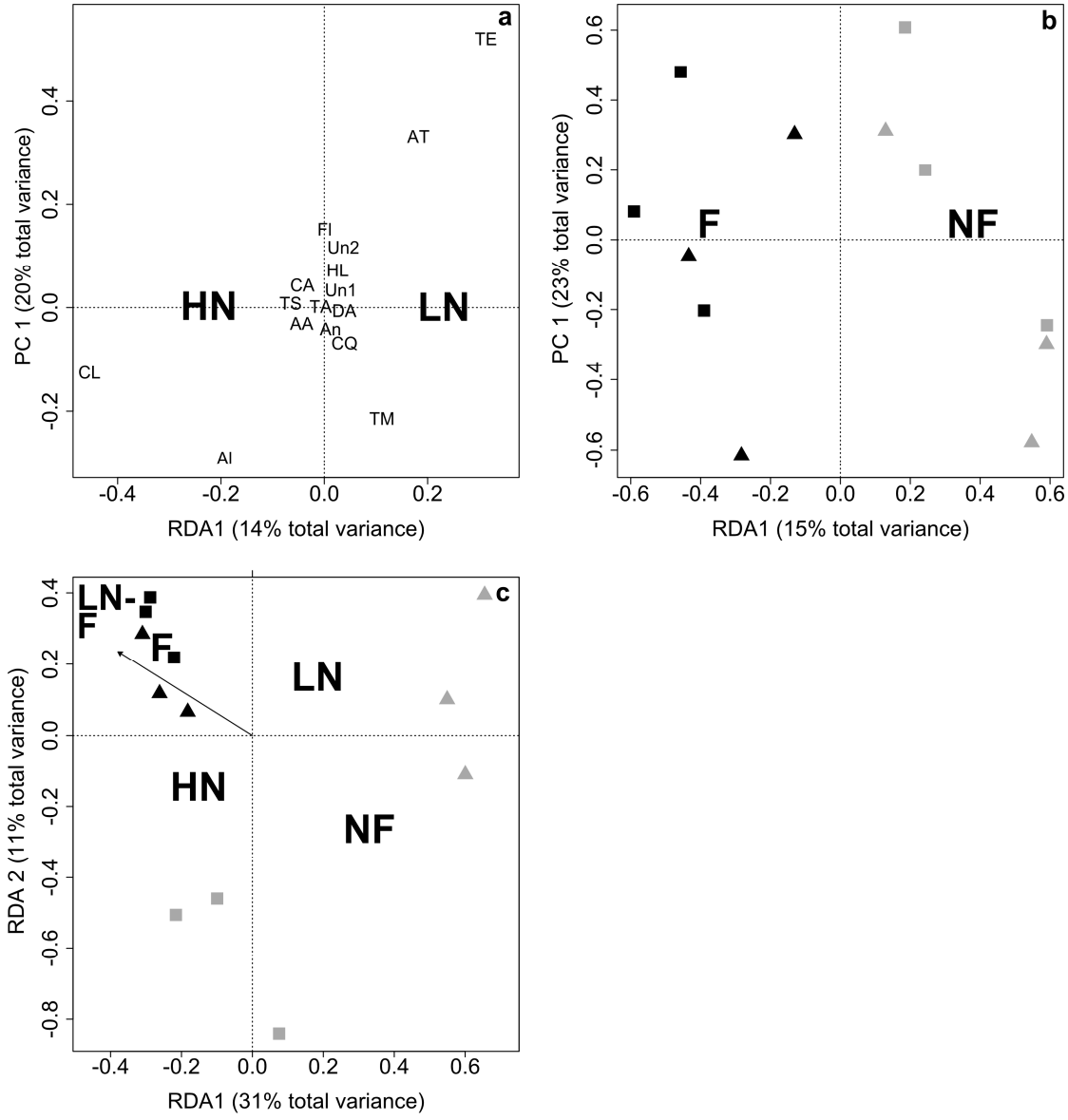

Figure 4.2: Triplots from redundancy analysis with fungal and bacterial species as response variables and the explanatory variables selected in automatic model building for a) fungal species (conidia data) from both runs after partialling out the effect of run. Species abbreviations: *Alatospora sp.* (Al), *Anguilospora sp.* (An), *Articulospora atra* (AA), *Articulospora tetracladia* (AT), *Clavariopsis aquatica* (CA), *Clavatospora longibrachiata* (CL), *Culicidospora aquatica* (CQ), Dactylella aquatica (DA), *Flagelospora sp.* (Fl), *Heliscus lugdunensis* (HL), *Heliscella stellata* (HS), *Lunulospora curvula* (LC), *Lemonniera terrestris* (LT), *Tricladium angulatum* (TA), *Tricladium splendens* (TS), *Tetracladium marchalianum* (TM), *Tetrachaetum elegans* (TE) and two unknown species (Un1 and Un2). b) Fungal species (DGGE OTUs) from first run (black symbols = fungicides, grey symbols = no fungicides, squares = high nutrients, triangles = low nutrients). c) Bacterial species (DGGE OTUs) from first run. Symbology as in 2b. Treatments: low nutrients (LN), high nutrients (HN), no fungicides (NF), fungicides (F).

4.4.2 Effects on invertebrates and LD

Invertebrate weight gain was similar across treatments (Figure 4.3a-c). Nonetheless, HN-NF and HN-F treatments resulted in a 61% and 37% higher body mass of *S. personatum* than in the control treatment, respectively (> 126% needed for statistical significance, Figure 4.3c). Similarly, leaf decomposition rate was similar across treatments, although it was 23% higher than the control in the HN-NF treatment (> 48% needed for statistical significance, Figure 4.3d).

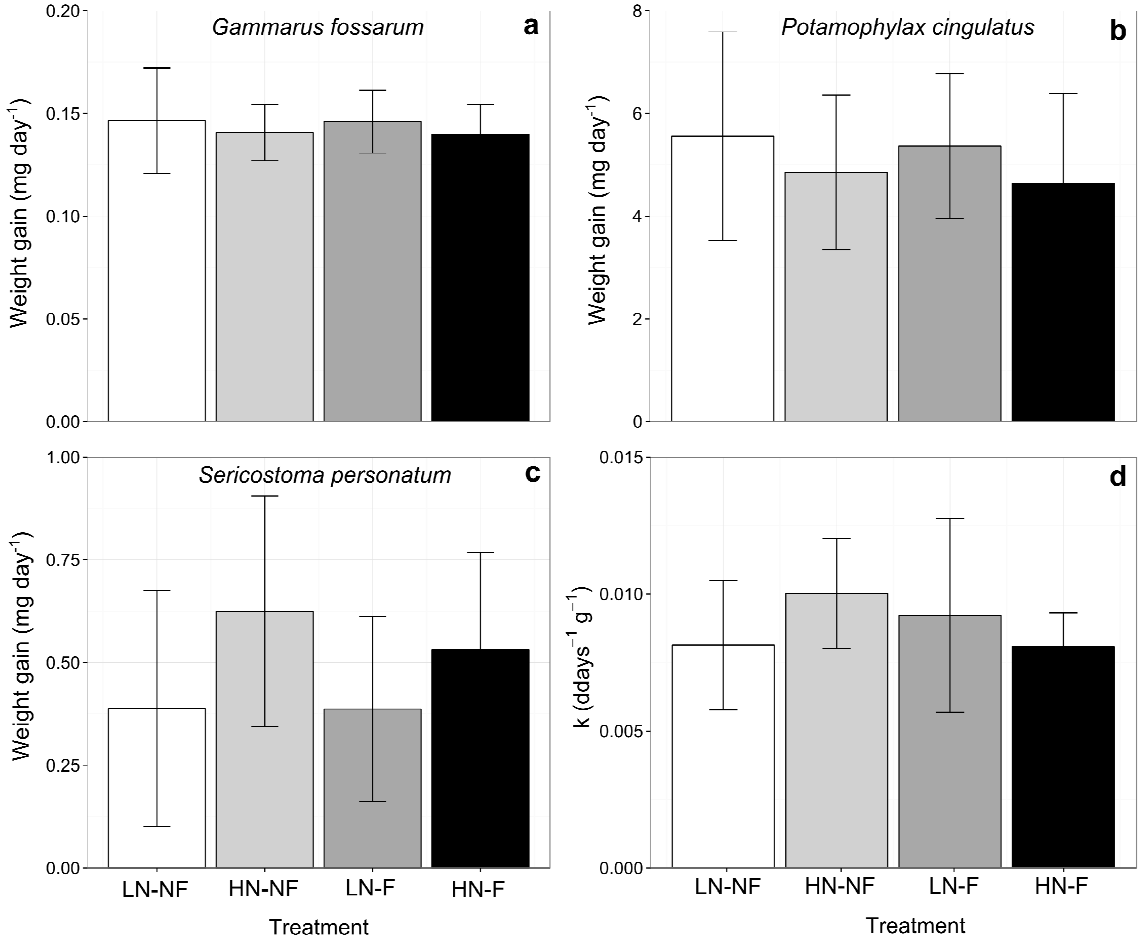

Figure 4.3: Mean value (with 95% CI) for weight gain of *Gammarus fossarum* (a), weight gain of *Potamophylax cingulatus* (b), weight gain of *Sericostoma personatum* (c) and leaf decomposition rate per gram of invertebrate biomass (d). Treatments: low nutrients and no fungicides (LN-NF, white), high nutrients and no fungicides (HN-NF, light grey,), low nutrients and fungicides (LN-F, dark grey) and high nutrients and fungicides (HN-F, black).

4.5 Discussion

4.5.1 Effects on microorganisms

Our first hypothesis that fungicides alter fungal community composition was supported by the community analyses of the DGGE data, which showed changes in the relative abundance of fungal species. Nevertheless, fungal sporulation showed no significant response to fungicides. Besides, fungal biomass did not decrease and algal biomass did not increase significantly under fungicide exposure, indicating no change in the outcome of competition between fungi and algae within the biofilm. On the contrary, higher fungal biomass (40%) was observed for the HN-F treatment, though not statistically significant. The lack of effects on sporulation contrasted with the results of Bundschuh et al. (2011), but they used 1 to 2 orders of magnitude higher fungicide concentrations. Moreover, microbial endpoints were analysed nine days after the end of fungicide exposure and recovery might have occurred, which can explain the non-response of fungal sporulation. Water renewal could also have contributed to recovery through the immigration of non-exposed microorganisms. Although algae might have also recovered to some extent, heterogeneous light conditions across mesocosms may explain the high within-treatment variability in algal biomass and masked potential effects of treatment factors. The increase in fungal biomass might be explained by a community change driven by the combination of fungicides and high-nutrient conditions leading to a community with faster growing species, resulting in higher fungal biomass. Other explanation for increased biomass is stimulation of fungal growth at low fungicide concentrations, a phenomenon generally termed hormesis (Calabrese et al., 1987), which has been recently reported for tebuconazole (Zubrod et al., 2015). In this regard, ergosterol protects fungi against oxidative stress (Dupont et al., 2012) and it might be that some fungicides stimulate ergosterol synthesis to some extent.

Considering that we have tested fungicide toxicity levels associated with effects on microbial and invertebrate decomposer communities, fungal biomass and LD in a field study (Fernández et al. 2014, 2015), we expected a similar response of the fungal community in our mesocosm study. However, in the mesocosms, community changes were less pronounced than those observed in the field and not found for sporulation. In addition, the decrease of fungal biomass was not observed. A major source of divergence when comparing mesocosms to the field situation may be that we tested a single fungicide pollution event, in contrast to the recurrent episodic pollution to which stream decomposer communities are exposed each year during the application period (Roßberg, 2010), which may result in more pronounced and more persistent community changes.

In contrast to our hypothesis, fungi did not show higher biomass under nutrient enrichment, whereas the hypothesised increase in bacterial density was observed. Bacterial community structure, however, was affected by both experimental factors and their interaction. The lack of a positive relationship between fungal biomass and nutrients contrasts with most studies (e.g. Pascoal and Cássio, 2004; Suberkropp et al., 2010). Our result can be explained by a higher invertebrate feeding activity that could mask nutrient stimulation of fungal biomass (Robinson and Gessner, 2000), though only *S. personatum* exhibited enhanced weight gain under high nutrient concentrations. Besides, phosphorous has been suggested to play a major role in enhancing microbial biomass (Connolly and Pearson, 2013) and its concentrations decreased in our study. Although fungal community composition based on DGGE data did not respond significantly to nutrients, fungal

sporulation did. *C. longibrachiata* released more spores under high-nutrient conditions, whereas *T. elegans* and *A. tetracladia* showed the opposite response. Although these results partly match those of other studies, comparisons are hampered by the fact that those studies were done under field conditions (e.g. Pascoal and Cássio, 2004) or used different leaf species (e.g. Gulis and Suberkropp, 2003). With respect to other components of the microbial community, our results matched the positive effects of nutrients on bacterial density reported in several studies (e.g. Fernández et al., 2015; Pascoal and Cássio, 2004) but did not agree with the typical increase in benthic algal biomass (e.g. Biggs, 2000; Dodds et al., 2002). This latter issue may be explained because the endpoint was analysed at the end of the experiment, more than a week after the second and last nutrient addition, when phosphate concentrations had decreased. Nonetheless, some authors suggest that hydrology and light exert stronger influence on benthic algae than nutrients (e.g. Figueroa-Nieves et al., 2006).

4.5.2 Effects on invertebrates

Contrary to our hypothesis, fungicides did not adversely affect invertebrate weight gain. This may be because our fungicide toxicity concentrations were below the effect threshold for invertebrates (Cuppen et al., 2000; Flores et al., 2014; Zubrod et al., 2015). Indirect effects may also have played a minor role because fungicide-driven changes in microbial biomass were minor. Regarding nutrients, we hypothesised that enrichment would increase microbial production and in turn enhance invertebrate weight gain. Nonetheless, only *S. personatum* showed increased weight gain under high-nutrient conditions. This result suggests a species-dependant response of the invertebrate community to nutrient enrichment that may have complex effects on LD. Besides and as discussed above, the increase in microbial production was rather moderate for fungi (19% and 40% in the HN-NF and HN-F treatments, respectively), though more pronounced for bacteria (67% and 74%). Finally, the heterogeneity in the size class of trichopterans in run 1 may have introduced variability in the weight gain of these taxa, due to the inverse relationship between body mass and growth rate (Campos and González, 2009; Scriber and Slansky, 1981). Besides, the predictive power of case opening for the weight of *P. cingulatus* (Appendix C, Figure C.3) was relatively low and resulted in additional variability (Figure 3). This suggests that this measure may require more replicates than body dimensions for certain species.

4.5.3 Effects on LD

In contrast to our first hypothesis, LD did not decrease in the fungicide treatments. This may be explained by the little change observed in the microbial community and the almost absent effect on invertebrates. Besides, considering that invertebrate LD can greatly exceed microbial LD (Hieber and Gessner, 2002), changes in the microbial community may have not propagated to total LD. However, contrasting results regarding the relative contribution of microorganisms and invertebrates to LD have been reported in the literature and differences have been suggested to originate from different invertebrate densities (Graça, 2001). Our results contrasted with laboratory studies reporting negative effects of fungicides on microbial LD (Artigas et al., 2012; Rasmussen et al., 2012a). Nonetheless, these studies used longer experimental periods and tested concentrations of fungicides in the order of tens of $\mu g\ L^{-1}$.

Our reference field study (Fernández et al., 2015), despite being associated with much

lower fungicide concentrations than those typically tested in the laboratory, did show a decrease in microbial LD along the toxicity gradient. On the contrary and in line with the results from this study, no relationship of water-borne fungicide pollution with invertebrate LD was observed. Whereas similar results were reported by Rasmussen et al. (2012b), Schäfer et al. (2007) found a negative effect of pesticides on invertebrate LD due to the decrease in the abundance of pesticide-sensitive species. However, in the latter two field studies not only fungicides but also herbicides and insecticides contributed to total pesticide pollution. Also here the comparison of the field situation with our mesocosm experiment is hindered by the lack of some processes typically occurring in the field, such as the above mentioned recurrent episodic exposure to fungicides or recolonisation. Thus, longer experiments addressing these shortcomings are required to predict long-term effects on decomposition and decomposers and to unravel underlying mechanisms.

An increase of LD was observed in the HN-NF treatment (23%), though not statistically significant. This weak effect may be attributed to the short duration of our study. Studies reporting increased microbial LD rates following nutrient enrichment (Gulis and Suberkropp, 2003; Pascoal and Cássio, 2004; Robinson and Gessner, 2000) are typically longer than two weeks. Another important aspect is the relative contribution of each nutrient to LD. Ferreira et al. (2006) reported a strong asymptotic relationship between nitrate and decomposition of alder leaves in Portuguese streams, in which nitrate highly stimulate LD within the range 0 – 1 mg nitrate L^{-1}. Minor improvement in LD is obtained over this threshold, suggesting a negligible role of nitrate enrichment in streams with natural concentrations matching the conditions of our experiment. On the other hand, Connolly and Pearson (2013) reported that phosphorous governed acceleration in LD and those effects were detectable in the range of tens of µg L^{-1}. Thus, phosphate may have caused the higher LD in the above mentioned treatment, although depletion may have limited the effect.

4.5.4 Conclusions

Despite the lack of significant differences on LD hindered the elucidation of potential compensatory effects of nutrients on fungicide-driven impacts, our experiment showed that both experimental factors affect the microbial community differently and can alter decomposer community structure. Specifically, fungal community structure responded stronger to fungicides than to nutrients, whereas the opposite pattern was found for sporulation. Bacterial community structure was affected by both factors, although only nutrients influenced bacterial density. Though not observed in this study, changes in the microbial community may propagate to negative effects on invertebrates and on LD, considering that stream decomposer communities are exposed to recurrent fungicide pollution and we did not test worst-case scenarios. Finally, longer experiments are needed to study the effects of community change on LD.

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5. General discussion

5.1 Episodic exposure to fungicides in agricultural streams

The two different techniques used in our field study to assess polar organic fungicide concentrations in stream water, namely event-driven water sampling (EDS) and passive sampling with styrene-divinylbenzene reverse phase sulfonated disks (SDB disks), were in good agreement (chapter 2). EDS exceeded time-weighted average (TWA) concentrations on average by a factor of 3 and these differences showed high variability among compounds. EDS exceedance was within the expected range (up to 12-fold, sensu Schäfer et al., 2008), as EDS was assumed to capture a few-hour peak, whereas passive samplers provided with a TWA concentration for 2 days (deployment duration) and in this period the concentration may drop to less than 10% with respect to the peak (Leu et al., 2004).

The calibration information generated for the SDB disks revealed a 5-day duration of the linear uptake for most compounds, what indicates suitability for characterising short-peak exposures. Nonetheless, these passive samplers can be used for longer monitoring periods by using a diffusion-limiting membrane, which will increase the time where the sampler operates in the linear uptake but also slow down the uptake of analytes (Schäfer et al., 2008a). Sampling rates were assessed under climatic and hydraulic conditions typical for small low-slope streams flowing across temperate agricultural areas and ranged from 0.26 to 0.77 Ld-1. Comparison with the few available sampling rates in the literature was not possible due to differences in sorbent material, sampling devices and environmental conditions. Apart from the valuable calibration information generated for the SDB disks, a free-software solution to derive sampling rates under time-variable exposure is provided as supplementary data of the manuscript that constitutes chapter 2 (Fernández et al., 2014).

Mean and maximum peak (EDS) concentrations were under 1 and 3 µg/l, respectively. This is in agreement with other studies reporting on our set of fungicides (Battaglin et al., 2011; Bereswill et al., 2012; Herrero-Hernández et al., 2013; Reilly et al., 2012; Wightwick et al., 2012), although most of them were not focused on rainfall-triggered contamination. In our study, these fungicide concentrations were associated with a relatively even distributed rainfall along the application period, whereas rain after long dry periods can lead to an order of magnitude higher concentrations (Bereswill et al., 2012).

Overall, we demonstrated that SDB disks can be used for the quantification of episodic exposure to fungicides. Monitored concentrations can be analysed jointly with biological endpoints *in situ* to generate valuable ecotoxicological information, as intended in this thesis. Besides, passive sampling can serve as alternative to flow-proportional sampling, which is generally costly because it requires on-site power supply. However, other approaches such as time-proportional sampling are required to properly cover the temporal variability of pollution (Bundschuh et al., 2014). In this regard, current monitoring approaches of fungicides and other chemical mixtures, such as the monitoring of pollutants within the Water Framework Directive (WFD) frame, can be optimised by selecting:

- Locations with high probability of exceedance of regulatory thesholds. Several criteria should be considered when performing this task, such as the percentage and type of agricultural land upstream a monitoring point and the application rates

and toxicity of the pesticides associated to those crops. For instance, the typical application rates for strobilurins and triazoles (0.13 to 0.25 kg/ha) are an order of magnitude lower than those of dithiocarbamates (1.5 – 3.5 kg/ha; Russell, 2005). Besides, threatened lotic ecosystems which are rare or have high ecological value deserve special attention.

- Representative monitoring points. Chemical mixtures which are frequent in time or space deserve prior attention. This task implies spatial analysis at scales that can exceed the boundaries of local administrations. Thus, coordination and collaboration between different administrative levels may be required.

- Sampling frequencies in agreement with the temporal variability of pollution. In the case of fungicides, monitoring efforts should focus on the fungicide application period and the following weeks.

5.2 Effects of fungicides on microbial decomposers

In the field study (chapter 3), changes in fungal communities were analysed via conidia identification. Sporulation was promoted in the laboratory from leaf material retrieved in the field. Differences in conidia abundance were found, suggesting differences in community composition (Bärlocher, 2005) despite this endpoint is poorly related with the mycelial biomass of the fungal species in the leaf material (Bermingham et al., 1997). Redundancy analysis showed that toxicity originating from polar organic fungicides was the major driver of these changes. The causality of this finding is supported by the additional laboratory experiment in chapter 3. However, in our mesocosm experiment examining potential interaction effects of nutrients and fungicides (chapter 4), nutrients governed differences in conidia abundance. In this latter experiment fungicide-driven changes may have been overruled by nutrient effects: whereas in the field changes were more pronounced because the fungal communities may respond to years of recurrent episodic fungicide pollution during the application period (Roßberg, 2010), the single episodic pollution event in the mesocosm experiment may have lead only to slight changes. This hypothesis is supported by the molecular analysis (DGGE), which provides a more accurate picture of the fungal community and the relative abundance of fungal species in comparison to conidia identification. DGGE revealed a minor but significant fungicide-driven change in the mesocosm experiment. Nonetheless, recurrent episodic fungicide pollution may also lead to pollution-induced community tolerance (Blanck et al., 1988) and this was not covered by the mesocosm experiment. Overall, these results suggested that fungal community structure respond to fungicides, while sporulation responded strongest to nutrients. Fungicide and nutrient-derived changes in conidia abundance partly matched those reported in other studies on fungicides (Bundschuh et al., 2011; Zubrod et al., 2015) and nutrients (Gulis and Suberkropp, 2003; Pascoal and Cássio, 2004), with differences originating probably from different experimental conditions.

Beside changes in fungal composition, fungicide toxicity in the field was associated with a decrease in fungal biomass, which has also been previously reported under laboratory conditions (e.g. Bundschuh et al., 2011). In the field experiment, linear regression models attributed this effect solely to fungicide toxicity, despite they included multiple physico-chemical and habitat variables as predictors. On the contrary, this negative effect of

fungicides was not observed in the mesocosm experiment. Instead, higher fungal biomass was observed when fungicides were combined with high nutrients. This may be explained by a fungicide-driven community change leading to a community with faster growing species, resulting in higher fungal biomass. Other explanation for increased biomass is stimulation of fungal growth at low fungicide concentrations, a phenomenon generally termed hormesis (Calabrese et al., 1987) (Calabrese et al., 1987), which has been recently reported for tebuconazole (Zubrod et al., 2015). In this regard, ergosterol protects fungi against oxidative stress (Dupont et al., 2012) and it might be that some fungicides stimulate ergosterol synthesis to some extent. Moreover, the fungal endpoints were analysed nine days after the end of the fungicide exposure and limited recovery might have occurred, which can explain the non-response of fungal sporulation. Finally, fungal communities in agricultural streams are exposed to recurrent episodic fungicide pollution during the application period (Roßberg, 2010), which may result in more pronounced and more persistent community changes, and explain the difference to the laboratory.

Bacterial density also increased along the gradient of fungicide toxicity covered in the field study, although linear regression showed that nutrients were stronger predictors of this endpoint. Specifically, bacterial density was positively associated with nutrients, as reported in several studies (e.g. Pascoal and Cássio, 2004). This was also observed in the mesocosm study, where the increase was slightly higher in the presence of fungicides, potentially because fungi become less competitive for nutrients under these conditions (Frey-Klett et al., 2011). Bacterial community structure was only analysed in the mesocosm study, where it was affected by nutrients and fungicides. Significant effects of nutrients in bacterial stream communities are frequent in the literature (see Zeglin, 2015), whereas little is know about the effects of fungicides. In contrast to our results, bacterial community did not significantly respond in a recent study on tebuconazole (Dimitrov et al., 2014).

Future research on fungicide effects on microbial decomposers should focus on the community level to address the shortcomings of single-species tests (e.g. McClellan et al., 2008). Because of the importance of species identity to provide with functional redundancy in processing stream litter, results may highly depend on community composition. Therefore, a deeper knowledge on the ecology and distribution of aquatic fungi may be necessary to select representative fungal communities in future studies. Traditional fungal identification techniques, such as those based on conidia identification, are able to detect changes in the structure of the community but fail on providing an accurate characterisation of those changes. Thus, the scientific community should take advance of available molecular techniques, such as DGGE and next-generation sequencing techniques, which can serve as an accurate and useful tool to describe the structure of fungal communities. Finally, taking mode of action into account when analysing ecotoxicological data (e.g. by aggregating toxicity only from fungicides with the same mode of action) should facilitate to unravel the effect mechanisms of chemical mixtures.

5.3 Effects of fungicides on invertebrate decomposers

The invertebrate shredder community in our field study was highly dominated by gammarids in most sampling sites. Though measured fungicide concentrations were below effect thresholds for gammarids (Zubrod et al., 2015, 2014), water-borne fungicide

toxicity was associated with the distribution of the three dominant gammarid species across the study area. However, it did not influence gammarid feeding rate. This latter endpoint was negatively associated with sediment-borne copper concentrations, which ranged from 100 mg Cu/kg sediment in unpolluted or slightly polluted sites to more than 400 mg/kg in polluted sites. Decreasing abundance and diversity of crustaceans and other macroinvertebrates have been reported in other studies at sediment copper concentrations detected also during the present study (Kraft and Sypniewski, 1981; Mebane, 2002). Nonetheless, the association should be interpreted with caution because sediment copper toxicity for aquatic organisms is governed by the availability of free copper ions in the water column (Kramer et al., 2004), which in turn depend on dissolved organic carbon and pH (De Schamphelaere and Janssen, 2004). In the mesocosm study, water-borne fungicides did not adversely affect weight gain of the three invertebrate species used in the experiment (one gammarid species and two trichoptera species). This may be explained by the fungicide concentrations used, which were below the effect threshold for invertebrates (Cuppen et al., 2000; Flores et al., 2014; Zubrod et al., 2015). Indirect effects may also have played a minor role because fungicide-driven changes in microbial biomass and microbial community structure were minor during this experiment.

5.4 Effects of fungicides on litter decomposition

A reduction of microbial-mediated litter decomposition of up to 40% was found along the water-borne fungicide pollution gradient covered in the field study. Observed changes in fungal community structure and the decrease in fungal biomass may explain this reduction. However, this explanation relies on the assumption that fungi are more important than bacteria for litter decomposition, which has been shown in several studies (Duarte et al., 2010; Pascoal and Cássio, 2004), though similar importance of fungi and bacteria has been reported occasionally (Hieber and Gessner, 2002). Thus, it remains also speculative whether the increase in bacterial density of up to a half logarithmic unit in sites with high fungicide toxicity partially buffered the decrease in microbial-mediated litter decomposition.

Regression models did not support the hypothesis that fungicide-driven changes in microbial communities propagate to a reduction in microbial-mediated litter decomposition. Instead, they indicated that microbial decomposition activity was largely driven by physico-chemical variables. This should be interpreted with caution and against the results from previous studies (Rasmussen et al., 2012b; Schäfer et al., 2012a) due to the relatively low sample size in our field study. This mismatch between changes in the structure of the decomposer community structure and the process of litter decomposition itself can be explained by (1) the integrative nature of ecosystem processes that are influenced by a magnitude of environmental variables (Tank et al., 2010) and by (2) the fact that the response of ecosystem processes depends rather on changes in the functional composition of communities than on the taxonomic composition (Vandewalle et al., 2010). Moreover, the toxic unit approach used in this study may be oversimplified and not accurately capture complex fungicide mixtures occurring in the field and their effect on ecosystem processes. For example, the sumTU assumes additive mixture effects (concentration addition), whereas non-additive effects of fungicide mixtures can occur (Zubrod et al., 2015).

In contrast to microbial decomposition activity, invertebrate-mediated decomposition was not related to water-borne fungicide pollution in the field study. This contrasts with our expectations, as fungal species preferred by gammarids were more abundant in non or low-polluted sites, which in turn indicates that food intake may not be affected by the fungal community composition if no alternative food is present (Arsuffi and Suberkropp, 1989; Jabiol and Chauvet, 2012). The absence of detectable effects may be explained by (1) the fact that measured fungicide concentrations were below effect thresholds for gammarids, as mentioned above and by (2) the temporal mismatch between pesticide sampling and litter decomposition assessment. Nonetheless, gammarid feeding measured *in situ* was similarly unrelated to fungicide toxicity either, despite being measured in concert with pesticide sampling. Our results contrast with previous field studies reporting a decrease in invertebrate-mediated decomposition because of the loss of pesticide-sensitive species (Schäfer et al., 2012a, 2012b). However, these studies reported data from agricultural areas with a more diverse shredder community, whereas a high dominance of gammarids was found in our study area. Another explanation for the lack of effects on invertebrate-mediated decomposition in our field study is that litter decomposition and decomposers were sampled in autumn, whereas effects could occur earlier in the year (Schäfer et al., 2012b). Thus, sampling in autumn implies that (i) insect shredders may have emerged and that the shredder community can be more heterogeneous and potentially more sensitive during other seasons, and (ii) the exposure to fungicides was lower than in summer. Our results match instead those of Rasmussen et al. (2012b), where a *G. pulex*-dominated shredder community in Danish agricultural streams was unrelated to a pesticide gradient of a comparable toxic unit range. In this latter study, they found a positive correlation of invertebrate-mediated decomposition with the density of gammarids, whereas in our field study this association was not statistically significant. Despite being unrelated to water-borne fungicide pollution, both invertebrate-mediated decomposition and the gammarid feeding rate were negatively associated with sediment-borne copper concentrations. This may be a consequence of the negative effects of copper on invertebrates discussed in the previous section.

In the mesocosm study treatments did not show statistically significant differences in litter decomposition, hindering the elucidation of potential compensatory effects of nutrients on fungicide-driven impacts. The lack of fungicide effects may partly be explained by the chosen level of fungicide toxicity, which was representative of the episodic fungicide exposure monitored in the field study but well below the concentrations used in laboratory studies reporting a fungicide-driven reduction in microbial decomposition activity (Artigas et al., 2012; Rasmussen et al., 2012a). These studies used longer experimental periods and tested concentrations of fungicides in the order of tens of µg L-1. The duration of the experiment can also explain the lack of statistically significant effects of nutrients. Studies reporting increased microbial decomposition rates following nutrient enrichment (Gulis and Suberkropp, 2003; Pascoal and Cássio, 2004; Robinson and Gessner, 2000) are typically longer than two weeks. Overall, the mesocosm experiment showed that both nutrients and fungicides affect decomposers differently and can alter decomposer community structure, although longer experiments are needed to study the effects of community change on litter decomposition.

5.5 Conclusions

Our results suggest that polar organic fungicides in streams change the structure of fungal communities. These changes may propagate to litter decomposition, as fungal species show different degradative capabilities (Duarte et al., 2006; Suberkropp and Arsuffi, 1984; Zemek et al., 1985). However, other studies suggest a certain level of functional redundancy at community level. In this respect, Ferreira and Chauvet (2012) performed a microcosm experiment in which community performance on litter decomposition was not related to the identity of the dominant species. Differences may ultimately originate from differences in the pool of fungal species involved in each study, which highlights the importance of species identity.

Whether other effects observed in our field study, such as reduced fungal biomass, increased bacterial density or reduced microbial-mediated litter decomposition can be attributed to fungicides remains speculative. Contrasting results on these issues have been reported in the literature and therefore further investigations are required. Effects may ultimately depend on the structure and composition of decomposer communities, the composition of the fungicide mixture, the exposure regime and the environmental factors influencing survival and recovery of decomposers. Thus, further studies should include representative field surveys in terms of fungicide pollution and physical, chemical and biological conditions. This should be combined with experiments under controlled conditions to test for the causality of field observations.

At management level, special attention should be paid to the environmental awareness of farmers and to the promotion of “precision agriculture”, in which crop management practises are optimised in time and space based on local characteristics such as topography, soil type, climate or drainage systems (Bramley, 2009). Despite technical advances, the increasing human pressure on ecosystems and resources worldwide jeopardize the health of nature and humans at global scale (Rockström et al., 2009). Therefore, the traditional approach of transforming natural resources to satisfy human needs should be merged with a new one, in which the size and behaviour of human population also take into account the boundaries of ecosystem resilience and natural resources.

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Appendix A

Supplementary information for chapter 2

Table A.1: Physicochemical properties of test pesticides.

Pesticide	Type	Log K_{OW} [a]	Log K_{OC} [a]	Molecular weight (g/mol) [a]	Water solubility (mg/L) [a]	Half-life (DT50; days) [a]	
						Photolysis	Hydrolysis
Azoxystrobin	F	2.50	2.77	403.40	6.70	8.7	stable
Boscalid	F	2.96	2.91	343.21	4.60	30	stable
Cyprodinil	F	4.00	3.23	225.29	13	7.5	stable
Dimethoate	I	0.70	1.04	229.26	39,800	175	68
Dimethomorph	F	2.68	2.54	387.86	28.95	97	70
Fenhexamid	F	3.51 (3.26)	2.68	302.20	20	0.05	stable
Fludioxonil	F	4.12	4.88	248.19	1.80	10	stable
Imidacloprid	I	0.57	2.34	255.66	610	0.2	stable
Indoxacarb	I	4.65	3.81	527.83	0.20	3	22
Iprovalicarb	F	3.20	2.03	320.43	17.8	stable	stable
Kresoxim-methyl	F	3.40	2.49	313.35	2	18.2	35
Metalaxyl-M	F	1.71	2.82	279.33	26,000	stable	stable
Metrafenone	F	4.30	3.49	409.3	0.49	6.2	stable
Myclobutanil	F	2.89	2.71	288.78	132	15	stable
Pyrimethanil	F	2.84	2.48	199.11	121	stable	stable
Quinoxyfen	F	4.66	4.36	308.13	0.05	0.8	stable
Tebuconazole	F	3.70	2.89	307.82	36	stable	stable
Tebufenpyrad	I	4.93	3.78	333.8	2.39	stable	stable
Tolyfluanid	F	3.90	3.51	347.27	0.90	stable	1.9

F: fungicide; I: insecticide.

[a] Data obtained from the Pesticide Properties Database (http://sitem.herts.ac.uk/aeru/footprint/index2.htm). Partition coefficient in brackets is normalized to the fraction of the neutral species at the pH of the river water used in the calibration experiment (pH 7), using the equation for the ionization corrected octanol-water partition coefficient $D_{OW} = 1/(1 + 10^{(7\text{-}pKa)})K_{OW}$. Dissociation constant value for fenhexamid available from: http://toxnet.nlm.nih.gov/index.html. Water solubility measured in water at 20°C. DT50 = half life time.

Table A.2: Environmental variables characterising the 17 sampling sites included in this study.

Variable	Minimum	Maximum	Median	Mean	SD
Stream width (m)	0.80	7.30	1.67	2.21	1.61
Stream depth (m)	0.07	0.43	0.15	0.19	0.10
Current velocity (m/s)	0.01	0.67	0.23	0.26	0.17
Temperature (°C)	11.21	13.77	12.62	12.50	0.81
pH	7.51	8.26	7.87	7.85	0.24
Oxygen (mg/L)	5.30	10.61	9.60	9.10	1.30
Conductivity (µS/cm)	110	1290	332	481	340
NO_2 (mg/L)	0.00	0.80	0.04	0.09	0.19
NO_3 (mg/L)	2	60	5	9	14
PO_4 (mg/L)	0.10	0.60	0.20	0.25	0.13
NH_4 (mg/L)	0.00	0.20	0.00	0.01	0.05
Riffles sections (%)	0	100	80	70	36
Pool sections (%)	0	100	20	30	36
Leaves and wood (< 10 cm dieameter)[a]	1	3	1	-	-
Wood (> 10 cm dieameter)[a]	1	2	1	-	-
Filamentous algae[a]	0	2	0	-	-
Macrophytes[a]	0	2	0	-	-
Shading[a]	2	5	4	-	-
Left bank cover[a]	0	5	3	-	-
Right bank cover[a]	1	5	2	-	-
Boulder (%)	0	65	5	13	18
Cobble (%)	0	60	15	18	17
Pebble (%)	0	50	5	14	16
Gravel (%)	0	30	0	5	9
Sand (%)	0	100	30	32	37
Silt (%)	0	100	0	18	37

[a] Measured using an ordinal scale indicating the coverage, ranging from 0 (absent) to 5 (very high)

Table A3: Characteristics of the rainfall events in which the pesticide sampling was carried out.

Starting date	Duration (days)	Days without rainfall before	Maximum intensity (mm/day)	Amount of rainfall (mm/event)
12/07/12	4	3	7 - 12	13 - 32
03/08/12	2	5	6 - 10	13 -18
21/08/12	2	5	2 - 22	6 - 23
22/09/12	3	12	10 - 13	20 - 29

Table A4: Analytical data for test pesticides (part 1).

Pesticide	Recovery (%)		2nd extraction MeOH (%)[b]	m/z[c]
	SDB disk[a]	EDS		
Azoxystrobin	69 ± 4	32 ± 16	2	404.12
Boscalid	100 ± 19	46 ± 8	3	343.04
Cyprodinil	60 ± 6	18 ± 10	NA	226.13
Dimethoate	62 ± 6	33 ± 23	2	230.07
Dimethomorph	85 ± 8	21 ± 10	2	388.13
Fenhexamid	127 ± 19	39 ± 6	3	302.07
Fludioxonil	101 ± 10	42 ± 8	4	266.07
Imidacloprid	71 ± 10	67 ± 12	2	256.06
Indoxacarb	68 ± 7	NA	2	528.08
Iprovalicarb	79 ± 2	53 ± 3	3	312.22
Kresoxim-methyl	71 ± 5	15 ± 10	3	314.14
Metalaxyl-M	63 ± 4	63 ± 6	4	280.15
Metrafenone	64 ± 5	11 ± 7	4	409.06
Myclobutanil	87 ± 10	48 ± 2	4	289.12
Pyrimethanil	43 ± 8	26 ± 11	NA	200.12
Quinoxyfen	63 ± 4	NA	17	308.00
Tebuconazole	71 ± 4	24 ± 2	9	308.15
Tebufenpyrad	66 ± 6	23 ± 9	4	334.17
Tolyfluanid	48 ±13	27 ± 8	1	346.99

NA = not applicable

[a] Recovery of the extraction assuming 100% of transference form the sorbent to the solvent.
[b] Analyte found in the SDB disks afeter a second extraction with MeOH (in percentage with respect with the analyte extracted in the first extraction).
[c] Specific transition masses of precursor ions

Table A4: Analytical data for test pesticides (part 2).

Pesticide	LOQ (ng/L)[a]		Ratio EDS/SDB [b]	Matrix effects (reduction in %)		
	SDB disk	EDS		SDB disk	EDS	Water
Azoxystrobin	6	1	1.36	-62	-78	<15
Boscalid	6	5	2.06	-60	-79	<15
Cyprodinil	6	1	11.82	-50	-83	<15
Dimethoate	10	1	NA	-48	-84	<15
Dimethomorph	6	1	2.53	-59	-83	<15
Fenhexamid	6	3	2.60	-49	NA	<15
Fludioxonil	5	1	2.19	-66	-77	+25
Imidacloprid	8	5	1.09	-38	-82	<15
Indoxacarb	7	NA	NA	-39	-76	-58
Iprovalicarb	7	2	0.58	-54	-76	<15
Kresoxim-methyl	6	20	2.34	-54	-85	<15
Metalaxyl-M	7	1	2.92	-49	-84	<15
Metrafenone	5	6	3.03	-46	-82	<15
Myclobutanil	6	1	1.69	-55	-71	<15
Pyrimethanil	9	1	4.15	-60	-88	<15
Quinoxyfen	5	NA	NA	-58	-76	+31
Tebuconazole	5	1	1.76	-41	-77	<15
Tebufenpyrad	4	5	NA	-37	-67	<15
Tolyfluanid	3	40	NA	-49	-78	<15

NA = not applicable

[a] LOQ = Limit of quantification for a sample obtained with the respective method.
[b] Ratio between the concentrations derived with EDS and SDB. Only those samples with values above the LOQ for the two methods were included in the assessment. Results are shown only for those fungicides with at least three available sample

Figure A.1: Land-use composition derived from Corine Land Cover and location of the sampling sites in the study area.

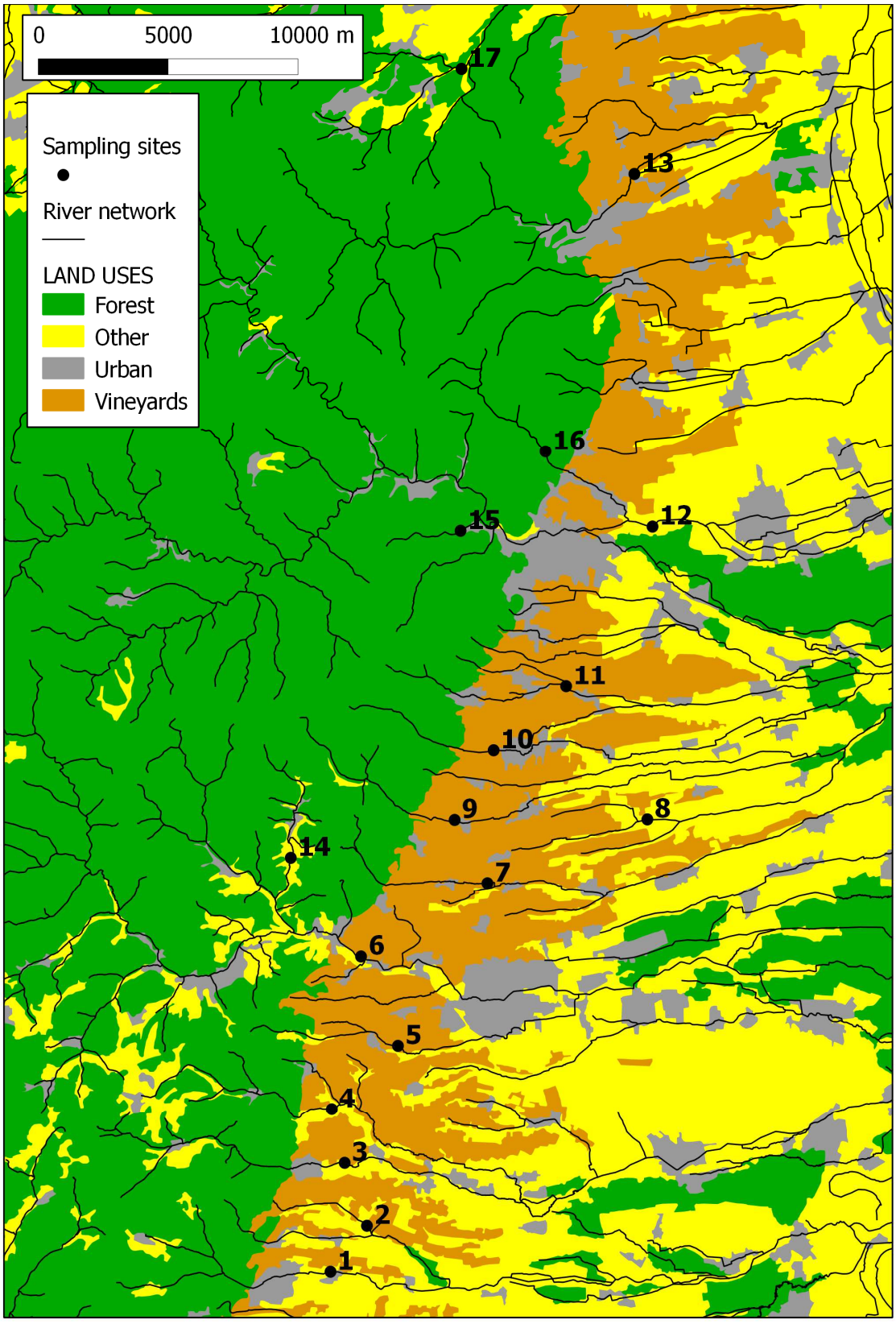
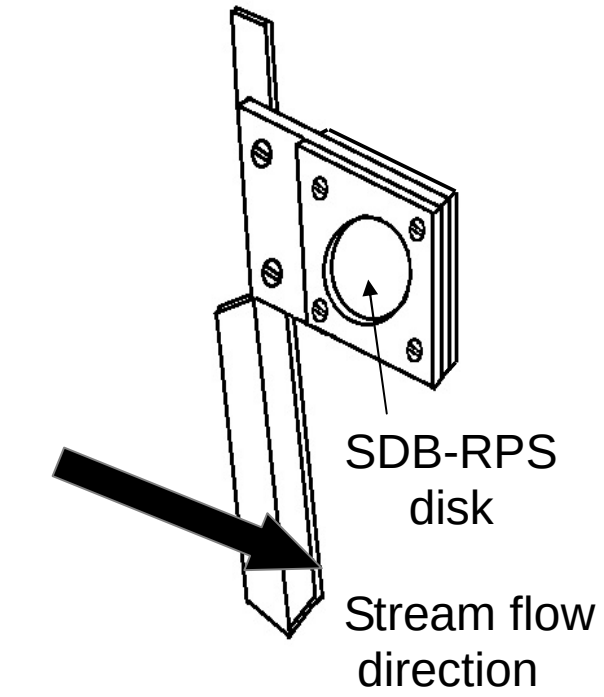


Figure A.2: Metal holder used to deploy SDB disks in the streams.



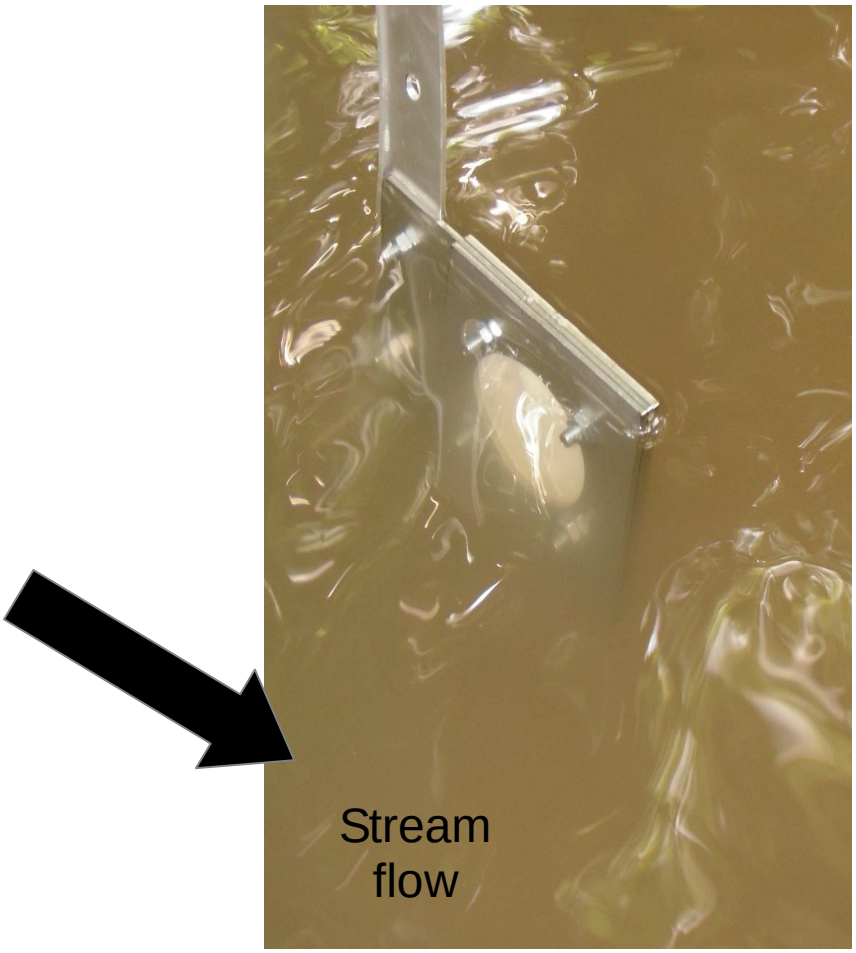


Figure A.3: Artificial channels used in the calibration experiment.

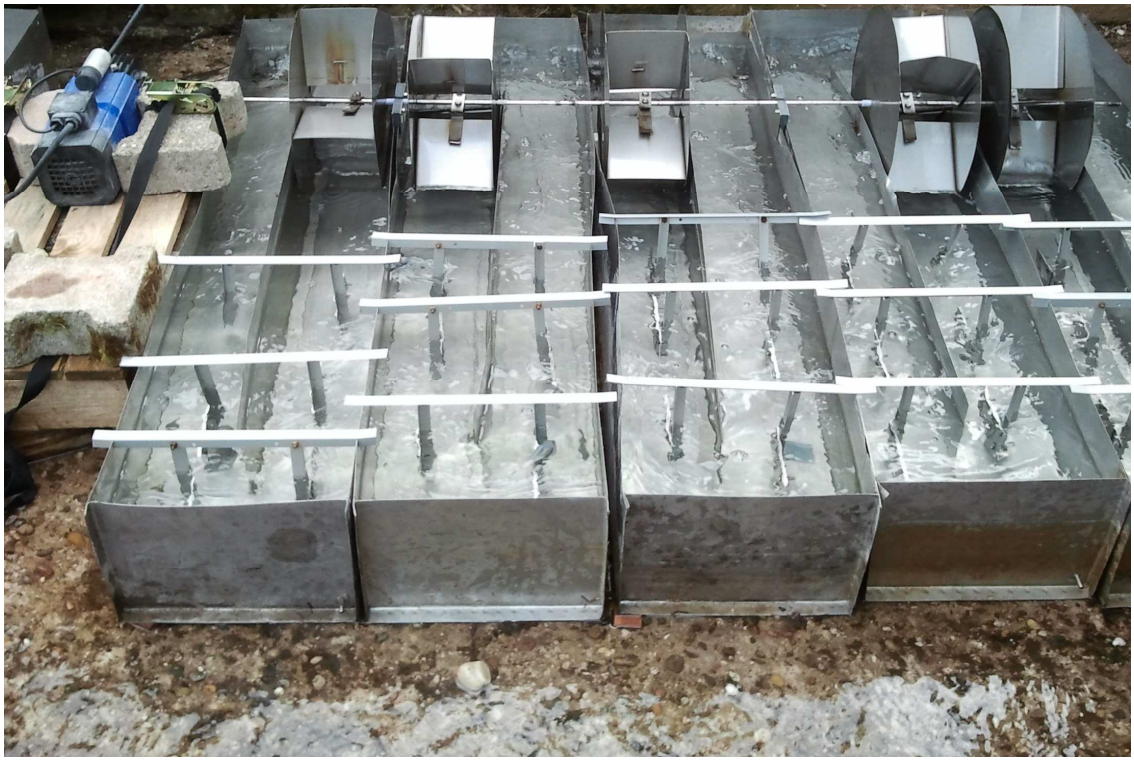


Figure A.4: Event-driven water samplers:

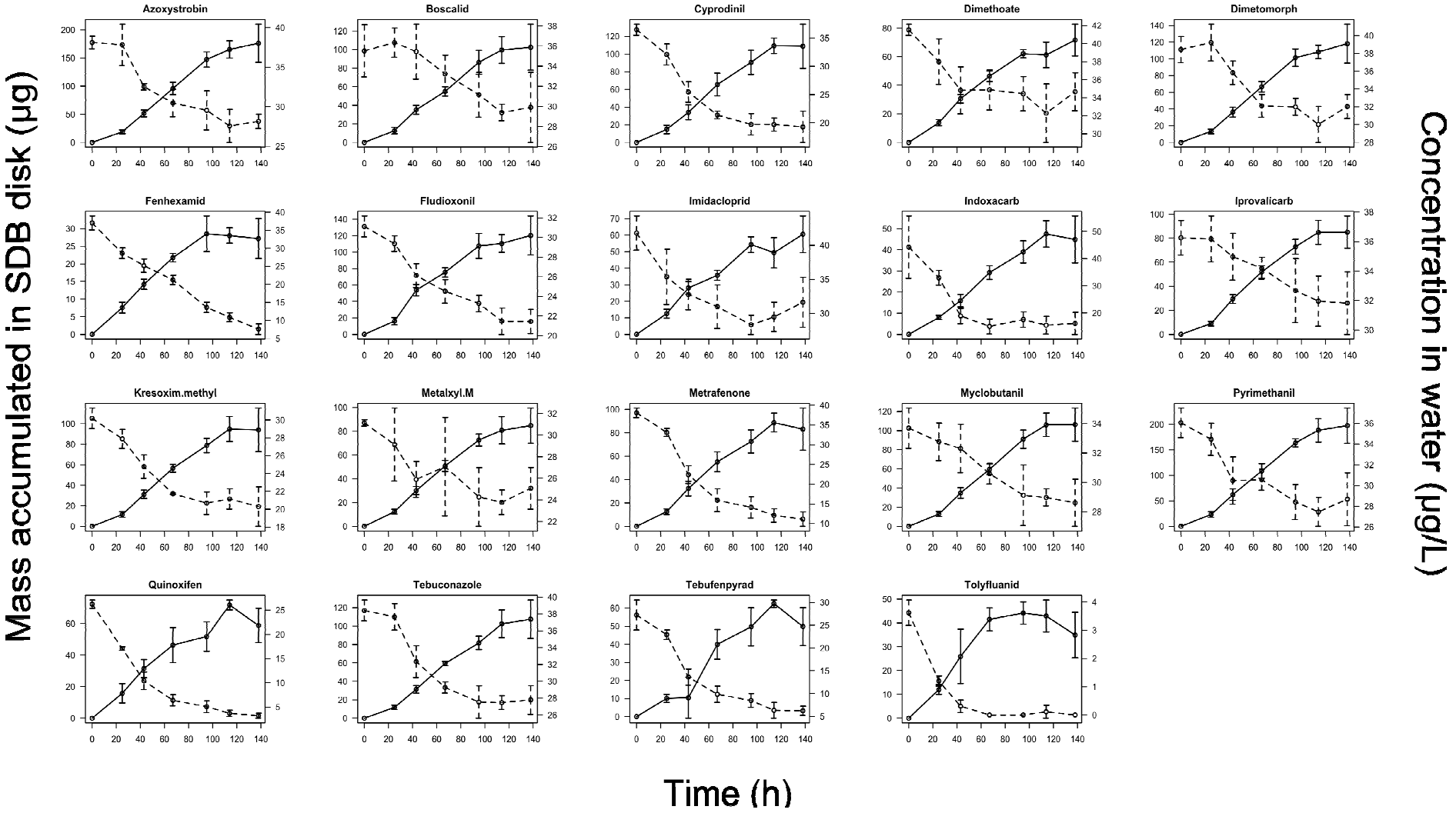


Figure A.5: Mass in sampler (solid line, left y axis) and water concentration (dashed line, right y axis) profiles during the 6-day sorption experiment. Error bars represent the standard error of the mean (n=4).

Figure A.6: Relationship between octanol-water partition coefficient (K_{OW}) and sampling rates for the analysed pesticides (except tolyfluanid).

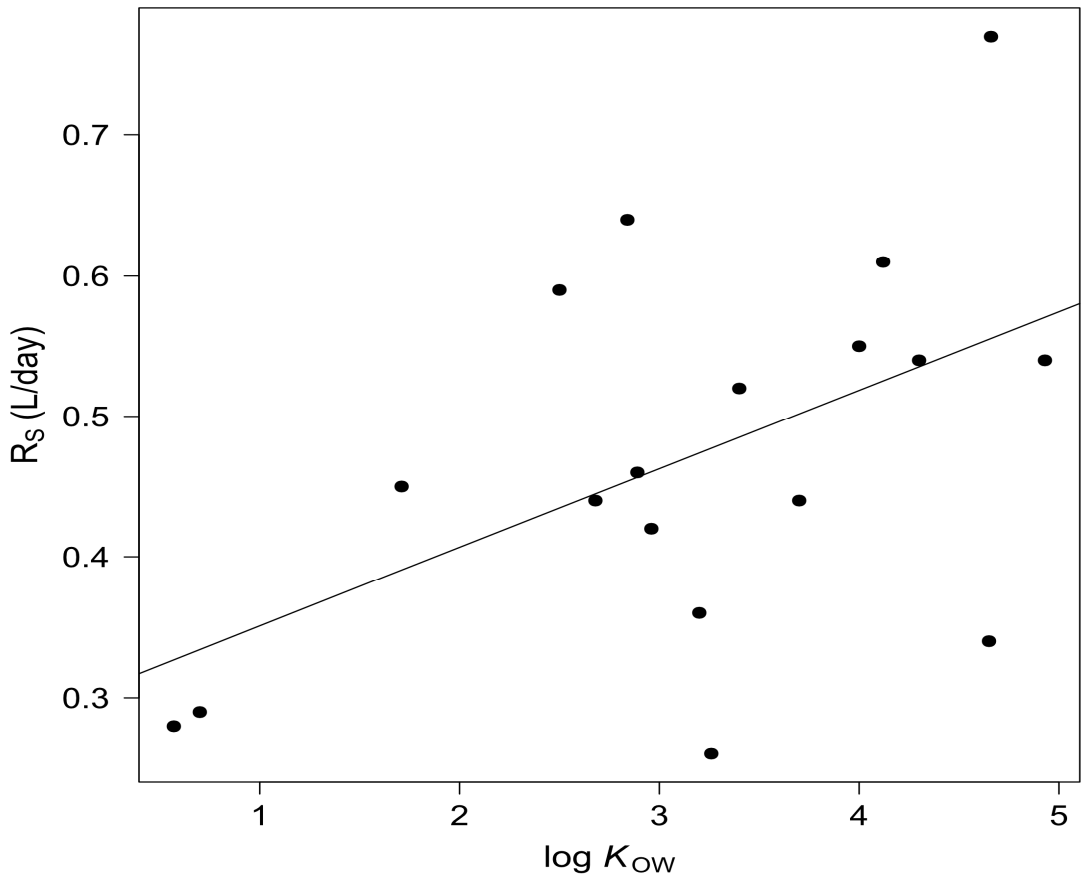
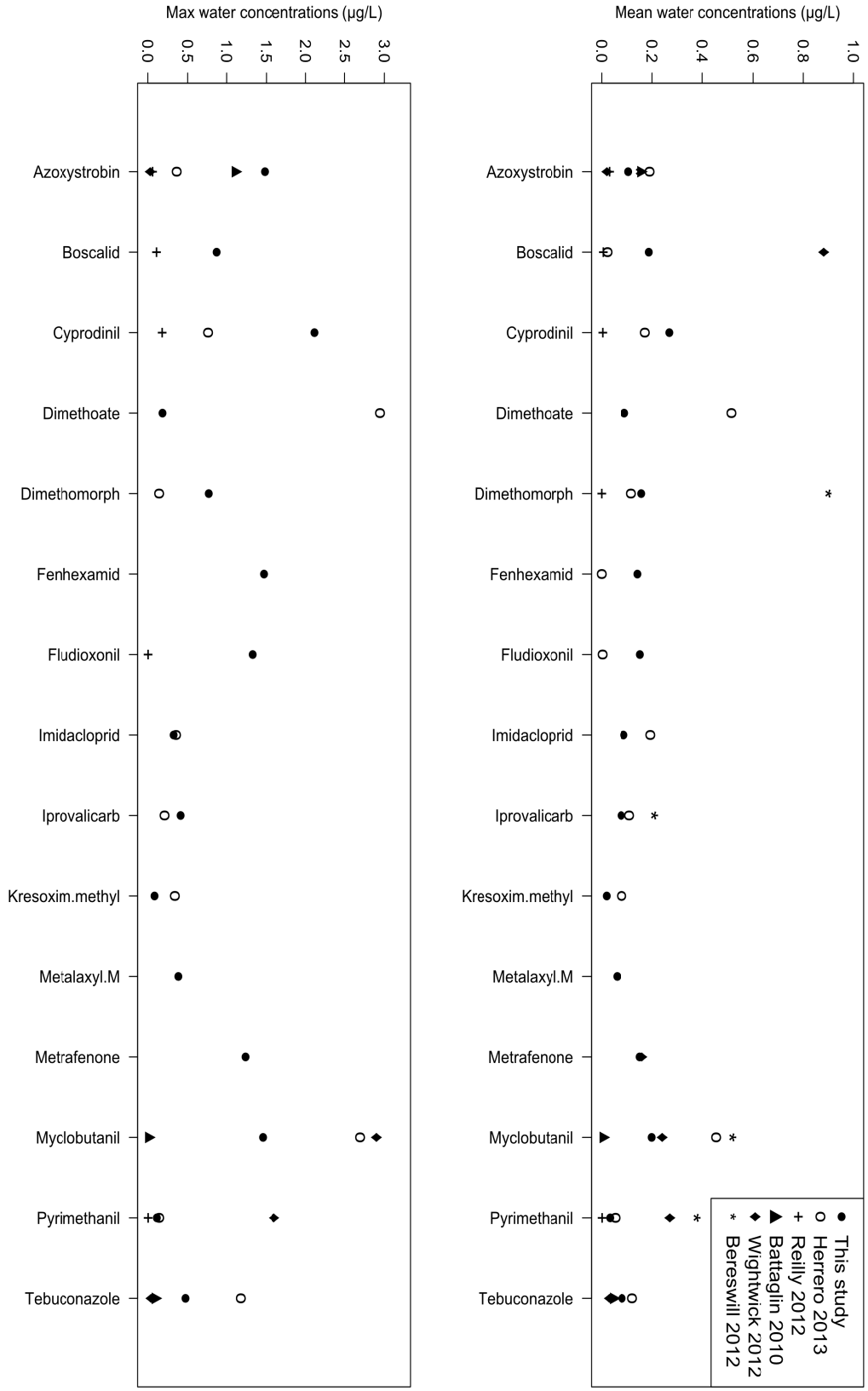


Figure A.7: Mean (top) and maximum (bottom) pesticide concentrations in water reported by different studies. For studies reporting concentrations in different years (Bereswill et al., 2012) or regions within the study area (Herrero-Hernández et al., 2013), the mean concentration is given.



Appendix B

Supplementary information for chapter 3

Figure B.1: Sampling schedule for the field study. Precipitation data taken from a weather station in the study area (Weinbiet/Pfalz).

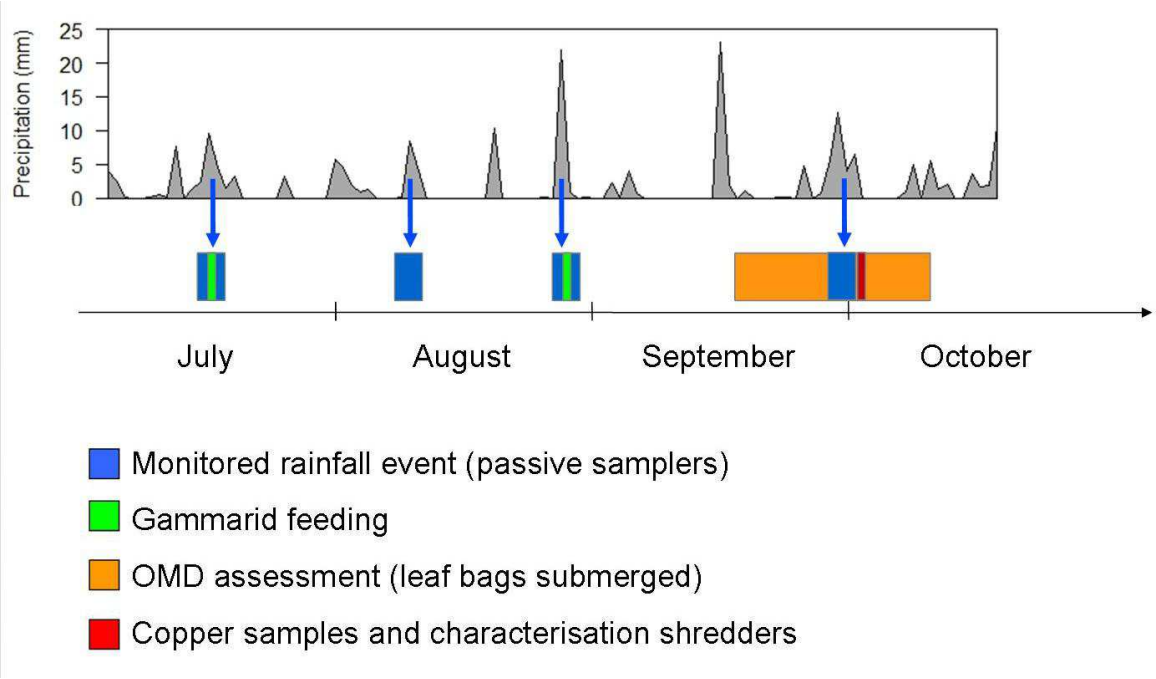


Figure B.2: Location of the study area and distribution of sampling sites.

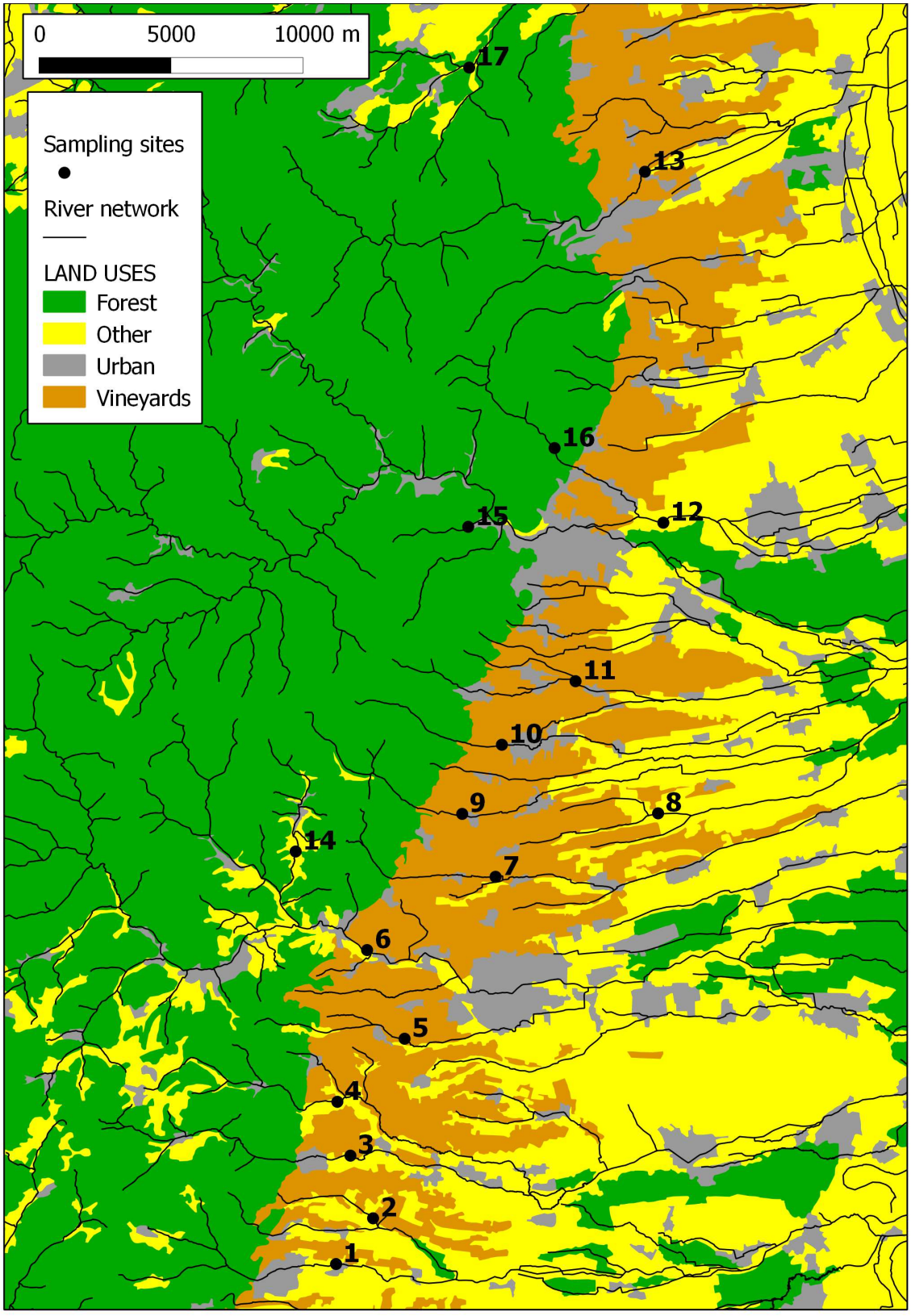


Table B.1: Pesticide toxicity, mean and maximum time-weighted average pesticide concentrations (TWA, only positive samples were considered in the assessment), frequency of detection found in vineyard sites, recovery of passive samplers and limit of quantification of the analytical method.

Pesticide	Type	$EC50_{P.\ subcapitata}$ (µg/L)	$EC50_{D.magna}$ (µg/L)	TWA mean (µg/L)	TWA max (µg/L)
Azoxystrobin	F	360	230	0.107	1.481
Boscalid	F	3,750	5,330	0.187	0.874
Cyprodinil	F	2,600	220	0.268	2.112
Dimethoate	I	90,400	2,000	0.091	0.184
Dimethomorph	F	29,200	10,600	0.158	0.775
Fenhexamid	F	26,100	18,800	0.143	1.469
Fludioxonil	F	183	400	0.153	1.327
Imidacloprid	I	10,000	85,000	0.088	0.324
Indoxacarb	I	110	600	0.018	0.047
Iprovalicarb	F	10,000	19,800	0.077	0.412
Kresoxim-methyl	F	63	186	0.020	0.084
Metalaxyl-M	F	36,000	100,000	0.061	0.383
Metrafenone	F	710	920	0.151	1.237
Myclobutanil	F	2,660	17,000	0.199	1.457
Pyrimethanil	F	1,200	2,900	0.033	0.113
Quinoxyfen	F	27	80	0.015	0.062
Tebuconazole	F	1,690	2,790	0.081	0.473
Tebufenpyrad	F	52	46	0.000	0.000
Tolyfluanid	I	1,500	190	0.000	0.000

F=fungicide; I= Insecticide.

Table B.2: Frequency of detection of pesticides found in vineyard sites, recovery of passive samplers and limit of quantification of the analytical method.

Pesticide	Detections (%)	Recovery (%)	LOQ (ng/L)
Azoxystrobin	62	69 ± 4	6
Boscalid	77	100 ± 19	6
Cyprodinil	31	60 ± 6	6
Dimethoate	23	62 ± 6	10
Dimethomorph	77	85 ± 8	6
Fenhexamid	69	127 ± 19	6
Fludioxonil	46	101 ± 10	5
Imidacloprid	23	71 ± 10	8
Indoxacarb	53	68 ± 7	7
Iprovalicarb	69	79 ± 2	7
Kresoxim-methyl	62	71 ± 5	6
Metalaxyl-M	85	63 ± 4	7
Metrafenone	69	64 ± 5	5
Myclobutanil	100	87 ± 10	6
Pyrimethanil	70	43 ± 8	9
Quinoxyfen	38	63 ± 4	5
Tebuconazole	76	71 ± 4	5
Tebufenpyrad	0	66 ± 6	4
Tolyfluanid	0	48 ±13	3

Table B.3: Pearson correlation between leaf decomposition rate (*k*) and fungicide toxicity in 17 German streams using time-weighted average concentrations. Toxicity is expressed in terms of toxic units for *P. subcapitata* and *D. magna* and related to microbial and invertebrate-mediated LD, respectively. For regression with microbial-mediated LD, sites 1 and 16 were excluded from the analysis because of undue influence according to Cook's distance. To aggregate the toxicity from different pesticides, the logarithmic sum of toxic units (sumTU) and the maximum log TU (maxTU) of all pesticides were calculated for each site and rainfall event, whereas pesticide concentrations from the four different rainfall events were integrated using mean and maximum values. The sumTU approach assumes a similar mode of action of compounds and represents an estimate of the mixture toxicity, whereas the maxTU is the toxicity of the most potent toxicant in a sample and ignores mixture effects.

Assessment of TU	Test organism	Integration	Correlation with *k*	
			r	p
sumTU	*P. subcapitata*	mean	-0.57	0.03
sumTU	*P. subcapitata*	max	-0.56	0.03
maxTU	*P. subcapitata*	mean	-0.50	0.06
maxTU	*P. subcapitata*	max	-0.49	0.06
sumTU	*D. magna*	mean	-0.03	0.91
sumTU	*D. magna*	max	-0.03	0.90
maxTU	*D. magna*	mean	-0.01	0.96
maxTU	*D. magna*	max	-0.03	0.91

Figure B.3: a) Correlation between invertebrate leaf decomposition rate and fungicide toxicity in terms of the sum of toxic units for *D. magna* (sumTU). b) Relationship between the feeding rate of *Gammarus fossarum* during the third monitored rainfall event and fungicide toxicity in terms of the sum of toxic units for D. magna during that event.

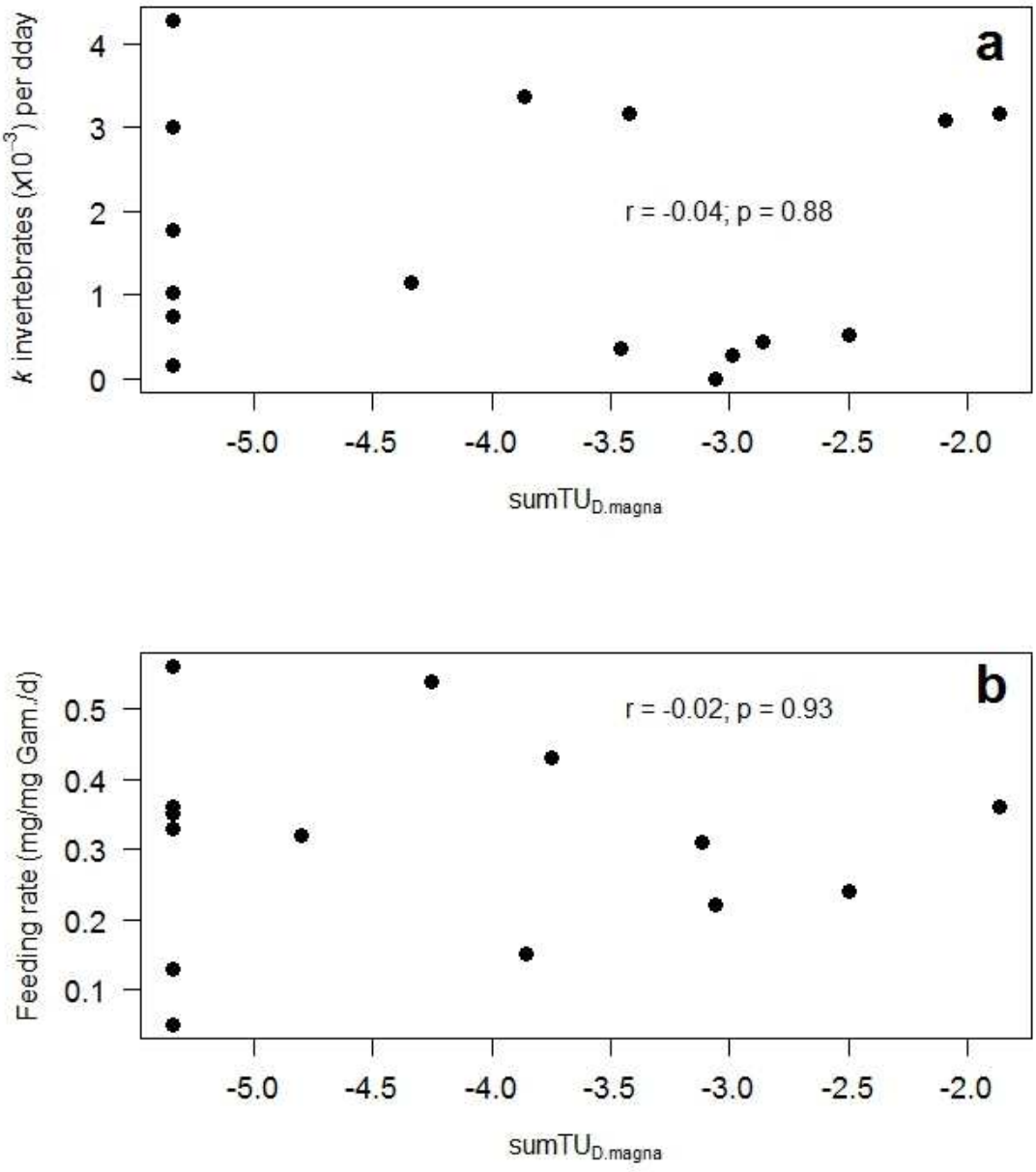


Figure B.4: Environmental variables with highest explanatory power for microbial and invertebrate-mediated LD when retaining copper as predictor variable, with metrics normalised to sum 100%. The proportion of variance explained by the model (R^2) is shown below the barplots.

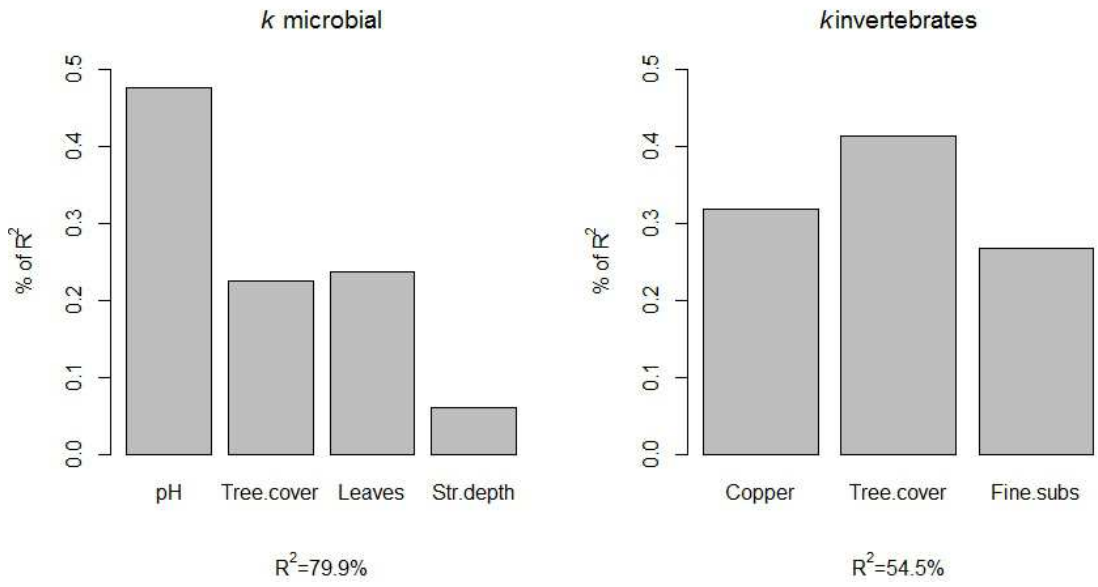


Figure B.5: Contribution of gammarids (in percentage) to the total abundance of leaf-shredding macroinvertebrates at each sampling site. Total shredder abundance is provided at the top of each bar.

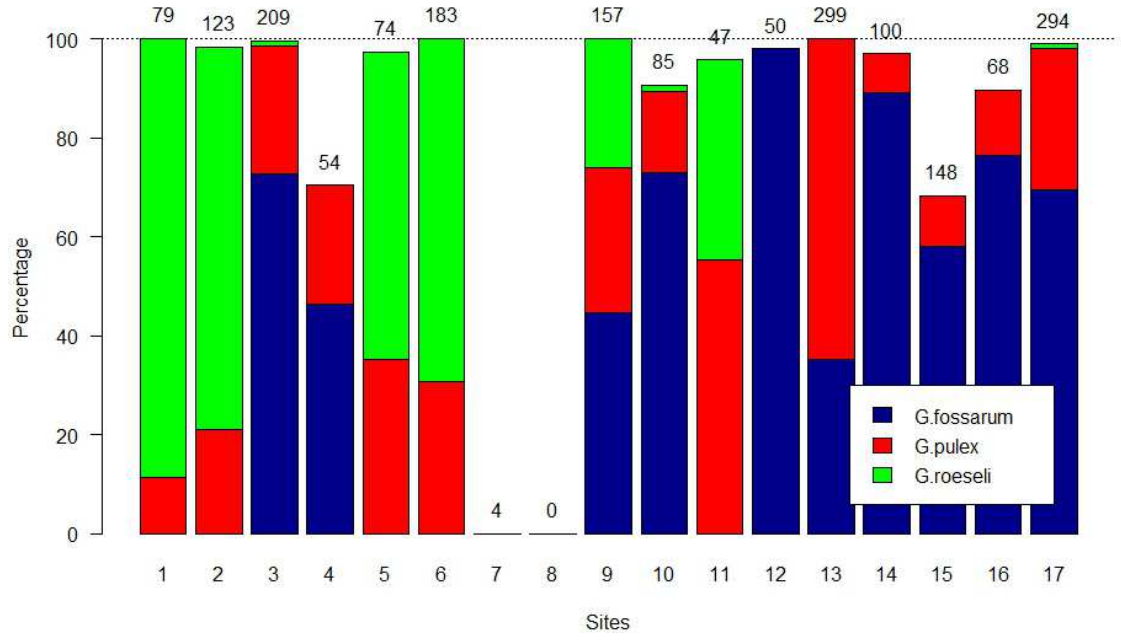


Figure B.6: Relationship between invertebrate leaf decomposition rate and the feeding rate of *Gammarus fossarum* during the first (a) and third (b) monitored rainfall events in the 17 monitored German streams.

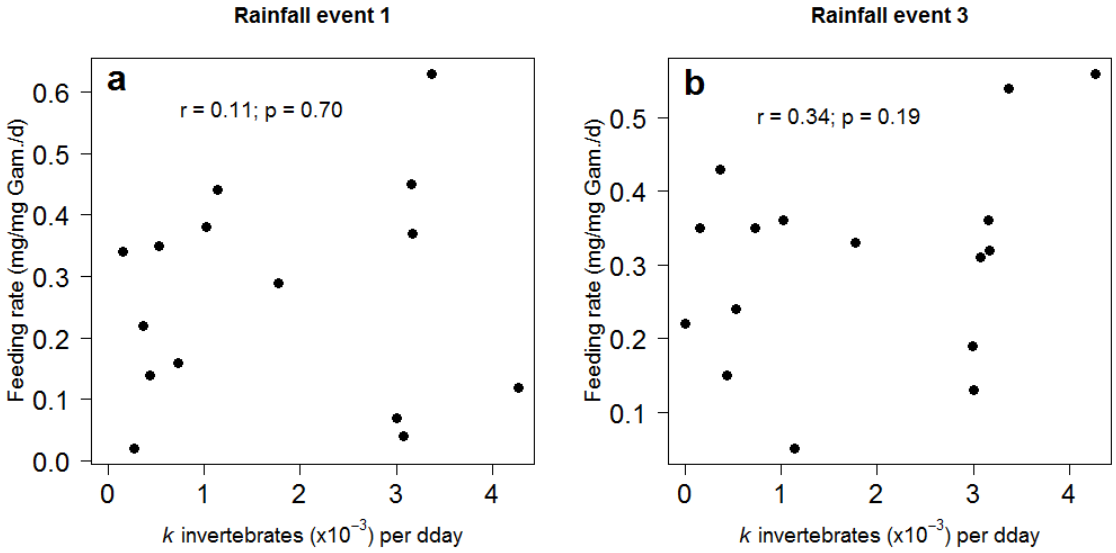
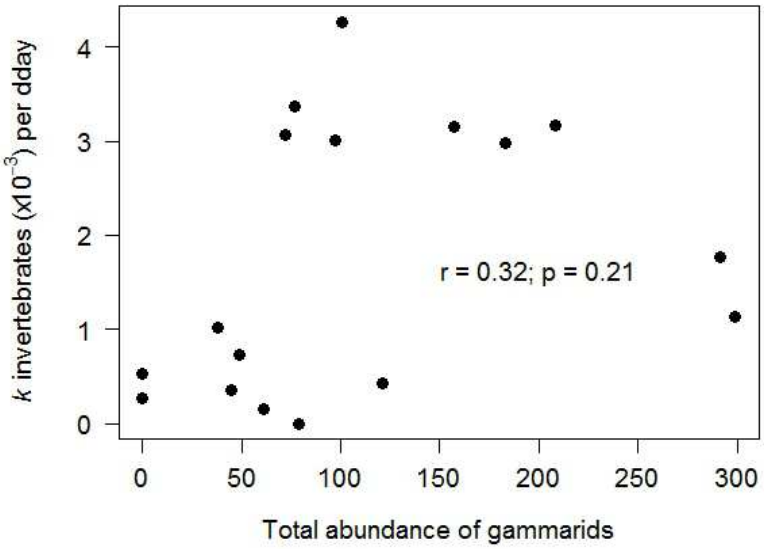


Figure B.7: Relationship between gammarid abundance and invertebrate leaf decomposition rate in the 17 monitored German streams.



Appendix C

Supplementary information for chapter 4

Figure C.1: Scheme of a mesocosm and experimental design used in this study.

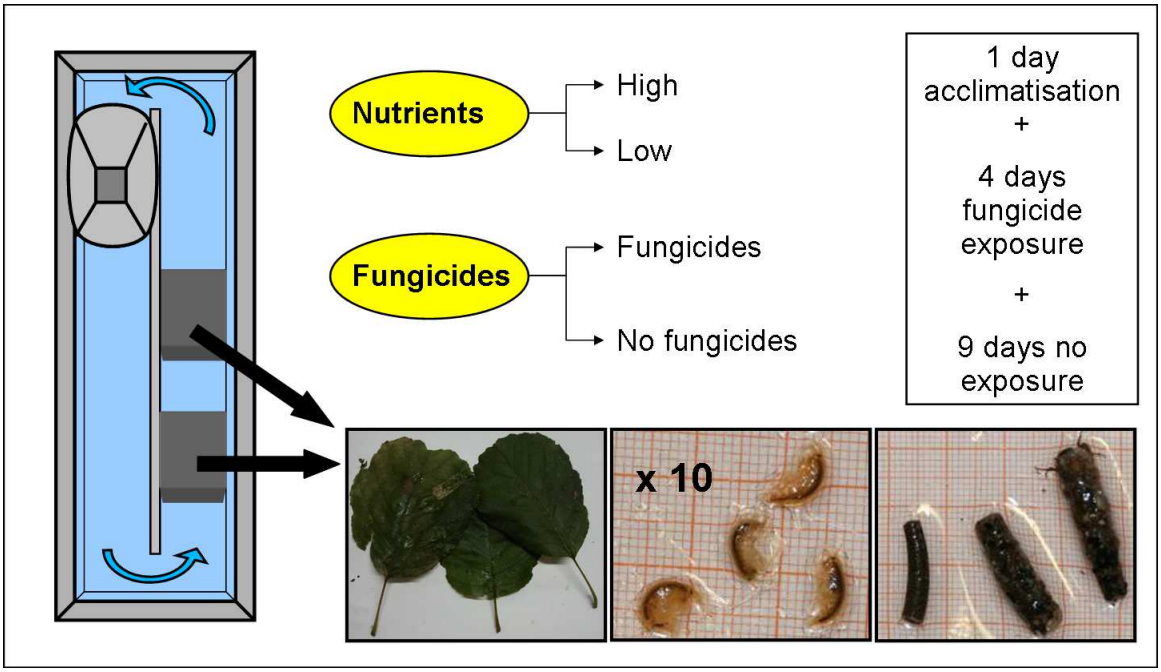


Figure C.2: Photo of the mesocosm and enclosures. Enclosures containing colonised leaves and invertebrates were covered with a net to prevent lost of trichopterans.

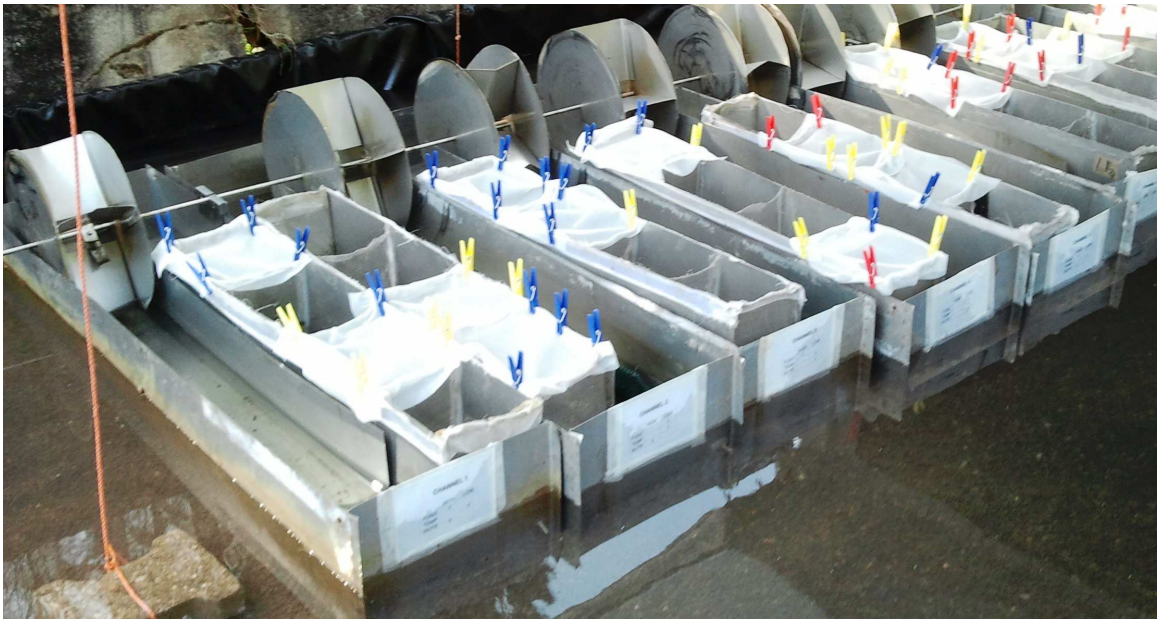


Figure C.3: Power functions used to predict initial weight of invertebrates.

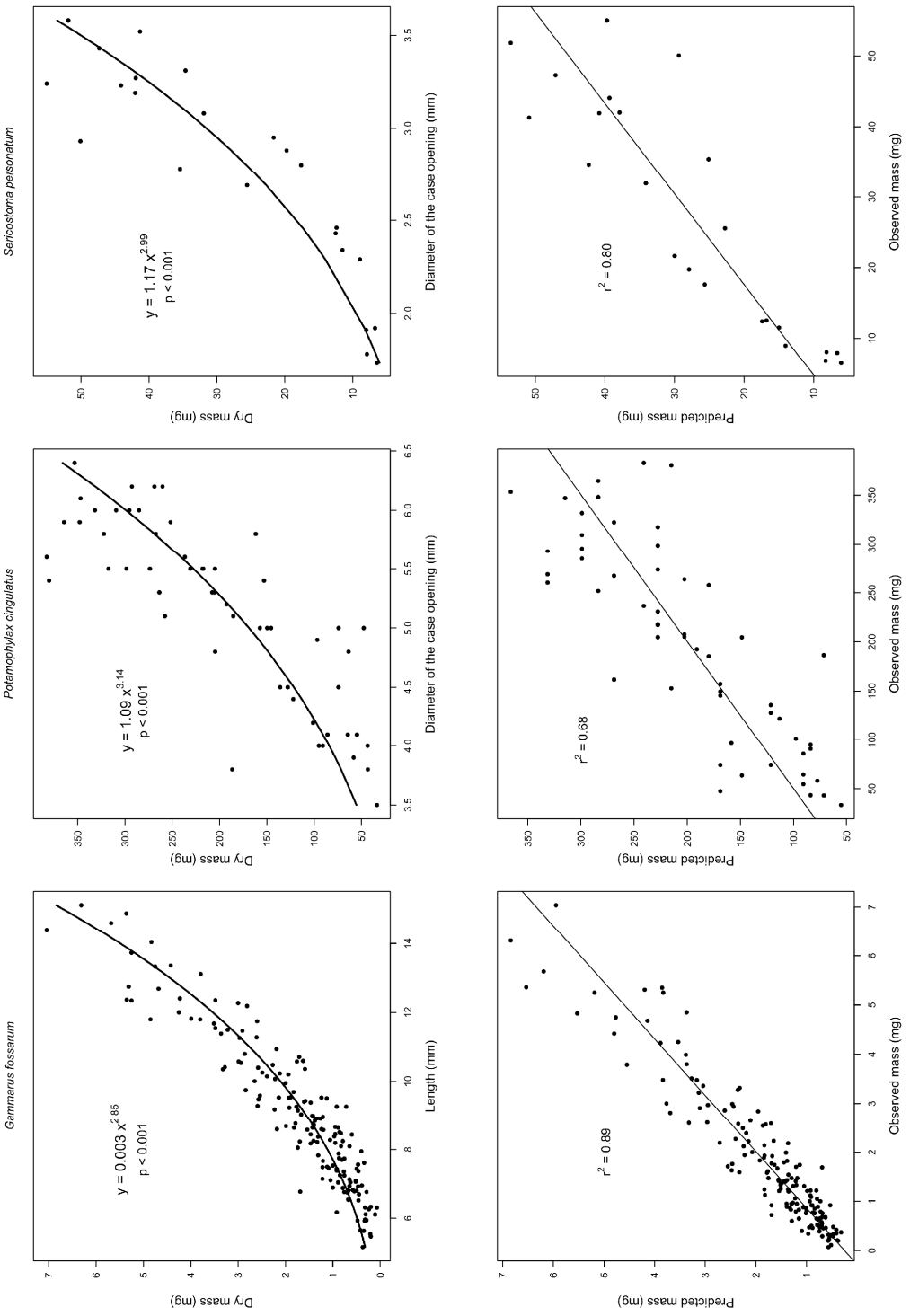


Figure C.4: Measured fungicide concentrations in mesocosms belonging to the fungicide treatment.

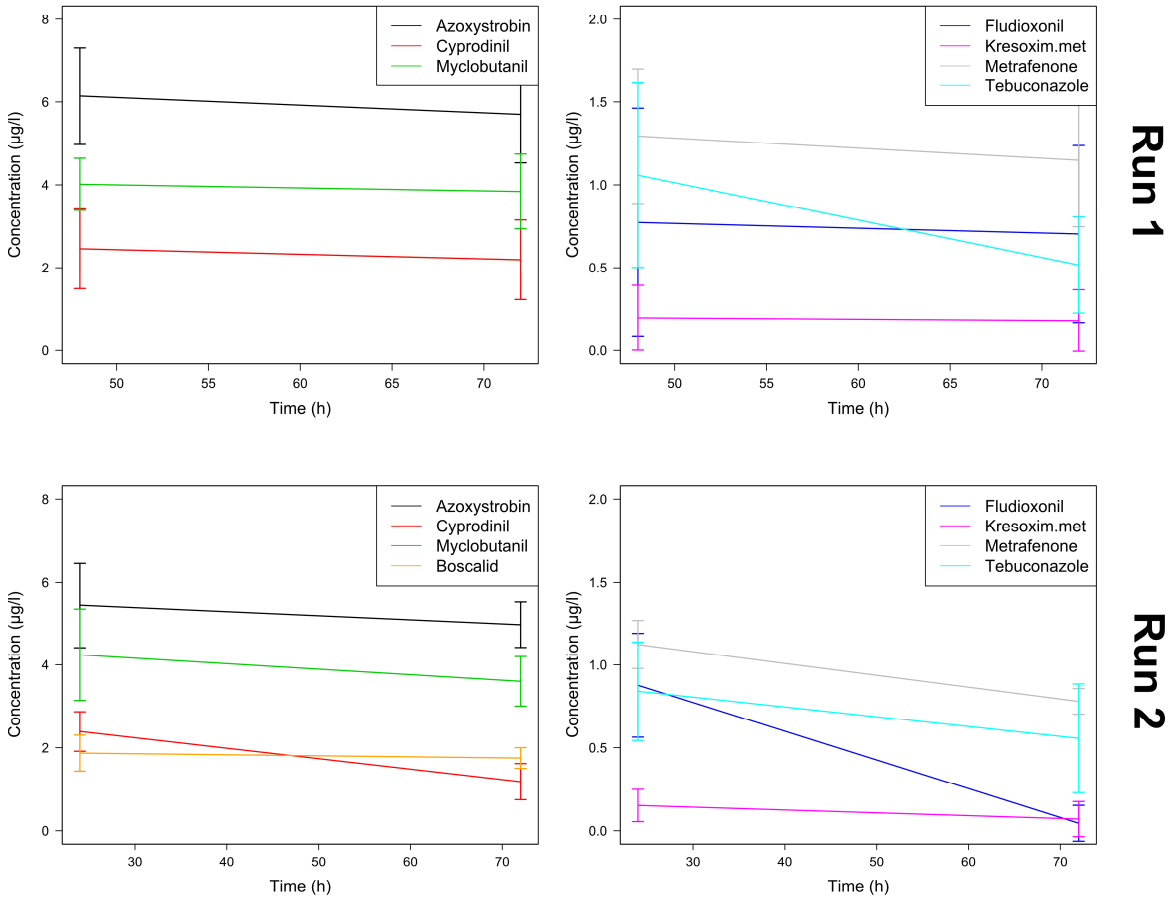


Figure C.5. RDA triplot using fungal and bacterial species (DGGE data) as response variables and the variables selected in the automatic model building as explanatory variables. Black symbols = mesocosms with fungicides, grey symbols = mesocosms without fungicides; squares = high nutrients; triangles = low nutrients

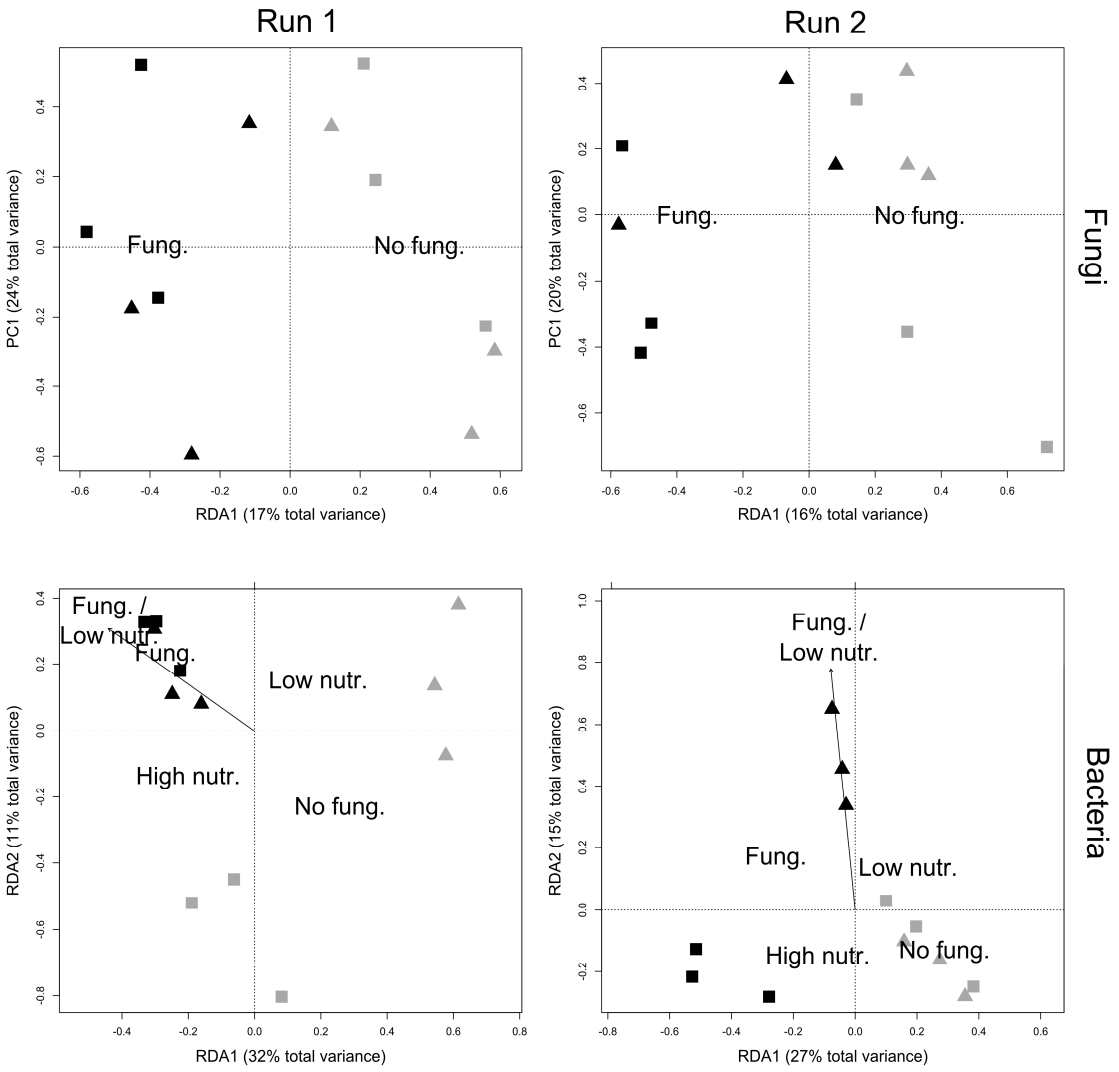


Figure C.6: Clustering of samples with fungal DGGE data using Unweighted Pair Group Method with Arithmetic Mean. The similarity matrix was computed using the Pearson coefficient.

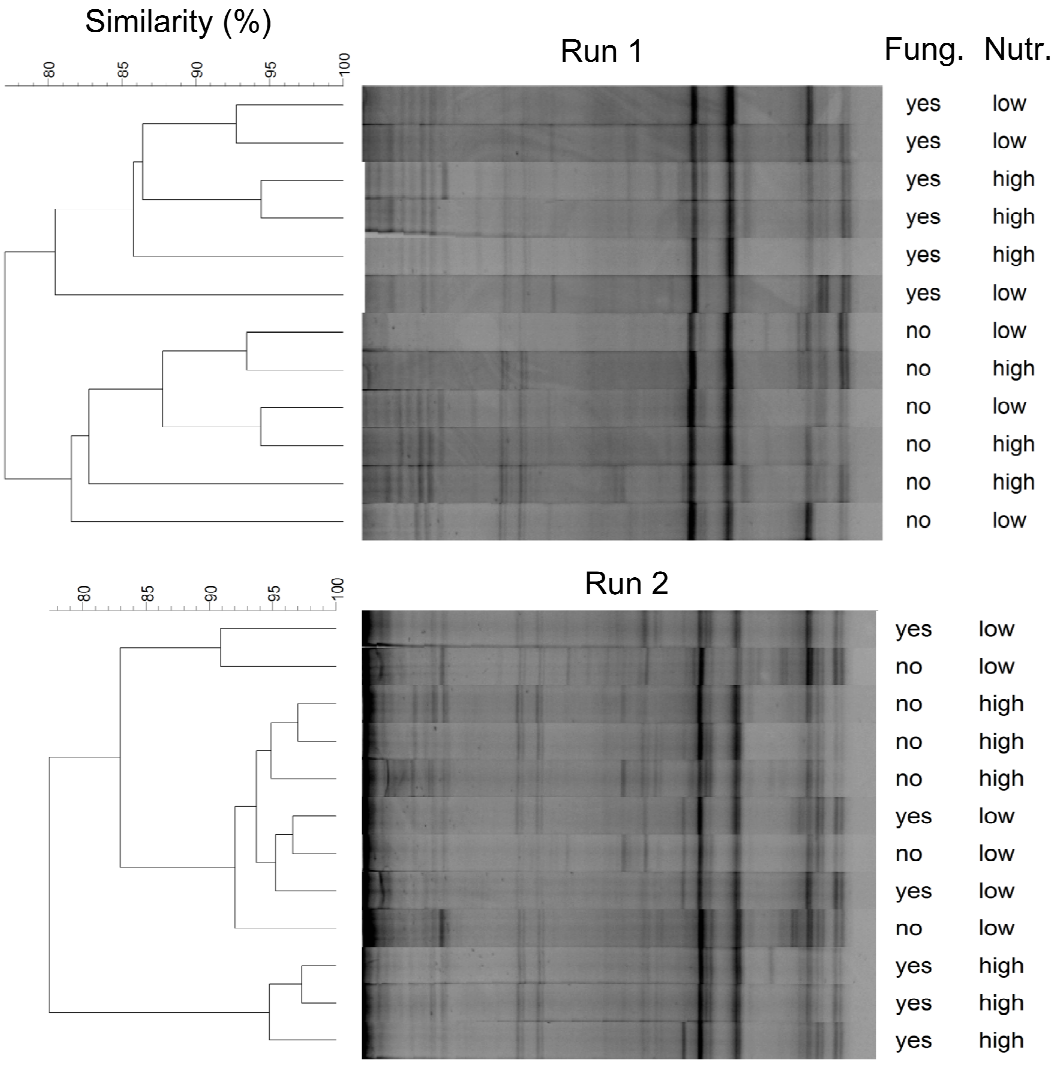
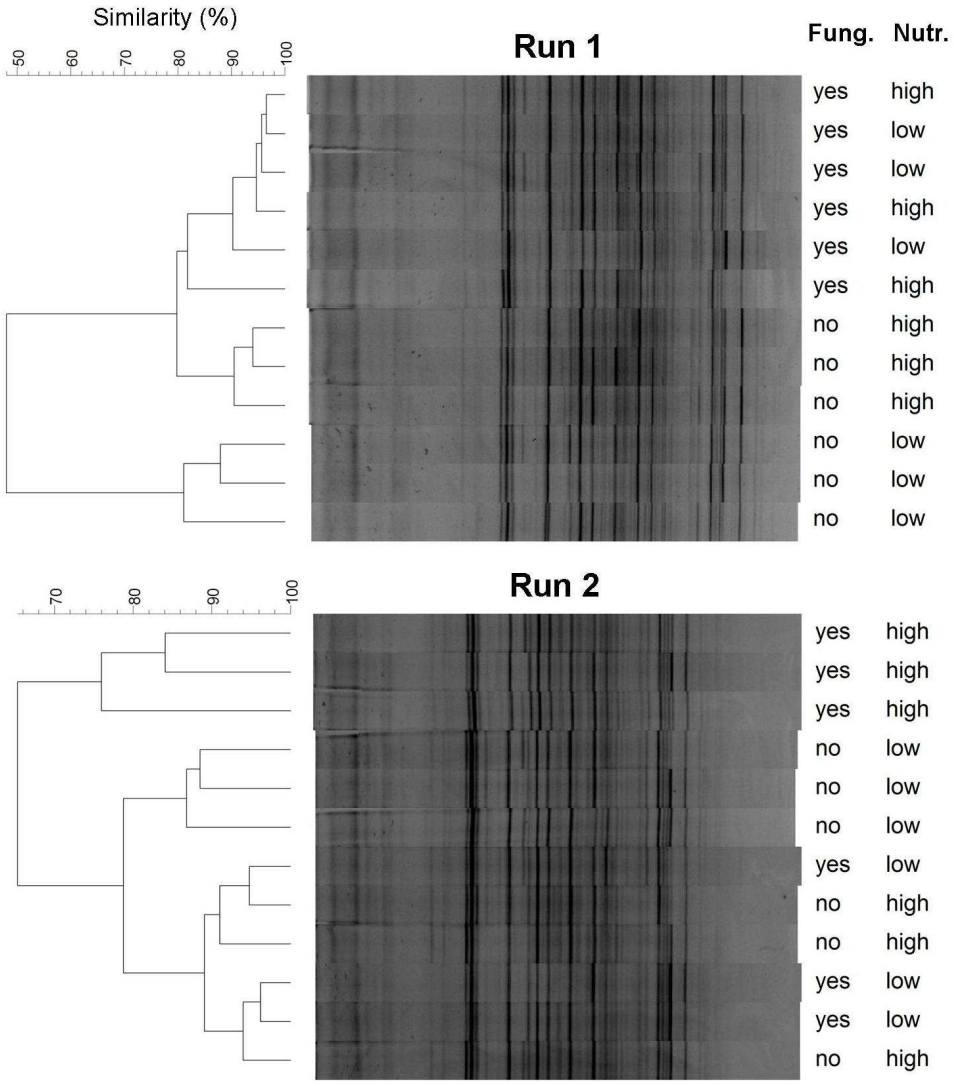


Figure C.7: Clustering of samples with bacterial DGGE data using Unweighted Pair Group Method with Arithmetic Mean. The similarity matrix was computed using the Pearson coefficient.



Author's Contributions

Paper I

TITLE	Calibration and field application of passive sampling for episodic exposure to polar organic pesticides in streams
AUTHORS	Diego Fernández, Etiënne L. M. Vermeirssen, Nicole Bandow, Katherine Muñoz and Ralf B. Schäfer
STATUS	Published in in 2014 Environmental Pollution, Vol. 194, pp 196–202
CONTRIBUTION	Fernández (70%) Designed research, Analysed data, Discussed results, Wrote manuscript Vermeirssen (5%) Designed research, Discussed results, Edited manuscript Bandow (5%) Analysed data, Discussed results, Edited manuscript Muñoz (10%) Analysed data, Discussed results, Edited manuscript Schäfer (10%) Designed research, Discussed results, Edited manuscript

Paper II

TITLE	Effects of fungicides on decomposer communities and litter decomposition in vineyard streams
AUTHORS	Diego Fernández, Katharina Voß, Mirco Bundschuh, Jochen P. Zubrod and Ralf B. Schäfer
STATUS	Published in 2015 in Science of the Total Environment, Vol. 533, pp 40-48
CONTRIBUTION	Fernández (70%) Designed research, Analysed data, Discussed results, Wrote manuscript Voß (10%) Analysed data, Edited manuscript Bundschuh (5%) Designed research, Discussed results, Edited manuscript Zubrod (5%) Discussed results, Edited manuscript Schäfer (10%) Designed research, Discussed results, Edited manuscript

Paper III

TITLE	Does nutrient enrichment compensate fungicide effects on litter decomposition and decomposer communities in streams?
AUTHORS	Diego Fernández, Mallikarjun Tummala, Verena C. Schreiner, Sofia Duarte, Cláudia Pascoal, Carola Winkelmann, Daniela Mewes, Katherine Muñoz and Ralf B. Schäfer
STATUS	In Review in Aquatic Toxicology
CONTRIBUTION	Fernández (55%) Designed research, Analysed data, Discussed results, Wrote manuscript Tummala (10%) Analysed data, Discussed results, Wrote manuscript Schreiner (10%) Analysed data, Discussed results, Edited manuscript Duarte (5%) Analysed data, Discussed results, Edited manuscript Pascoal (2.5%) Analysed data, Discussed results, Edited manuscript Winkelmann (2.5%) Designed research, Analysed data, Discussed results, Edited manuscript Mewes (2.5%) Analysed data, Discussed results, Edited manuscript Muñoz (2.5%) Analysed data, Discussed results, Edited manuscript Schäfer (10%) Designed research, Discussed results, Edited manuscript

Diego Fernández González

Resume

PERSONAL DATA

Born	12 October, 1983 in Burgos (Spain)
E-Mail	diegofgrm@gmail.com

ACADEMIC EDUCATION

05/2012 – present	PhD student on Ecotoxicology Koblenz-Landau University, Landau in der Pfalz, Germany Research topic: "Quantification of the influence of current-used fungicides on allochthonous organic matter decomposition in streams"
10/2007 - 05/2009	Master's degree in Environmental Water Systems Management University of Cantabria, Cantabria, Spain
09/2001 - 12/2005	Bachelor's degree in Environmental Sciences University of León, León, Spain

PROFESSIONAL EXPERIENCE

06/2008 – 05/2012	**Environmental Technician at Environmental Hydraulics Institute of Cantabria, Spain** Worked on several projects related with natural resources management and ecology, especially in fluvial ecosystems. Performed activities: field work, data analysis, GIS work, report writing and contributions to research papers.
11/2006 – 09/2007	**Environmental Management Department Assistant at Bridgestone Hispania S.A., Spain** Worked on maintaining an Environmental Management System that complies with the ISO 14001 2004 standard. Worked on developing several environmental awareness programs aimed at factory staff.
04/2006 – 11/2006	**Environmental educator at Granja Escuela Arlanzón S.L., Spain** Led outdoor environmental education activities for children.
07/2004 – 10/2004	**Fire Lookout for the local government in Castilla y León, Spain**

SCIENTIFIC STAYS ABROAD

2014	CBMA - Centre of Molecular and Environmental Biology. University of Minho (Portugal). Topic: performing denaturing gradient gel electrophoresis with microbial DNA. Duration: 3 weeks.

PROFESSIONAL TRAINING

2016	GIS Akademie, Berlin, Germany "GIS Analyst"
2015	alfatraining, Mannheim, Germany "Object-oriented programming with Java" "Relational databases – SQL"
2012	Institut of Geoinformatic – University of Münster, Münster, Germany "GEOSTAT: analysis of spatiotemporal data with open-source GIS software"
2010	Environmental Agency, Warrington, United Kingdom "River Habitat Survey Trainer"
2010	Environmental Hydraulics Institute of Cantabria, Santander, Spain "Training course for sampling and determination of river macroinvertebrates"
2008	Environmental Agency, Warrington, United Kingdom "River Habitat Survey Surveyor"
2006	Burgos Chamber of Commerce, Burgos, Spain "Integrated Management Systems: Quality, Environmental and Occupational Health and Safety" (500 hours)

COMPUTER SKILLS

GIS software	ArcGIS, QGIS
Business software	Microsoft Office, OpenOffice
Statistical software	R, Statistica
Programming	R, SQL, Java
Citation man. soft.	EndNote, Zotero

OTHER INFORMATION

Languages	Spanish (mother tongue) English (fluent) German (fluent)
Driving license	Class B

PROFESSIONAL MEMBERSHIPS

Since 2010	Member of the Iberian Association of Limnology.
Since 2014	Member of the Society of Environmental Toxicology and Chemistry

PEER – REVIEWED PUBLICATIONS

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.

Voß, K., **Fernández, D.** & Schäfer, R.B. 2015. Organic matter breakdown in streams in a region of contrasting anthropogenic land use. Science of the Total Environment. 527–528, 179–184.

Fernández, D., Vermeirssen, E. L. M., Bandow, N., Muñoz, K. & Schäfer, R. B. 2014. Calibration and field application of passive sampling for episodic exposure to polar organic pesticides in streams. Environmental Pollution. 194, 196–202.

Fernández, D., Barquín, J., Álvarez-Cabria, M. & Peñas, F.J. 2014. Land-use coverage as an indicator of riparian quality. Ecological indicators. 41, 165–174.

Fernández, D., Barquín, J., Álvarez-Cabria, M. & Peñas, F.J. 2012. Quantifying the performance of automated GIS-based geomorphological approaches for riparian zone delineation using digital elevation models. Hydrology and Earth System Sciences.16, 3851–3862.

Barquín, J., Ondiviela, B., Recio, M., Álvarez-Cabria, M., Peñas, F.J., **Fernández, D**., Gómez, A., Álvarez C. & Juanes, J.A. 2012. Assessing the conservation status of alder-ash alluvial forest and Atlantic salmon in the Natura 2000 river network of Cantabria, Northern Spain. In: P. J. Boon and P. J. Raven, editors. River conservation and management. Wiley-Blackwell.

Fernández, D., Barquín, J. & Raven, P.J. 2011. A review of river habitat characterisation methods: indices vs. characterisation protocols. Limnetica. 30 (2), 217-234.

Barquín, J., **Fernández, D**., Álvarez-Cabria, M. & Peñas, F.J. 2011. Riparian quality and habitat heterogenety assessment in Cantabrian Rivers. Limnetica. 30 (2), 329-346.

Naura, M., Sear, D., Álvarez-Cabria, M., Peñas, F.J., **Fernández, D.** & Barquín, J. 2011. Integrating monitoring, expert knowledge and habitat management within conservation organisations for the delivery of the water framework directive: A proposed approach. Limnetica. 30 (2), 427-446.

CONFERENCE PRESENTATIONS

Fernández, D., Voss, K., Zubrod, J.P., Pascoal, C., Duarte, S., Bundschuh, M. & Schäfer, R.B. Effects of fungicides on decomposer communities and leaf decompostion in streams. Platform presentation. SETAC Europe 25th Annual Meeting, Barcelona, Spain, May 2015.

Fernández, D., Peters, K., Zubrod, J.P., Vermeirssen, E.L.M., Bundschuh, M. & Schäfer, R.B. Effects of fungicides on leaf decompostion in vineyard streams. Platform presentation. SETAC Europe 24th Annual Meeting, Basel, Switzerland, May 2014.

Fernández, D., Peters, K., Schadt, S., Vermeirssen, E.L.M. & Schäfer, R.B. Effects of fungicides on leaf decompostion in vineyard streams. Platform presentation. XXXII Congress of the International Society of Limnology (SIL) Budapest, Hungary, Aug. 2013

Fernández, D., Barquín, J., Álvarez-Cabria, M., Peñas, F.J., Juanes, J.A., Ondiviela, B. & Fernández, F. Environmental factors governing riparian habitat distribution in an Atlantic river from the Cantabrian region, Northern Spain. Poster. 7 th Symposium for European Freshwater Sciences, Girona, Spain, Jun. 2011.

Fernández, D., Barquín, J. & Raven, P.J. Riparian quality modelling from habitats composition. Platform presentation. XV Conference of the Iberian Association of Limnology, Ponta Delgada, Portugal, Jul. 2010.