

## Abstract

The fate of aquatic ecosystems is of vital importance for the future of the environment and mankind. Yet, these ecosystems are under constant threat of degradation through human behavior like water usage, climate change and exposure to xenobiotics. Of these xenobiotics, pesticides are among the most severe influences threatening aquatic biodiversity. To further investigate the role of xenobiotics, the NiddaMan project was conducted between 2015 and 2018 in the Horloff catchment area in the Wetterau in Hesse, Germany. A sub-project of NiddaMan was to study the connection of run-off effects following heavy rainfall events and the resulting influxes of pesticides in aquatic water bodies. Heavy rainfall events are unique in their characteristics as being a one-time event leading to a pulse discharge of contaminants from soils and plant covers into nearby streams. Typically, the effects caused by these events are either studied with regard to the effects directly observed in the water bodies through the utilization of active or passive biomonitoring, chemical analysis or laboratory testing with the substances found in the chemical analysis. However, these methods do not present a comprehensive image of the situation found in the natural environment. In the natural environment different kinds of compounds made from a wide array of xenobiotics interact with natural factors, directly influencing the mechanisms of action of the aquatic ecosystem.

The aim of this thesis is to analyze the effects of compounds found in water samples through a chemical analysis and to compare these results with those from in-vivo and in-vitro assays. These water samples were collected after heavy rainfall events in three small streams in the Horloff catchment area. This combinatory method shall provide a more comprehensive representation of the situation found in the natural environment. *Daphnia magna* was used as a proxy for in-vivo testing, whereas the cellulose degrading, GC Gram positive bacteria *Cellulomonas uda* was used for in-vitro testing. The application of *C. uda* is based upon the supposition that higher concentrations of pollutants lead to an inhibition of the exoenzyme  $\beta$ -glucosidase and an interruption of the growth of *C. uda* depending on the severity of the toxic compound. In conclusion, the chemical analysis shows a seasonal increase of pesticides in the water samples, whereas no effects on *D. magna* could be observed in acute toxicity tests that ran for 48 h. Utilization of *C. uda* as a proxy offered varying results that showed the sensitivity of the bacteria to the toxic compounds, albeit in a narrow spectrum that often did not directly represent the findings of the chemical analysis.

## Zusammenfassung

Obwohl das Schicksal aquatischer Ökosysteme von zentraler Bedeutung für die Zukunft von Umwelt und Menschheit ist, geraten diese Ökosysteme immer mehr unter Druck durch menschliche Einflüsse. Wassernutzung, Klimawandel und die Exposition von Xenobiotika wie Pestiziden stellen dabei die größten Gefährdungen für die aquatische Biodiversität dar. Im Rahmen des von 2015 bis 2018 durchgeführten NiddaMan Projektes wurde die Relevanz der Exposition von Xenobiotika im Einzugsgebiet der Horloff (Hessen) untersucht. Eines der Unterprojekte war dabei die Untersuchung von Run-Off Effekten in Oberflächengewässern, die als Folge von Starkregenereignissen auftreten. Im Nachklang dieser Starkregenereignisse werden große Mengen von Schadstoffen in Einmaleffekten aus dem Boden und der Ackervegetation mit dem Abflusswasser in die angrenzenden Oberflächengewässer gespült. In der Wissenschaft werden diese Effekte meist isoliert betrachtet, indem entweder aktive oder passive Biomonitoring Untersuchungen durchgeführt werden oder das Wasser chemisch analysiert wird. Falls Labortests durchgeführt werden, werden meist nur wenige Substanzen isoliert oder in Verbindung mit Stellvertreterarten untersucht. In der Umwelt ist jedoch meist eine Mischung verschiedenster Xenobiotika anzutreffen, die mit einer weiten Bandbreite von natürlichen Faktoren interagieren, die von den Standarduntersuchungen aber meist nicht abgebildet werden.

Zur Berücksichtigung dieser verschiedenen Ebenen sollen im Rahmen dieser Thesis deshalb die Effekte der in Wasserproben gefundenen Substanzmischungen mit einer chemischen Analyse und parallel mit in-vivo und in-vitro Testverfahren untersucht und verglichen werden. Hierzu wurden für die Thesis in drei Flüssen im Einzugsgebiet der Horloff nach Starkregenereignissen Wasserproben gesammelt und in-vivo mit *Daphnia magna* getestet. Die in-vitro Tests wurden mit Cellulose abbauenden GC Gram positiven Bakterien der Gattung *Cellulomonas uda* durchgeführt. Das Testverfahren basiert auf der Annahme, dass mit höheren Konzentrationen von Schadstoffen die Expression des Exoenzyms  $\beta$ -Glucosidase unterbrochen wird und der Punkt dieser Unterbrechung photometrisch festgestellt werden kann. Die chemische Analyse zeigte eine saisonal geprägte Verteilung der Einträge nach Starkregenereignissen, deren Effekte aber in 48 h Akuttests für *D. magna* keine Auswirkungen zeigten. Die Ergebnisse für *C. uda* waren hingegen uneinheitlich - einerseits zeigte sich eine Hemmung des Wachstums durch die toxischen Substanzen, andererseits erfolgte diese Hemmung in einem sehr engen Spektrum, welches keine Rückschlüsse auf toxische Werte zuließ und mitunter auch nicht im Einklang mit der chemischen Analyse stand.

**Declaration of Academic Integrity**

I hereby confirm that the present thesis is solely my own work and every source of information, reference or data used within has been acknowledged and is fully cited.

The presented figures and tables have been created by myself or otherwise specified with the source.

This thesis, in same or similar form, has neither been published nor presented to any other authority before.

*Frankfurt am Main, 03<sup>rd</sup> May 2019*

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## List of Abbreviations

4	<b>4-NOPG</b>	4-Nitrophenyl-β-D-Glucopyranosid
A	<b><i>A. fischerii</i></b>	<i>Allivibrio fischerii</i>
B	<b>BfG</b>	Bundesanstalt für Gewässerkunde (German Federal Institute of Hydrology)
	<b>BMBF</b>	Bundesministerium für Bildung und Forschung (German Federal Ministry of Education and Research)
	<b>BMEL</b>	Bundesministerin für Ernährung und Landwirtschaft (German Federal Ministry of Food and Agriculture)
	<b>BMU</b>	Bundesministerium für Umwelt, Naturschutz und Reaktorsicherheit (German Federal Ministry for Environment, Nature Conservation and Nuclear Safety)
	<b>BQE</b>	Biological Quality Elements
	<b>BZL</b>	Bundesinformationszentrum Landwirtschaft (Federal Office for Agriculture and Food)
C	<b><i>C. riparius</i></b>	<i>Chiromonus riparius</i>
D	<b>CI</b>	95% Confidence Interval
	<b><i>C. uda</i></b>	<i>Cellulomonas uda</i>
	<b><i>D. magna</i></b>	<i>Daphnia magna</i>
D	<b>DMSO</b>	Dimethyl sulfoxide
	<b>DDT</b>	Dichlordiphenyltrichlorethan
E	<b>EC</b>	European Commission
	<b>EC50</b>	Effective Concentration 50% (in-vitro assays)
	<b>EEA</b>	European Environment Agency
	<b>EEC</b>	European Economic Community
	<b>EQS</b>	Environmental Quality Standards
	<b>EFSA</b>	European Food Safety Authority
	<b>ES</b>	Extract Samples
	<b>EU</b>	European Union
F	<b>FAU</b>	Formazin Attenuation Units
	<b>FFH</b>	Fauna-Flora-Habitat
H	<b>HLNUG</b>	Hessisches Landesamt für Naturschutz, Umwelt und Geologie (Hessian Agency for Nature Conservation, Environment and Geology)
	<b><i>H. azteca</i></b>	<i>Hyalella azteca</i>
I	<b>IPCC</b>	Intergovernmental Panel on Climate Change
	<b>ISO</b>	International Organization for Standardization

	<b>LAWA</b>	Bund/Länder-Arbeitsgemeinschaft Wasser (German Working Group on Water Issues)
<b>L</b>	<b>LC50</b>	Lethal Concentration 50 % (in-vivo tests)
	<b>LF</b>	Langder Flutgraben
	<b>LOQ</b>	Limit of Quantification
<b>N</b>	<b>NAP</b>	National Action Plan
	<b>NC</b>	Negative Control
<b>O</b>	<b>OD</b>	Optical Density
	<b>OECD</b>	Organization for Economic Co-Operation and Development
	<b>PB</b>	Photometric Blank
<b>P</b>	<b>PC</b>	Positive Control
	<b>PPDB</b>	Public Pesticide Data Base (University of Hertfordshire)
	<b>PPP</b>	Plant Protection Products
	<b>PR</b>	Previous Row
	<b>RA</b>	Risk Assessment
	<b>RAC</b>	Regulatory Acceptable Concentration
	<b>RBMP</b>	River Basin Management Plan
<b>R</b>	<b>REF</b>	Relative Enhancement Factor
	<b>ReWaM</b>	Regionales Wasserressourcen-Management für den nachhaltigen Gewässerschutz in Deutschland (Regional Water Resources Management for Sustainable Protection of Waters in Germany)
	<b>RP</b>	Regierungspräsidium (Regional Administrative Council)
	<b>SC</b>	Solvent Control
	<b>SMR</b>	Substrate Metabolic Rate
<b>S</b>	<b>SOP</b>	Standard Operation Protocol
	<b>SPE</b>	Solid Phase Extraction
	<b>SPEAR</b>	Species at Risk
	<b>SWB</b>	Surface Water Body
<b>T</b>	<b>TR</b>	Test Run
	<b>TU</b>	Toxic Unit
	<b>WB</b>	Waschbach
<b>W</b>	<b>WFD</b>	Water Framework Directive (in German: WRRL – Wasserrahmenrichtlinie)
	<b>WG</b>	Weidgraben



## 1. Introduction

River systems today face a multitude of challenges from many different stakeholders from sectors like agriculture, forestry, fishery and transport (Rinaldo et al. 2018). These challenges increased in the last two centuries in intensity as well as complexity with often negative ecological consequences, leading to degraded river systems which have lost their ability to support ecosystem functionality and human activities (Giller 2005). River systems support the growth and function of urban and rural areas by supplying water, production of energy or cooling of power plants and many more functions (Carvalho et al. 2019). Following the advent of environmental awareness in the late 20<sup>th</sup> century, policy makers themselves became aware that the long-held view of river systems as usable resources for human demands like damming or transportation of goods could not be continued endlessly without taking into consideration the ecological side as well. First steps to alleviate the worst effects were taken in the area of the European Union (EU) in the 1970s by issuing standards for the quality of drinking water (Hering et al. 2010). In the 1990s further phases of water legislation tried to fill in gaps concerning topics like waste water treatment and pollutions stemming from agriculture. While these steps were important to realize that river systems provide multifaceted ecosystem services they did not “[...]address the increasing awareness of citizens and other involved parties for their water” (EC 2016). To take into consideration these different aspects of water usage, river system functionality and differing stakeholder interests, the EU initiated the European Water Framework Directive (WFD) ( Directive 2000/60/EC (EP 2000) in the year 2000 (Hering et al. 2010) stating that the best management option for water systems would be: “a single system of water management”. Applying this option would place managing of an entire river basin at the heart of water managing options, thereby considering river systems as entities that are intertwined at different sections like surface water, groundwater or chemical and physical structure. The directive aimed to change water management measures in all member states by placing aquatic ecology at the center of all decisions regarding management of water systems. The EU also defined several objectives for surface and groundwater systems that shall be reached by 2027, with the key aspect being to achieve good ecological and chemical status in all surface waters (Hering et al. 2010). Yet, despite all efforts and improvements undertaken so far, the EU stated in 2012 that more than half of all

European surface waters were unlikely to reach a good ecological status by 2015 and that for more than 40% of all surface waters the ecological status had not been determined (Hering et al. 2010). These findings did not improve in the meantime as a new assessment by the EU in 2018 (EEA 2018) found that only 40% of all European surface water bodies (SWB) are in good ecological health, whereas 38% of all SWBs reach good chemical status. To monitor the processes set in motion by the WFD the EU initiated the implementation of river basin management plans (RBMPs) that have been published every 6 years from 2003 onward by the member states of the EU ever since (EEA 2018). These RBMPs also include updates on anthropogenic pollutants such as organic chemicals, pesticides and heavy metals. Among these pollutants the EU first identified 33, now 45, priority substances that are considered to be extremely hazardous for the aquatic environment. For these substances the EU established an environmental quality standards (EQS) classification system that includes concentration thresholds, which in the end define if a good chemical status is reached by a water body (EC 2012). However, the number of chemicals and combinations thereof are considered potentially infinite (Kienzler et al. 2016), making it highly impractical to test large quantities of substances and their combinations. Still, due to the different degradation rates and the constant entry of new pollutants, combinations of substances are the most commonly found type of chemicals in nature to which humans and the environment are constantly exposed, often with unknown consequences (Kienzler et al. 2016). In water systems these mixtures are the results of different ways of entry like surface water run-off (Corada-Fernández et al. 2017; Meite et al. 2018), precipitation (Langer et al. 2017; Topaz et al. 2018), drainage of fields (Bundschuh et al. 2013), storm water overflow charge (Birch et al. 2011), discharges from waste water treatment plants (Corada-Fernández et al. 2017) and leaching from soils (Meite et al. 2018). Most often pesticides are part of the mixture found in surface waters (Reineke and Schlömann 2015; Langer et al. 2017; Brinke et al. 2017). Plant protection products (PPP) in all forms (i.e. herbicides, pesticides, fungicides) are an integral part of conventional agriculture (UBA 2018). They ensure that crops are more stable and larger quantities can be harvested as pesticides substantially reduce threats to the crop like fungi, competing plants and pest. In 2016, 753 different types of pesticides with 270 active substances were sold in Germany alone. Since 2006 between 30.000 to 35.000 tons were sold annually, demonstrating the huge importance of this market for the chemical industry (UBA 2018). However, the usage of pesticides

is connected to biodiversity loss (Beketov et al. 2013) and degradation of aquatic ecosystems (Liess and Ohe 2005) among other severe consequences for the environment. In 2012 and 2013, these consequences have also been recognized by the German government (Brinke et al. 2017), which issued several laws to constrict the usage of pesticides to sustainable levels and initiated the National Action Plan (NAP) on the sustainable use of PPP in 2013 (Brinke et al. 2017). A main objective of the NAP is to monitor and analyze the pesticide load in small river streams, which are often not represented in the monitoring system of the WFD but are prone to high short peak pesticide loads that surpass non-hazardous levels (Brinke et al. 2017). These high pesticide loads are frequent results of discharges from diffuse (i.e. agriculture) as well as point sources (i.e. waste water treatment plants or storm water overflow charges) (EEA 2018). As recognized in article 10 of the WFD (EP 2000), discharges from these sources shall be controlled. However, discharges from these sources are still among the most significant pressures faced by European SWBs (EEA 2018). A main trigger for the release of these discharges are heavy rainfall events (Chiovarou and Siewicki 2008) whose increase has been considered a likely future scenario in the northern hemisphere in all Intergovernmental Panel on Climate Change (IPCC) reports since 2001 (Alexander 2016). Due to spray drift, surface run-off (Cold and Forbes 2004) or preferential flow from soils (McGrath et al. 2010; Meite et al. 2018), heavy rainfall events lead to short term peaks of pesticide loads in SWBs especially during the main planting and spraying season (Liess and Ohe 2005). A future risk assessment (RA) for the possible hazards to these effects is complicated by the often unknown or highly debated combinatory effects of climate change, neobiota or abiotic stressors (Field et al. 2013; NiddaMan 2018). Nevertheless, since pesticides are regularly detected in Hessian rivers (HLNUG 2018a), it is relevant to assess the risks connected to peak pesticide loads in connection with the toxicological effects of the combination of substances found in river systems (Kienzler et al. 2016).

Filling some of the gaps related to this situation was one of the objectives of the “NiddaMan” project, which was initiated in May 2015 and ran until April 2018 as one of 14 projects in the BMBF (Federal Ministry of Education and Research) initiative ReWaM (Regional Water Resources Management for Sustainable Protection of Waters in Germany) (BfG 2015). The catchment area of the river Nidda in Hesse was chosen as a model region due to its exemplary challenges for a typical central-European river system, as exemplified by the land-use conflict

between agriculture and nature conservation in this region. Like many other German river systems, the Nidda and most of its tributaries will most likely not reach good ecological and chemical status in the WFD until 2027 (NiddaMan 2018), leading to a dilemma for policy makers who must make decisions regarding the implementation of water management schemes, but also face challenges from agriculture, climate and land-use change as well as demographic transformations and alterations in water usage (NiddaMan 2018).

To study the relevance of combinatory effects resulting from heavy rainfall events and subsequent pesticide discharges from point and diffuse sources, a joint venture between the Department of Aquatic Ecotoxicology of the Goethe University, Frankfurt and the German Federal Institute for Hydrology (BfG) in Koblenz was conducted as part of the NiddaMan project from spring until summer 2018. During this campaign time water samples were taken after heavy rainfall events in several small streams in the Wetterau Region of Hesse, Germany and then analyzed chemically by the BfG. Through the sampling process and the chemical analysis, the first hypothesis:

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**H1: Temporal variations of peak pesticide loads with varying compounds can be identified in surface water bodies after heavy rainfall events**

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was developed.

This hypothesis tries to connect the statement of the NAP (Brinke et al. 2017) that small river streams with a catchment area of < 30 km<sup>2</sup> are underrepresented in the monitoring process of pesticide loads, with the subsequent finding that further monitoring campaigns are required to discern seasonal changes and trends of chemical compounds (Brinke et al. 2017). Sudden peaks of pesticide loads present unusually high toxic stress factors for the aquatic environment (Liess and Schulz 1999; Brinke et al. 2017; Meite et al. 2018) regarding heavy rainfall events (Langer et al. 2017). The effects of these xenobiotic stress factors are often poorly understood (NiddaMan 2018), yet require constant monitoring due to their ubiquitous occurrence (Kienzler et al. 2016) and dangerous side effects for aquatic biodiversity and ecosystem health (Malaj et al. 2014). The assessment of ecotoxicological risks due to the occurrence of toxic compounds in SWBs is usually derived from testing one or several chemical substances in the laboratory, a method that does

not factor in the challenges induced by sudden pulse events in natural settings (Malaj et al. 2014). Fast and reliable approaches to assess the risk of toxic compounds are therefore required to provide starting-points for the analysis of chemical mixtures. As part of this thesis a multilevel approach using in-vivo and in-vitro test assays has been applied, leading to the second hypothesis:

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**H2: It is possible to assess the toxicity of high pesticide loads consisting of varying compounds through the utilization of *Daphnia magna* and *Cellulomonas uda***

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Developing a toolbox consisting of a range of in-vivo and in-vitro assays that can be easily used to deliver reliable first indications concerning the toxicity of chemical mixtures would be enormously helpful to assess the risks faced by ecosystems in the aftermath of sudden peak pesticide loads. To test the applicability of this method 48 h acute toxicity in-vivo tests with the crustacea *D. magna* were conducted according to the Organization for Economic Co-Operation and Development (OECD) guideline 202 (OECD 2004). *D. magna* is the standard test organism for pesticide tests in the phylum Arthropoda (Köhler and Triebeskorn 2013) and tested by default for a wide range of pesticides, including DDT (Crosby and Tucker 1971), organophosphorus and carbamate pesticides (Barata et al. 2004), as well as neonicotinoids (Takács et al. 2017). For this reason, *D. magna* was chosen as the test organism for in-vivo assays conducted as part of this thesis, despite other organisms that may react more sensitively to toxic substances like *Chironomus riparius* (Barden 2001) which was tested in a different thesis (Ratz 2019). *D. magna* can be found in waterbodies all across Europe and plays a vital role in the aquatic food chain as prey to invertebrates and fish (Ebert 2005). To assess the vulnerability of the test organism, tests were conducted with native, filtered, water samples collected after heavy rainfall events and, in a second phase, *D. magna* was placed in a microtiter plate to test extracts that were enriched concentrations of the native water samples.

For in-vitro testing the cellulolytic, facultative anaerobic, bacteria (Poulsen et al. 2016) *C. uda* was chosen. Members of this genus can be found in soils (Blankinship et al. 2014), where they use different exoenzymes like  $\beta$ -glucosidase to splice cellulose and chitin (Reguera and Leschine 2001) into their basic monomers (Poulsen et al. 2016). In-depth studies to analyze the metabolism rate

of *C. uda* and thereby the metabolic growth of the bacteria have been conducted since the 1980s (Chosson 1987; Coninck-Chosson 1988; Dermoun and Belaich 1985; Dermoun et al. 1988) with the aim to use *C. uda* in varying fields that require the biodegradation of cellulose (Poulsen et al. 2016). Cellulose is the most common biopolymer and can be find in all types of soil and aquatic water systems (Poulsen et al. 2016; Chae et al. 2017). Using cellulase producing microorganisms has been studied with varying interests ranging from the development of chemicals (Dermoun et al. 1988), detection of soil pollution (Brohon et al. 2001), remediation of soils contaminated with heavy metals (Chae et al. 2017) to contributing to the synthesis of biofuels (Poulsen et al. 2016). Most research for exoenzyme production as a by-product of microbial activity was conducted in these scientific fields but *C. uda* has also been studied in regard to the analyzation of water related issues (Admiraal and Tubbing 1991; Clinton et al. 2010; Obst et al. 2012). As part of her dissertation in 2003 Kuhbier analyzed sediments in a contaminated stream (Horloff) to study the role of microorganic exoenzymatic activity in the self-purification process (Kuhbier 2003). A first method for the application of exoenzyme testing in water samples was developed at the end of the 1990s (Obst et al. 2012). In 2017 and 2018 this method has been enhanced in two different master theses (König 2017; Langner 2018) conducted in the department of aquatic ecotoxicology of the Goethe-University, Frankfurt. Both analyzed water samples of small surface streams in the Horloff region, Wetterau, Germany and used extracts made of these water samples (König 2017; Langner 2018). König (2017) modified the earlier test design developed by Obst et al. (2012) to establish a basic, exoenzyme based test method, aiming to analyze the effectiveness of self-purification measures in a river system by using *C. uda* as the test organism.

This test method was then enhanced by Langner (2018) with the aim to develop a standardized test method for the analysis of toxic compounds in waste water of sewage treatment plants. The aim for this master thesis is to further develop these tests to study if *C. uda* can be used as a suitable option for analyzing pesticides in water samples.

In a final step the outcome of these tests shall be assessed by combining the findings of the chemical analysis with the results of the in-vivo and in-vitro tests, directly leading to hypothesis three:

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### H3: Toxicity levels in the compounds found in water samples after heavy rainfall events correspond with the biological response

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Establishing links for interactions between heavy rainfall events and the subsequent pesticide loads, created by run-off effects from point and diffuse sources, is central to assess the toxic effects of pesticide compounds. Yet, the ever-changing circumstances and possible interferences from other environmental factors during a field study make it challenging to estimate the relevance of a single contributor (Liess and Ohe 2005) in this cause- and effect-relationship. In a first step Liess and Ohe (2005) combine the calculated toxic units (TUs) with the species at risk (SPEAR) index to account for correlations between the abundance of biodiversity and the occurrence of pesticides (Liess and Ohe 2005). Due to the interactions of a multitude of factors in a natural environment neither the results of the chemical analysis nor the findings of single biological quality elements (BQE) alone reveal reliable patterns concerning the toxicity of chemical compounds (Ohe et al. 2009b). To bridge the gap between these different sectors and to assess the stress caused to stream dwelling communities due to pesticide exposure, Liess and Ohe (Liess and Ohe 2005; Ohe et al. 2009a) refined the TU calculation to identify single stressors by usage of BQE-specific TUs. As part of this thesis BQE-specific TUs were calculated for the reference species *D. magna* and compared to the results of the in-vitro tests with *C. uda*. In a final step these results were then compared with the findings of the chemical analysis to account for emerging patterns.

## 2. Material and Methods

For this thesis a multilevel approach was tested that began with collecting water samples after heavy rainfall events through event-driven samplers in the spring of 2018. The sampled water was then filtered, chemically analyzed and used for 48 h acute toxicity tests with the native samples and *D. magna*. 500 mL of the sampled water were then utilized for extracts and used for in-vivo tests with higher concentration as well as for in-vitro assays with *C. uda*. In the end the toxicity of the river systems was calculated by plotting the REF50 values of the *C. uda* tests against the TUs based on LC50 values for *D. magna*.

## 2.1. Timeframe for the Sampling Campaign and Definition of Heavy Rainfall Event

Water samples were collected between 30<sup>th</sup> March and 15<sup>th</sup> August during the spring and summer 2018 at three different drainage channels or small creeks in the Horloff catchment area in the Wetterau, Hesse, Germany (see chapter 2.2 and 2.3). This time frame fit with the spring and summer growth period of grains (BZL 2019) and rapeseed (Deutscher Landwirtschaftsverlag GmbH 2017), during which farmers mostly spray pesticides and do the weeding (Rudel and Perovich 2009; UBA 2018). Despite being unusually warm and dry for long stretches of the year (see Figure 1) 20 heavy rainfall events with precipitation rates of more than 25 mm per hour occurred in the state of Hesse in 2018, a number only outmatched by the year 2006 (HLNUG 2018b).

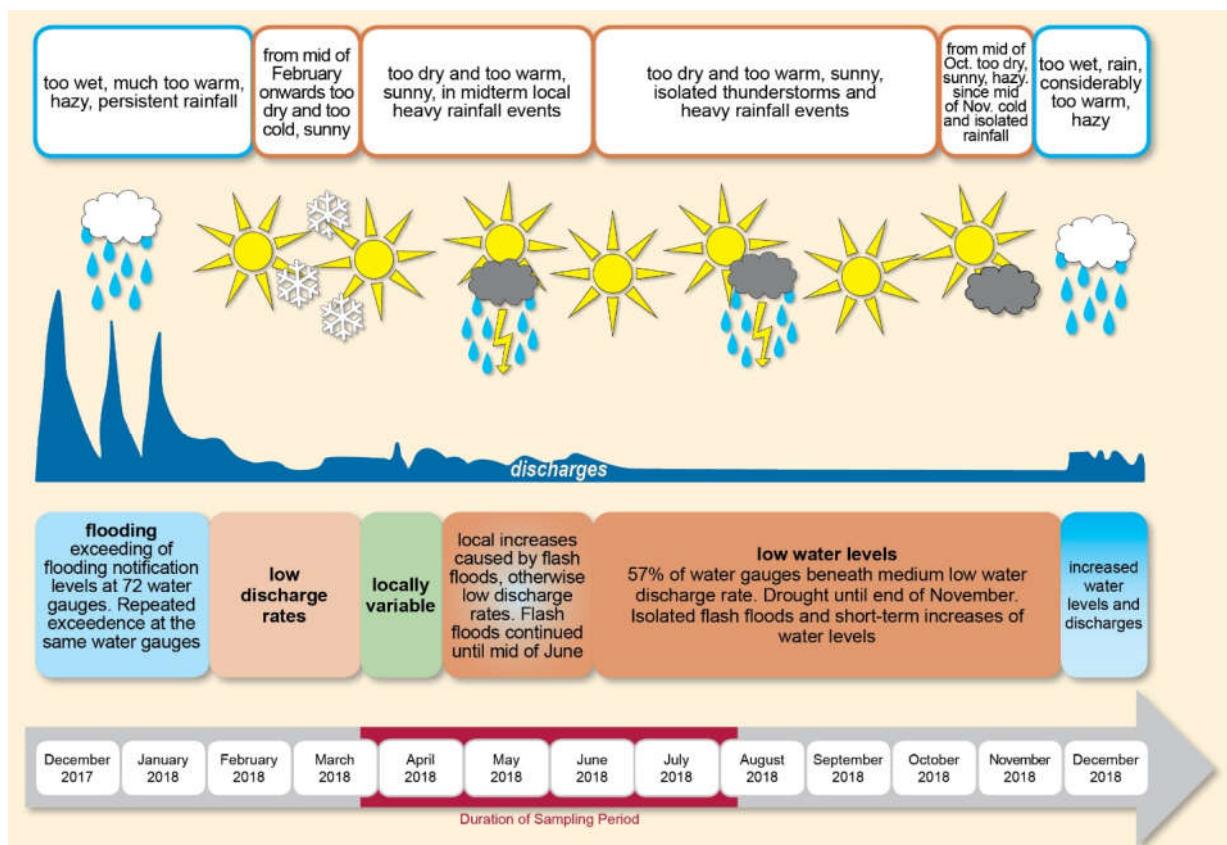


Figure 1 – Weather condition and water situation in Hesse from December 2017 until December 2018. The red marking shows the sampling period. Figure adapted from (HLNUG 2018b).

Water levels were measured constantly from 19<sup>th</sup> April to 25<sup>th</sup> September 2018 with a sensor placed on the river bed that registered changes in water height every 5 minutes. The sensor at the Langder Flutgraben (LF) (see chapter 2.3) was defunct after 20<sup>th</sup> June. The flow charts shown in figure 8 presents the changing water levels that occurred after heavy rainfall events during the

sampling timeframe. Sampling was induced by rising water levels that triggered the swimmer in the stream and thereby initiated the water sampling process (see chapter 2.4).

## 2.2. Characterization of Nidda and Horloff Catchment Area

The Nidda Man project was divided into several minor projects, which were all conducted in the name giving Nidda region in the central to the eastern area of Hesse in Germany.

As can be seen in figure 2 the source of the Nidda lies near Schotten in the Vogelsberg mid-mountain range at about 720 m above sea level. It then flows in a south western direction for 89.7 km through Hesse until it reaches the Main at Frankfurt-Höchst at 89.2 m above sea level. The catchment area of the Nidda entrenches a surface area of 1.942 km<sup>2</sup> and supplies water to a landscape inhabited by 1.3 million people and used very diversely, as demonstrated in table 2: for forestry near the source and agriculture (mainly wheat, canola, beet and vegetables) in the middle section between Nidda and Bad Vilbel as well as for the supply of freshwater (RP Darmstadt 2015). The withdrawal of freshwater occurs throughout nearly the whole length of the river but intensifies in the lower section near Frankfurt (RP Darmstadt 2015).

The geology in the catchment area is mostly made up from tertiary rock formations near the surface that were, in the Vogelsberg region, made up from volcanic activity in the Miocene (age between 18 and 14 million years) (Agricola 2009). The main rock types therefore are extrusive volcanic rocks like basalt but also, due to sedimentation, sands, silt and clay as well as limestone and lignite. Following the river downstream more remnants from glacial processes in the form of loess, sand and gravel can be found especially in the Wetterau region (HLNUG 2007). Since the end of the last ice age the loess cover has been downgraded to luvisol soils due to human agricultural activities. Among the main tributaries of the Nidda are the Horloff, the Wetter, the

*Table 1 – Land use designation in the Nidda catchment area (RP Darmstadt 2015).*

Land Use Designation	Area [km <sup>2</sup> ]	Percentage
<b>Forest</b>	628	32.3%
<b>Agriculture</b>	1030	53.0%
<b>Settlement</b>	171	8.8%
<b>Culture and Services</b>	24	1.2%
<b>Industry</b>	36	1.9%
<b>Green Space</b>	33	1.7%
<b>Traffic</b>	5	0.3%
<b>Other Usages</b>	10	0.5%
<b>Water Body</b>	6	0.3%
<b>Sum</b>	<b>1943</b>	<b>100.0%</b>

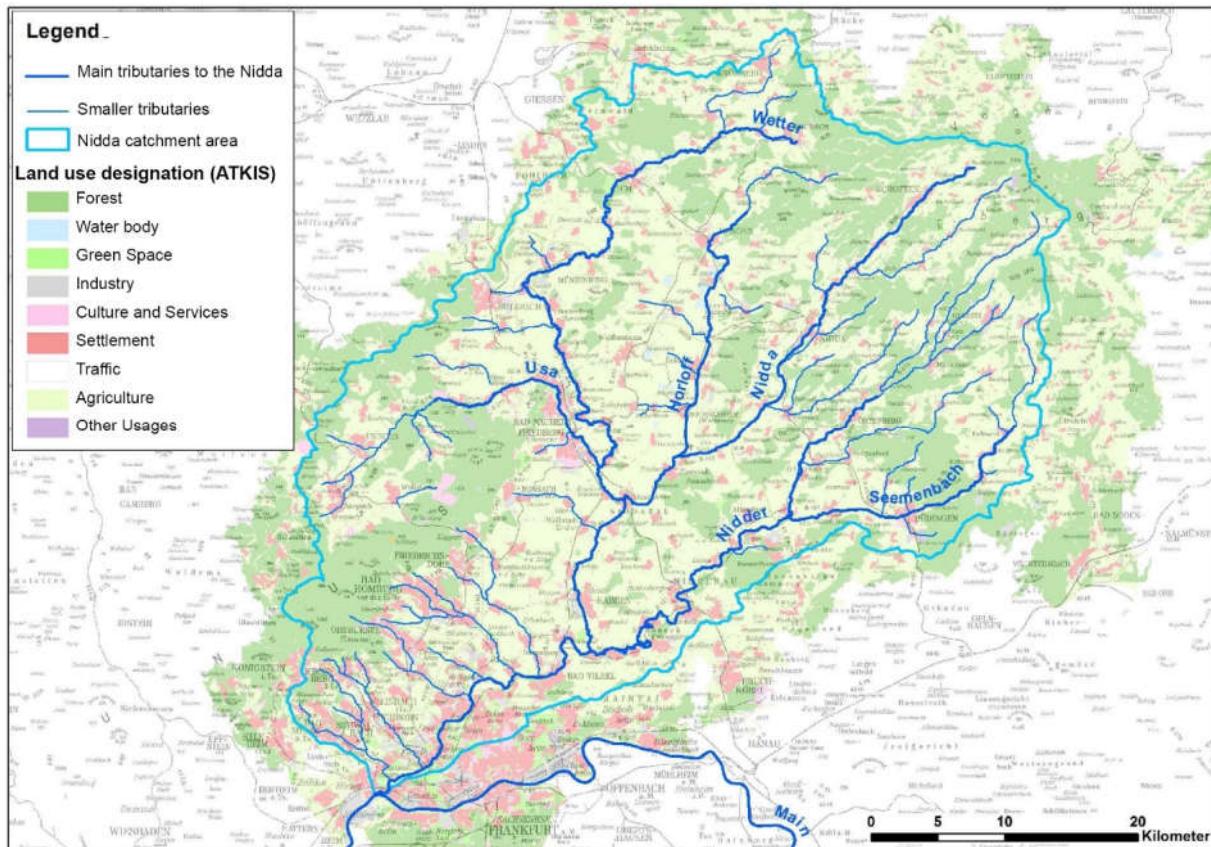


Figure 2 – Map of the Nidda catchment area including Horloff Adapted from (RP Darmstadt 2015).

Usa and the Nidder (ranked according to the point of influx). The Wetter, the Nidder and the Horloff also originate from the Vogelsberg Region, whereas the Usa has its source in the Taunus. After Nidder and Wetter, the Horloff is the third largest tributary to the Nidda with a length of 44.5 km and a catchment area of 279.2 km<sup>2</sup> (HLNUG 2007). The spring of the Horloff can be found north of Schotten, the confluence with the Nidda is near Ober-Florstadt in the Wetterau Region in Hesse. For most of its length the Horloff runs through areas that are protected for environmental reasons: either as a natural preserve, fauna-flora-habitat (FFH) territory, bird protection area or a mixture of those (RP Darmstadt 2015). The Horloff Aue near Trais-Horloff is an important station for migratory birds. Yet, the ecological conditions of the Horloff are still not satisfactory in many areas and do not match the requirements of the WFD at this point (WRRL-Viewer (HLNUG 2015)). The conditions for macrobenthos are still good in the upper reaches of the Horloff but then change fast to levels characterized as unsatisfactory and bad due to human activities like farming, traffic and settlement. Similar results can be seen for the fish population, the situation of diatoms and the contamination with chemical substances (HLNUG 2015). The

combination of these factors leads to an evaluation of the status of the waterbody that changes from moderate to bad near Inheiden at about water kilometer 20.0. From this point downstream the Horloff is, in most of its sections, still completely degraded with settlements and agricultural landscapes forcing it into an artificial riverbed. The contradictory situation of the existence of an important bird migration area, a Nature 2000 reserve and an FFH on the one hand and the strong degradation status of its riverbed on the other hand led to the decision to convert the stream into a more natural state. As a result of the Natura 2000 decree in 2008, the state of Hessen is liable to preserve and enhance the state of the habitats and species in the Horloff region (RP Gießen 2016). The action plan for the FFH area envisages a renaturation of the stream in its full course with the restitution to a good state of preservation regarding the Habitats-Directive (92/43/EEC) and the Bird-Protection-Directive (VS-RL) (2009/14 EG) (RP Gießen 2016).

### 2.3. Characterization of Sampling Sites

To study the effects of heavy rainfall events and the subsequent influx of PPP on aquatic sampling ecosystems three sites in three different river systems were selected jointly under the directive

*Table 2 – Coordinates of sample sites.*

River System	Coordinates (N)	Coordinates (E)
<b>Langder Flutgraben (LF)</b>	50°27'36.8"N	8°56'07.0"E
<b>Waschbach (WB)</b>	50°24'37.2"N	8°53'26.6"E
<b>Weidgraben (WG)</b>	50°24'14.6"N	8°54'41.7"E

of the BfG. The coordinates for these systems are shown in table 2 and their course on a map in figure 3. Due to the outline of the NiddaMan Project these sites had to be chosen in the Horloff Catchment Area.

Requirements for choosing the sites included the factors “vicinity of agricultural landscapes” for the possibility of influx of pesticides, “accessibility” due to the nature of the equipment, “stable water levels that rise significantly in case of rainfalls” and diversity between the different sampling sites. Artificial structures that might alter the water flow (i.e. dams, water overflow discharges etc.) were avoided as much as possible. A map for the land use designation in the vicinity of the streams can be found in figure 4.

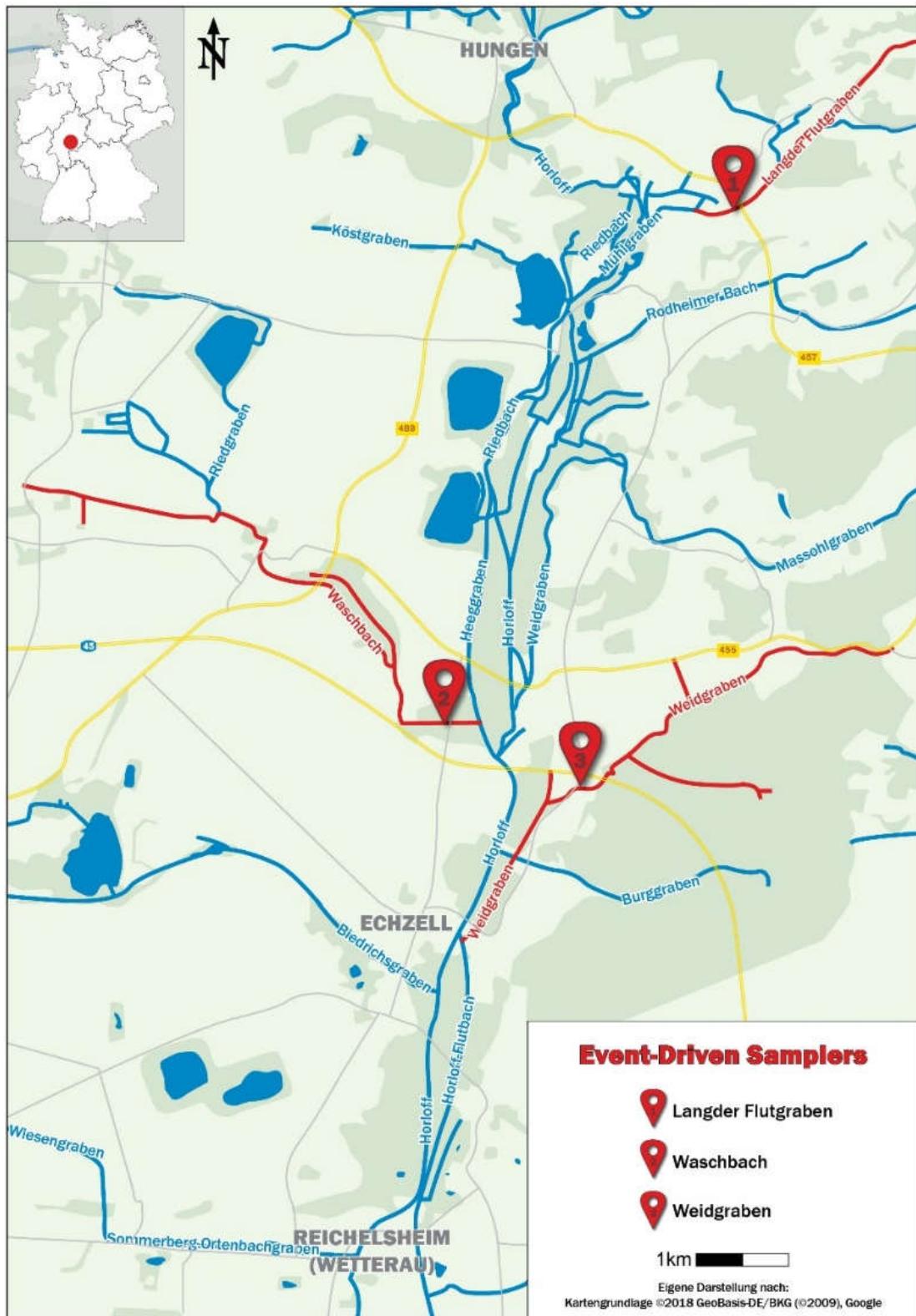


Figure 3 – Map of the research area between the towns of Hungen and Echzell. Sampled rivers are shown in red, the Horloff can be seen in the middle.

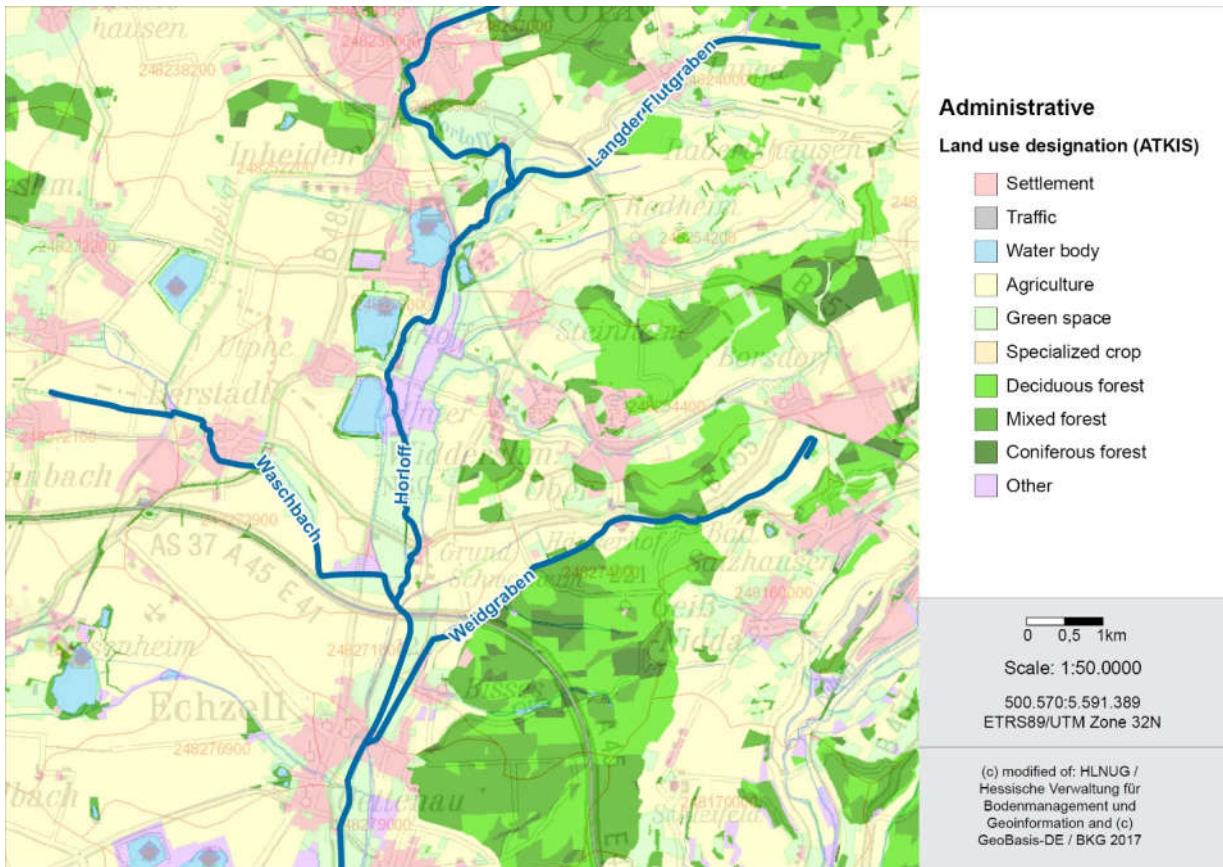


Figure 4 – Land use designation of the three streams in the area of investigation. Similar to table 1 that shows the land use for the Nidda catchment area, the catchment area of the three streams consists mostly of fields and, especially at the Weidgraben, of forest. Map adapted from (HLNUG 2015).

### 2.3.1. Langder Flutgraben

The Langder Flutgraben (location and course see figure 5) is a 5.40 km long left side tributary to the Horloff that does not have its own catchment area. Also known as Bach- and Biebergraben it belongs to the river type five, defined as a silicate small upland stream, rich in coarse material (LAWA: Grobmaterialeiche, silikatische Mittelgebirgsbäche), characterized through riverbeds that flow rather slowly in more shallow valleys. Typical for this river type is an alternating of rapids and slow flowing sections. The source of the Langder Flutgraben can be found north east of the village of Langd in the Wetterau in a small forest. It then flows through Langd and the surrounding fields, crossing underneath the B 487, a highly frequented country road, before leading into the Horloff near Trais Horloff. Several even smaller but unnamed ditches contribute to the Langder Flutgraben which is structurally completely altered from the town of Langd until its influx into the Horloff.

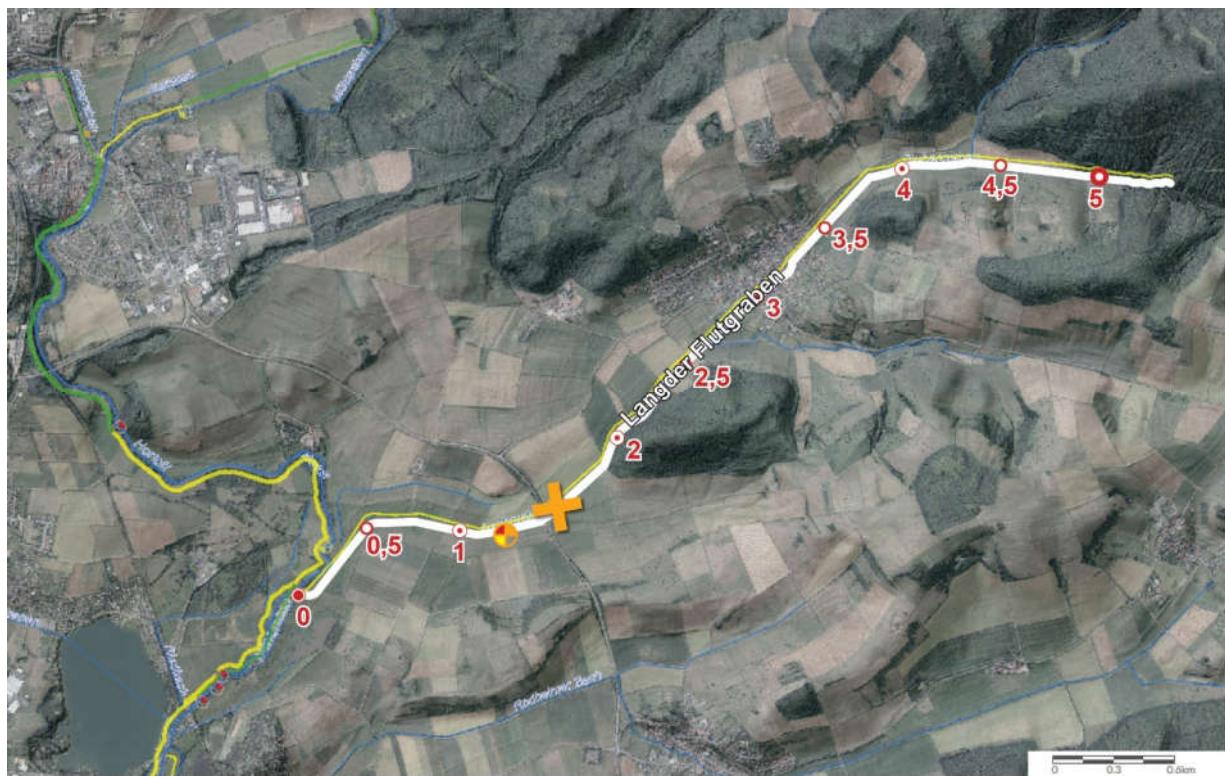


Figure 5 – Course and location of the Langder Flutgraben near the town of Langd (km 3.00).

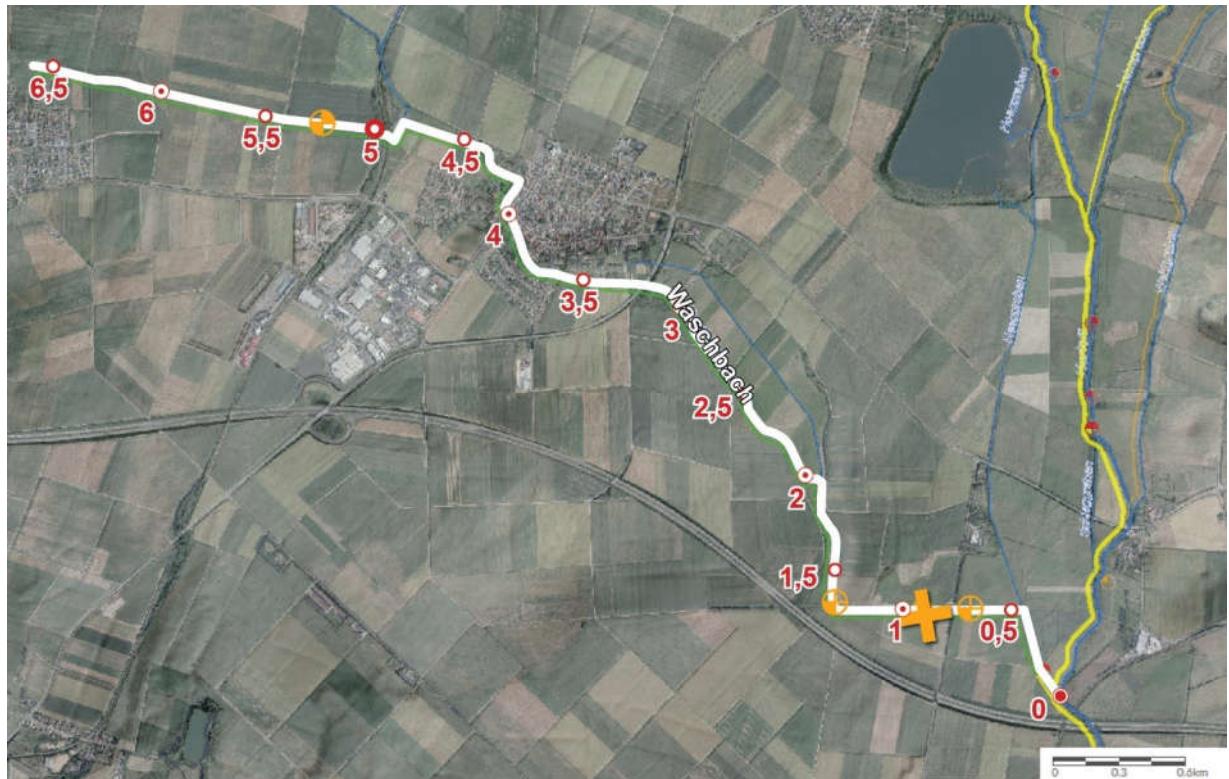
Location of the event-driven sampler ( $50^{\circ}27'36.8''N$   $8^{\circ}56'07.0''E$ ) at river km 1.5 is marked by an orange cross. Length of river in km, water quality level is moderate (yellow lines). Diagram at km 1.2 shows that state of fish is bad (red slice), for macrobenthos not satisfying (orange slice) and for diatoms moderate (yellow slice). The Horloff can be seen on the left – Map and Data adapted from (HLNUG 2015).

The quality of the structure changes from category 4 (clearly altered) to category 6 (extremely altered) and 7 (fully altered) from the village of Langd onwards. The effects of this status are confirmed by the German saprobic index, in which the Langder Flutgraben is categorized as level 3 (moderate). The fish quality has been judged as bad, whereas the status of diatoms is considered moderate and for macrobenthos not satisfying. The sampler at Langder Flutgraben was placed about 50.0 m before the ditch runs underneath the B487, in a location that is surrounded by fields.

### 2.3.2. Waschbach

The Waschbach (WB) (location and course see figure 6) is a river type 5 (HLNUG 2015) and 9.75 km long, right side tributary to the Horloff, which it joins south of Grund-Schwalbach, beginning near the small village of Wohnbach in the Wetterau. It flows through fields until it passes the town of Berstadt, where it runs partly underground, passing underneath the B455 and flowing

through an FFH and bird protection area. Several smaller ditches are tributaries to the Waschbach, with only two, the Riedgraben and the Heeggraben being named.



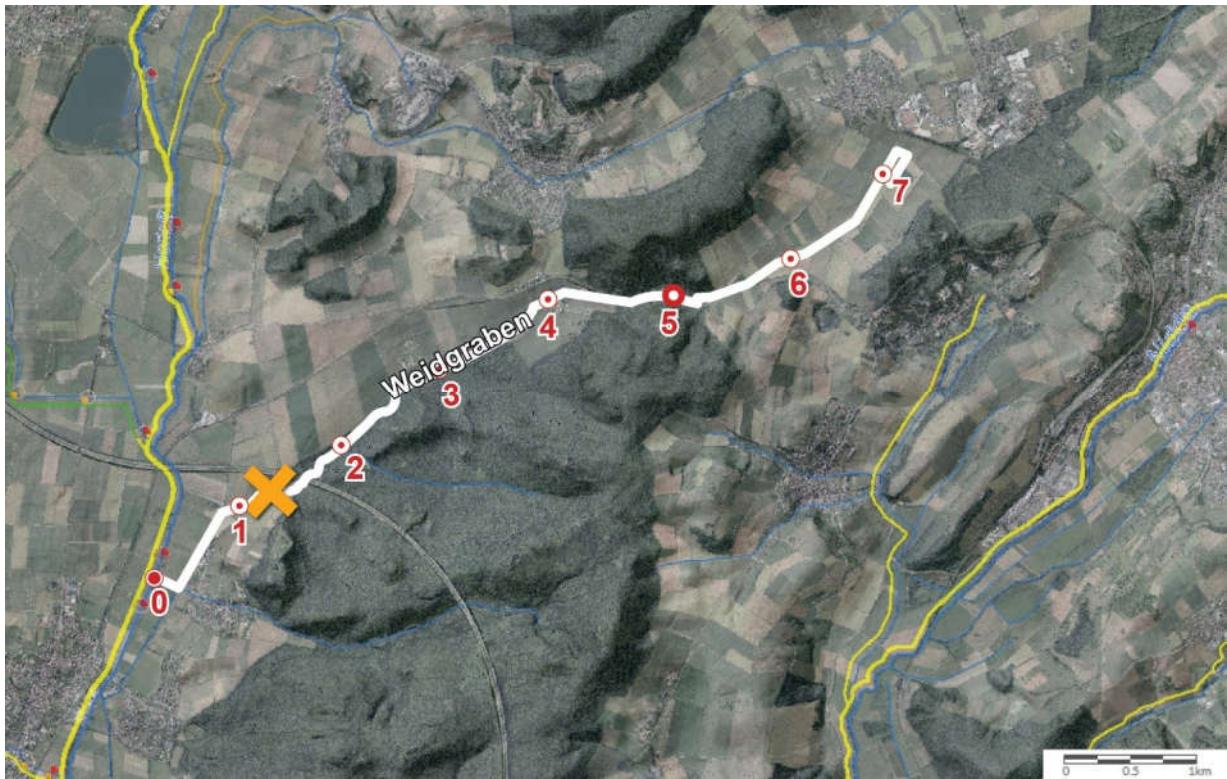
*Figure 6 – Course and location of the Waschbach near the town of Berstadt (km 4.50). Location of the event-driven sampler ( $50^{\circ}27'36.8''N$   $8^{\circ}56'07.0''E$ ) at river km 0.90 is marked by an orange cross. Length of river in km, water quality level is good (green lines). Diagrams show that state of macrobenthos and diatoms is not satisfying (orange slices). The Horloff can be seen on the right – Map and Data adapted from (HLNUG 2015).*

The catchment area of the Waschbach is about  $8.1 \text{ km}^2$ . The Waschbach is categorized as level 2 (good quality) in the German saprobic index with quality for diatoms and macrobenthos resulting in not satisfying levels, whereas fish quality has not been assessed. The structure of the creek has been described as drastically altered (HLNUG 2015) for most of its length and is worst in the town of Berstadt, where it does not flow in a natural riverbed anymore. In the FFH area the quality improves again to level four: clearly altered. Fields surrounded (see figures 4 and 6) the location of this sampler, except at one small patch at the end, where the Berstädter Kist is located, a wetland and important bird migration spot.

### 2.3.3. Weidgraben

With a length of  $8.40 \text{ km}$  the Weidgraben (WG) (location and course see figure 7) is the second longest creek that has been chosen for sampling, with the largest catchment area of  $12.7 \text{ km}^2$ . It

is a river type 9 (HLNUG 2015) silicate small upland stream with the source north-east of the town of Bad Salzhausen.



*Figure 7 – Course and location of the Weidgraben.*  
The Weidgraben does not cross any town till it flows into the Horloff-Flutgraben near Bisses. Location of the event-driven sampler ( $50^{\circ}24'14.6"N\ 8^{\circ}54'41.7"E$ ) at river km 1.2 is marked by an orange cross. Length of river in km. No data was available for water quality and biology. The Horloff can be seen on the left. - Map and Data adapted from (HLNUG 2015.)

The Weidgraben then runs in a south eastern direction through forests and fields until it flows into the Horloff-Flutbach near the village of Bisses. The most distinctive structure constructed above the river is a bridge over which runs the Autobahn A 45. The structure of the creek is category 7 upstream and improves to category 4 in the forest parts, before declining again from the Autobahn bridge downstream.

Neither the German saprobic index nor the quality of fish, diatoms or macrobenthos has been analyzed for this river system. The sampler was deployed in a small stretch of riparian forest that was enclosed by fields on both banks.

#### 2.3.4. Weather Conditions at Sampling Sites

Water levels were constant for most of the investigation period (see figure 8). Extreme changes in water levels occurred in May, most notably on the Waschbach on 31<sup>st</sup> May, when the most

significant rise of water levels was observed. After May the weather became drier (see figure 1), which is reflected in the lower water levels from June onwards.

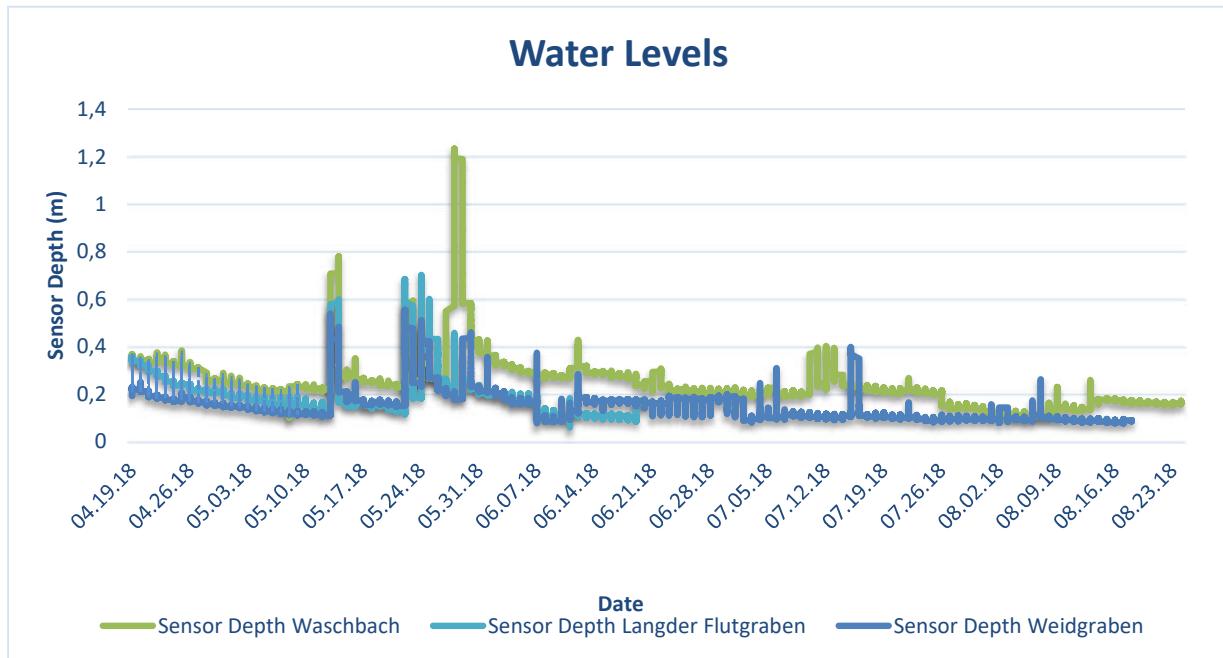


Figure 8 – Flow chart of the water levels of LF, WB and WG between 19<sup>th</sup> April and 23<sup>rd</sup> August.

In total, 8 heavy rainfall events occurred during the spring campaign 2018 that led to sampling for a total of 132 water samples. Table 3 shows the total number of samples available for native testing, chemical analysis and extract preparation, required for in-vitro testing.

Table 3 – Compilation of Heavy Rainfall Events that led to, at least, sampling in one site.

(First number in brackets: Number of samples analyzed by the BfG – Second number: Number of extracts)

\* Severe heavy rainfall event that led to flooding and blockage of the samplers. No water samples were taken by the samplers. Instead 8 water samples were collected manually at site 36 hours after the event.

\*\* Samples at Waschbach were taken at 14<sup>th</sup> June, from this date onward only 3 samples were used for elution (always samples numbered: II, V and VII).

\*\*\* Sampler at Langder Flutgraben defunct due to desiccation.

Sampling Date 2018	Langder Flutgraben	Waschbach	Weidgraben
30 <sup>th</sup> March	8 (8;0)	8 (8;0)	-
14 <sup>th</sup> April*	3 (0;3)	4 (0;2)	8 (0;3)
15 <sup>th</sup> May	8 (8;8)	8 (8;8)	8 (8;2)
24 <sup>th</sup> May	8 (7;7)	8 (8;8)	8 (0;2)
31 <sup>st</sup> May	7 (6;7)	-	3 (0;3)
12 <sup>th</sup> to 14 <sup>th</sup> June**	8 (8;3)	8 (8;3)	8 (8;3)
24 <sup>th</sup> July***	-	8 (0;3)	8 (0;3)
15 <sup>th</sup> August***	-	-	3 (0;0)
<b>Sum</b>	<b>42 (37;28)</b>	<b>44 (32;24)</b>	<b>46 (16;20)</b>

Of these 132 water samples 85 were analyzed by the BfG for its chemical compound and 72 were eluted for in-vitro testing. Samples from 24<sup>th</sup> July and 15<sup>th</sup> August as well as the manually collected samples from April 14<sup>th</sup> were not analyzed chemically. 46 samples were taken by the event-driven sampler at the Weidgraben, of which 16 were chemically analyzed and 20 used for the extracts. The sampler at the Waschbach provided 44 samples in total of which 32 were chemically analyzed and 24 eluted, whereas the sampler at the Langder Flutgraben provided 42 samples (37 chemically analyzed, 28 eluted), until it became defunct after June 12<sup>th</sup>.

## 2.4. Sampling Process

It has been shown that heavy rainfall events can lead to an increased influx of pesticides from fields (Meite et al. 2018) into small streams, creeks or drainage trenches (Langer et al. 2017). Samples taken during or shortly after a heavy rainfall events showed spikes in certain types of pesticides in swiss rivers in 2017 (Langer et al. 2017) as well as in tributaries to the Horloff (Wick 2018). Samples analyzed by the BfG from a test campaign in the fall of 2017 showed elevated levels of several pesticides and drugs (Wick 2018). The sampling campaign of the fall 2017 was continued in the spring 2018. As in the fall 2017 three event-driven samplers of the type P6 L (MAXX Mess- u. Probenahmetechnik GmbH, Rangendingen, Germany (MAXX Mess- und Probenahmetechnik GmbH 2015)) were deployed by the BfG at the described small river streams in the catchment area of the Horloff. Similar to the approach of Langner et al. (2017) these event-driven samplers were installed to take samples during the course of 24 hours after a heavy rainfall event took place. The analytical process included a chemical analysis of the samples by the BfG as well as in-vivo and in-vitro analysis by the Department of Aquatic Ecotoxicology of the Johann-Wolfgang-Goethe University. The method described in this section is based on an ideal sampling process, meaning that all glass bottles were equally filled with 300 mL collected water.

### 2.4.1. Event-Driven Sampler - Structure

In the course of the NiddaMan project the BfG developed an event-related sampling technique that included the installation of portable samplers of the type P6 L. One sampler each was deployed (see also chapter 2.3) at the Langder Flutgraben, the Waschbach and the Weidgraben. In this section the basic functionality of the sampler will be explained.

The most important parts of the sampler included the housing with the electronics (see figures A1 to A4) in the upper housing and the 24 glass bottles containing the sampled water in the bottle compartment, both outside of the water. Rising water levels were measured by using a swimmer that hovered over the water surface and was activated as soon as the water levels rose sufficiently to cover it with water. Sampled water was collected through tubes connecting the stream with the vacuum dosing vessel. For outdoor utility the sampler was powered by a battery, sufficient for one 24 hours load and the stand-by time in-between different events, and a mobile box. The mobile box was activated by an impulse from the sampler at the point of time at which collection of the water started. It then sent an SMS to inform that the water sampling had started after which it was required to wait 24 hours for the sampling process to finish.

#### 2.4.2. Settings of Event-Driven Sampler and Collection of Water



Figure 9 – Bottle compartment of the sampler at Weidgraben, syringe filter, syringe and hydraulic sensor. As can be seen not all glass bottles are filled, a recurring situation at this location.

As shown in figure 9 the bottle compartment contained 24 bottles with a capacity of 300 mL each. The event-driven sampler was set to collect 100 mL of water according to the plan for sampling devised by the BfG (see table 4). After the end of the sampling process the compartment section had to be emptied quickly to prevent loss of volatile substances.

Table 4 – Sampling program used for the sampling campaign at all sampling sites.

Samples were taken with shorter durations of 20 minutes at the beginning and longer durations of 2 hours for one compound in the end. The target volume per compound was 300 mL. Three samples were joined together in continuous order in 1L glass bottles to receive the target volume of 900 mL for biological tests.

Sampling Program No.	1	2	3		
Duration Sampling Cycle (h)	3	9	12	24	Total Duration of Sampling Cycle (h)
Length per Compound Sample (h)	0.333	1	2		
Number of Compound Samples	9	9	6	24	Total Number of Compound Samples
Gap between Sampling (h)	0.083	0.25	0.5		
Gap between Sampling (min)	5	15	30		
Number of Compound Samples for Biology	3	3	2	8	Total Samples for Biology
Duration of Samples for Biology (h)	1	3	6		

Target Volume per Compound (mL): 300

Target Volume per Compound for Biology (mL): 900

Combined Samples per Compound for Biology: 3

The sampled water was divided into several vessels: 15 mL of each glass bottle were filtered with a syringe filter (0.45 µm) and filled into a micro tube labelled with Arabic numbering from 1 to 24. Afterwards three glass bottles were poured together into one 1 L brown glass bottle labelled with Roman numerals from I to VIII. These 8 bottles from each sampler were later filtered in the laboratory by using grade 696 qualitative filter paper with a retention size of 1.5 µm (VWR International bvba, Leuven, Belgium). From the water filtered in the lab, 25 mL were sent to the BfG for chemical analysis and 330 mL were available for acute toxicity in-vivo testing with *D. magna*, *Hyalella azteca* and *C. riparius*. In contrast to the samples that were tested directly after an event, the bottles for 30<sup>th</sup> March were not filtered directly after the heavy rainfall event and used de-frozen and unfiltered samples instead.

The remaining 500 mL were used for solid phase extraction (SPE), drawn on a C18 column (Telos™ SPE Columns, Kinesis Inc., Vernon Hills, Illinois, USA) and enriched by the factor 1000 to be used later for in-vivo testing with *D. magna* and *H. azteca* and in-vitro testing with *C. uda* and *Allivibiro fisherii* (Ratz 2019) (for procedure and results of tests with *H. azteca*, *C. riparius* and *A. fisherii* see Ratz (2019)). The Telos™ C18 column type was chosen as it promised the highest chance for transferring and enriching of the substances found in the sampled water to the extract vials. For each day of sampling one to two additional columns with laboratory grade purified water have also been created and used as an SPE blanket.

## 2.5. Preparation of Extract

The preparation of the extracts used for the in-vivo and in-vitro testing required the following steps:

1. Activation of the molecule chains on the C18 column with 2 mL heptane, 2 mL acetone and 6 mL methanol
2. Drawing of the substances in the sampled water onto the columns
3. Freeze-drying of the columns with nitrogen

At a later point the frozen columns had to be eluted to solve the stored substances into a vial that would contain the extracts:

1. Starting the elution process by filling 5 mL methanol and 5 mL acetone into the columns and collection the eluted solution in 15 mL brown glass vials
2. Evaporation of the solution with nitrogen until until a final volume of 500 µL remains
3. Transferring this amount into vials and continuation of the evaporation until the vial could be filled with 500 µL DMSO

The DMSO (Dimethyl sulfoxide, CAS Number 67-68-5, Sigma-Aldrich/ Merck KGaA, Darmstadt, Germany) had to be applied to avoid full evaporation of the possibly volatile substances, as well as to make the elution stable for freezing and de-freezing. 500 mL of sample water were thereby enriched to a 1000-fold concentration in the 500 µL extract volume.

## 2.6. Chemical Analysis of Water Results and Assessment of Toxicity

Chemical analysis of the water samples was conducted by the BfG. For the chemical determination a sample catalogue consisting of 40 pesticides and 17 metabolites was used (see figure 14 and tables TA2 to TA5). The results were then itemized into the dates of a heavy rainfall event for each stream accordingly to the number of the taken samples and categorized into the respective substance found. For each sample the level of each substance was determined (in ng/L). To further evaluate the significance of the findings in the chemical analysis the results of the chemical analysis were analyzed with MS-Excel<sup>©</sup> (Microsoft, Redmond, Washington, USA) for a range of values, like days above limit of quantification (LOQ), most toxic events and the regulatory acceptable concentration (RAC) of each pesticide. RAC values are predefined thresholds for pesticides that aim to transfer laboratory findings of organism tests to real outdoor situations by extrapolating results from tests conducted with the most susceptible aquatic species amplified by a security factor to account for unknown uncertainties (WBF 2018). RAC values are issued by governmental bodies, like the Umweltbundesamt (UBA), who update the RAC values continuously, most recently in 2019 (UBA 2019). To assess the impacts of the chemical analysis these values were compared with the literature LC50 values for *D. magna* which were also required for calculating the TUs and were obtained from the Pesticide Properties DataBase (PPDB) (University of Hertfordshire 2016).

## 2.7. *Daphnia magna* Acute Toxicity Tests

The acute toxicity test with *D. magna* was conducted with native water samples as well as with extracts. Subsequent testing with extracts was conducted to determine if higher concentrations of the compounds would have more severe effects on the survival rate than tests with native samples. The test structure was adapted from the OECD Guideline 202 “*Daphnia sp.*, Acute Immobilization Test and Reproduction Test” (OECD 2004).

### 2.7.1. Characterization of *Daphnia magna*

The genus Daphnia (scientific classification in table 5) has been studied for 250 years and describes a class of planktonic crustacea consisting of 100 known species of freshwater

Table 5 – Scientific classification of *Daphnia magna*. (WoRMS - World Register of Marine Species - *Daphnia magna* Straus, 1820)

Scienc. Classification	
Kingdom	Animalia
Phylum	Arthropoda
Subphylum	Crustacea
Class	Branchiopoda
Order	Cladocera
Family	Daphniidae
Genus	<i>Daphnia</i>
Species	<i>Daphnia magna</i>

organisms. While species of this genus are found worldwide in all but the most extreme limnic water systems, only three are native to Europe, *D. magna* being one of them (Ebert 2005). Most commonly found in areas that rarely see freezing temperatures, *D. magna* inhabits ponds, lakes (Storch and Welsch 2014) and rocky water pools near the sea (Elenbaas 2018). They best dwell in water temperatures ranging from 18°C to 22°C but are known to withstand wider temperature amplitudes as well.

A restricting factor for the occurrence of *D. magna* consists in the number of fish found in the specific habitat. In cases of large fish populations *D. magna* is absent due to being preyed upon (Ebert 2005). *D. magna* is a planktonic crustacean species that, as a filter feeder, consistently creates a running water stream to transport oxygen and food to their mandibles. The most characteristic feature for all Daphnia species (schematic description of body parts in figure 10) is the carapax, a transparent shell with two flaps made of chitin that encompasses the whole body. The carapax is continued by the head plates, a characteristic for distinguishing different species of *Daphnia*. Typical for *Daphnia* species are their two different eyes, the compound, a large black eye used for basic vision and orientation and the much smaller ocellus. The only parts not encompassed by the carapax are the rudder antennae at the head, used for locomotion. Further extremities are the first antennae for sensory effects above the mouth, used for sensory effects and the five thoracic appendages (= phyllopods) used to produce the water stream through which

the filtered food and oxygen is transported to the mouth respectively the gills (Ebert 2005). Food consists mostly of phytoplankton, detritus (Ebert 2005) or even bacteria in the size range from 1 µm to 50 µm (Elenbaas 2018). After the food enters through the mouth, described by (Ebert 2005) as „food groove“, it is guided to the gut, which consists of three parts: the esophagus, the midgut, and the hindgut.

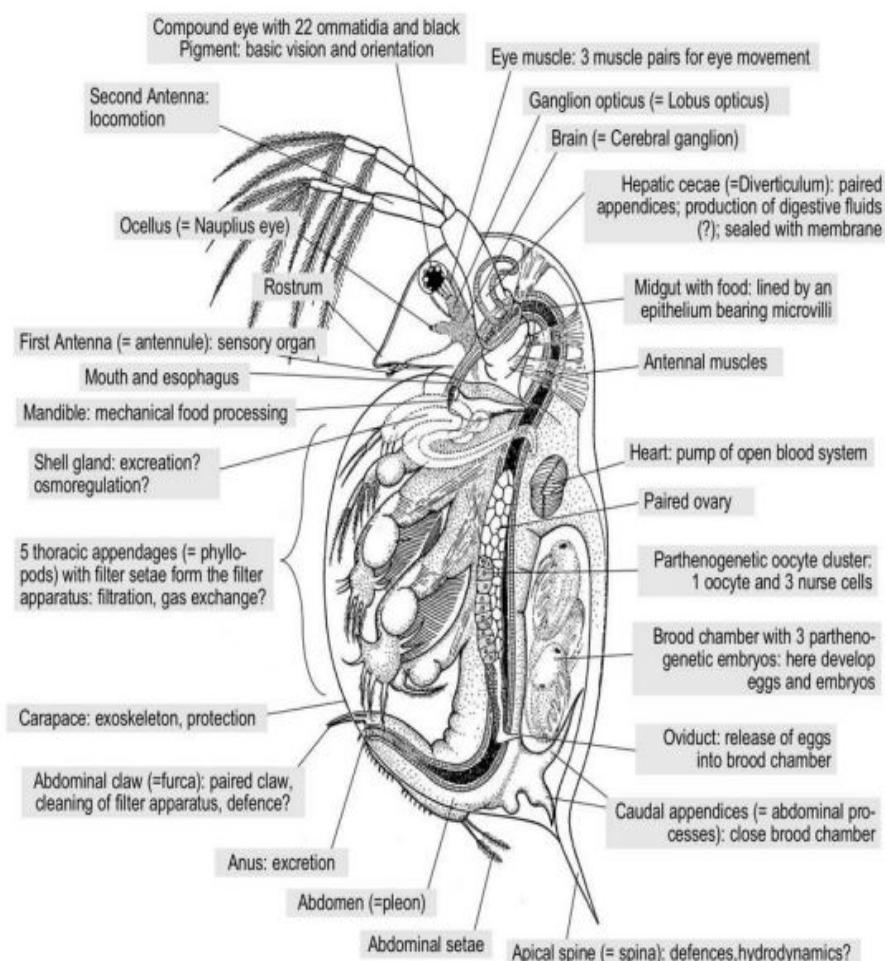


Figure 10 – Schematic display of *Daphnia magna* Strauss showing all relevant external and internal body parts (Ebert 2005).

The basic gender state for *D. magna* is female with males only occurring in case of inadequate environmental conditions and solely for reproduction matters (Ebert 2005). Males are therefore much smaller in size (ca. 2 mm), whereas females range from 3 to 5 mm. Resulting from environmental conditions reproduction is normally parthenogenetic, with females above an age of 10 days producing diploid eggs in an asexual process which then develop into exact female

clones of the mother (Ebert 2005). In cases of sexual reproduction (which did not occur in our laboratory environment) females produce haploid eggs that must be fertilized by the male and will be transported on the back of the female. After the next ecdysis the eggs are released. Since sexual fertilization mostly occurs during autumn, a population explosion occurs at the beginning of the following spring. In laboratory settings females have a life expectancy of about 40 days and produce offspring every 3 to 4 days (Ebert 2005).

Due to the facile handling in laboratory settings and practical characteristics like their transparent shell, the feeder filter *D. magna* has been used for a long time as a test organism to analyze all kinds of environmental pollutants. These factors played a vital role in defining *D. magna* as the standard invertebrate test organism for the analysis of pesticides (Köhler and Triebeskorn 2013). A similar standard has been set in the EU which defined *D. magna* as the standard test-organism in the arthropod's phylum for the risk assessment of insecticides and substances with insecticide activity (Köhler and Triebeskorn 2013).

As a result, these aspects were essential for choosing *D. magna* as the standard test-organism for the analysis of the collected samples for the in-vivo tests.

### 2.7.2. 48 h Acute Toxicity Test with Native Samples

The acute immobilization test was run for 48 hours with immobilization recorded at 24 hours and 48 hours intervals as mandated by the OECD. Conical sample containers made of glass with a size of 10 mL were used as test vessels and covered with snap-on lids. Test organisms were less than 48 hours old neonata (all daphnids in all test settings from: Department of Aquatic Ecotoxicology, Johann-Wolfgang-Goethe University, Frankfurt am Main), stemming from second to fourth brood

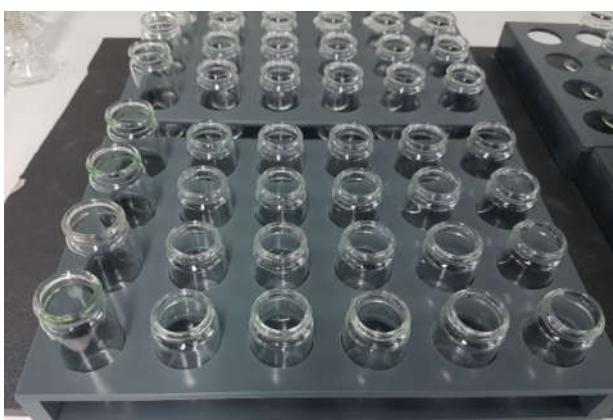


Figure 11 – Test setting for tests with native water samples showing one plate with 24 conical containers without lids.

progeny. Diverging from the OECD guideline 202 one daphnid was placed in one conical sample container with 2 mL test solution, an amount pre-determined through an earlier test (Reutter 2018). Native samples were tested with the samples that had been collected according to the intervals stated in table 4. For each interval 8 replicates were provided. Sample containers were placed

inside a stand according to their number (see figure 11). Negative controls (NCs) were chosen according to the general numbers of samples available but usually ranged from 24 to 36 negatives. Elendt M4 Medium was used for the NCs (Elendt and Bias 1990) as proposed by OECD guideline 202. In a solution containing 3.5 M potassium dichromate 12 positive controls (PCs) were placed. All test subjects were placed in a room that followed a 16-hour light and 8-hour dark cycle and had a temperature of 23°C. No feeding was conducted during the test and subjects were counted for immobilization after 24 hours and 48 hours.

### 2.7.3. 48 h Acute Toxicity Test with Extracts on 96-Well Microtiter Plate

To assess if higher concentrations of the chemical mixture found in the samples would lead to a higher mortality rate a second test phase was conducted with extracts on a microtiter plate (Anicrin S.r.l., Scorzè (VE) Italy) with 96 cavities. These tests were based on the findings of the chemical analysis and were based on earlier tests that showed that *D. magna* neonata can be used as test organisms on microtiter plates with 24 and 36 cavities (Baumann et al. 2014). Based upon these finding pre-tests with Elendt M4 medium (Elendt and Bias 1990) confirmed that microtiter plates with 96 cavities and 200 µL medium can be used for in-vivo acute tests with *D. magna* and that the maximum threshold for DMSO for these organisms is 2 % only allowing testing of a concentration 20 times higher than the native samples. DMSO was used for the solvent controls (SCs), whereas 3.5 M potassium dichromate was used for the PC. Four extracts were chosen for testing, which were assumed to contain either a highly toxic (LF V and LF VIII 24<sup>th</sup> May) or very low toxic compound (WB V and WB VIII 15<sup>th</sup> May). The microtiter plate was divided into NCs, SCs and samples, whereas PCs were placed on a separate plate (for schematic display of plates see figures A7 – A10, for dilution series see table 7). On a composition plate the dilution series was prepared by first diluting 9.6 µL extract with 231.4 µL M4 medium in columns 2 to 9 in row “A” as 8 replicates for each concentration were required. For the SCs the same amount of DMSO was diluted with M4 Elendt medium. To continue the dilution series 120 µL M4 Elendt medium were filled into each well in rows “B” to “H” and diluted with 120 µL of the compound from the previous row (PR) until the lowest concentration of 1.17 times of the native sample was reached. Afterwards 100 µL of the dilution compound in the composition plate was transferred into the corresponding row on the test plate. On the test plate one daphnid was pipetted with 100 µL M4 medium into each well with extract or SC. For each well of the NCs, placed on columns

1 and 12, one daphnid was transferred with 200 µL M4 medium, whereas the daphnids on the PC plate were pipetted with 100 µL M4 medium in wells that contained 100 µL 3.5 M potassium dichromate. In the end each well contained 200 µL of the respective substance. 16 individuals were tested for NC, PC and SC each with test subjects being controlled for immobilization after 24 hours and 48 hours. The statistical analysis of the in-vivo tests with *D. magna* can be found in A3.

## 2.8. In-vitro Test with *Cellulomonas Uda*

*C. uda* (scientific classification in table 6 - DSM 20108/ATCC 21399 (DSMZ 1990)) belongs to the bacteria genus *Cellulomonas*, a high-GC gram positive bacterial genus that belongs to the phylum

*Table 6 – Scientific classification of Cellulomonas uda.* Actinobacteria. So far, this genus is the only known and (DSMZ 1990)

Scienc. Classification	
Kingdom	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Order	Actinomycetales
Suborder	Micrococcineae
Family	Cellulomonadaceae
Genus	<i>Cellulomonas</i>
Species	<i>Cellulomonas uda</i>

described one whose members are cellulolytic facultative anaerobes. Therefore, bacteria of this genus can be found in all environments where the degradation of cellulose takes place. Mostly found in soils, they also inhabit the bark of trees, wood, sewage sludge and water systems (Poulsen et al. 2016; Reguera and Leschine 2001; Crecchio et al. 2001). *C. uda* is known for degrading cellulose aerobically by usage of different enzymes like  $\beta$ -glucosidase which is secreted to  $\beta$ -D-glucose molecules (Clemmer and Tseng 1986; Dermoun and Belaich 1985; Haggett et al. 1979). To determine the metabolic rate of the substrate 4-Nitrophenyl- $\beta$ -D-glucopyranoside (NPG), the  $\beta$ -glucosidase activity is measured photometrically through the colorant 4-nitrophenol.

### 2.8.1. Culturing of Bacteria

The original strain 20107 of *C. uda* was initially ordered by the “Leibniz Institute DSMZ – German Collection of Microorganism and Cell Cultures” in 2017. Since then this strain has been cultured several times from cryo cultures in the working group of the department for aquatic ecotoxicology, using the method outlined by König (2017). For the conducted research two strains, re-cultured from the original strain on 11<sup>th</sup> November 2017 and 05<sup>th</sup> September 2018, were used.

As an exponentially growing bacteria, *C. uda* shows the typical phases of kinetic growth, lag phase, log phase, stationary phase and death phase (Cypionka 2006; Reineke and Schrömann 2015). Since the bacteria can only be used in its most exponential growing phase during the log phase, it was necessary to calculate the exact point of time at which the bacteria enter the transition phase from exponential (log phase) to stationary growth. Culturing of the bacteria required 50 µg *C. uda* to be spiked into 50 mL of growth medium 53 (for formulation see table TA 1 and König (2017)) in a small Erlenmeyer flask and incubate on an analog mini shaker (Orbital shaker, Advanced Mini Shaker 15, VWR International GmbH, Darmstadt Germany) at 30°C and 150 orbital rpm.

To define the exact point at which the log phase begins, a pre-test was conducted to develop a kinetic growth chart (see figure 12). In 2-hour intervals the optical density (OD) 595 was measured by using a biospectrophotometer (Eppendorf BioSpectrometer<sup>©</sup> basic, Eppendorf AG, Hamburg, Deutschland). Based on the data received from the measurement of the extinction factor it was possible to calculate the FAU (Formazin Attenuation Units) value according to ISO-Guideline 11350 (ISO 2012). The extinction of bacteria cultures can be converted to FAU and depends on the opaqueness of a solution, whereas the opaqueness itself depends on the number of particles, proteins or bacteria included in a solution. The higher the FAU the higher will be the number of bacteria in the solution. As can be seen in figure 12 the phase transition from log to stationary phase happens approximately after 20 to 22 hours, therefore samples of the bacteria had to be applied before that point at about 19 h +/- 1 h after incubation.

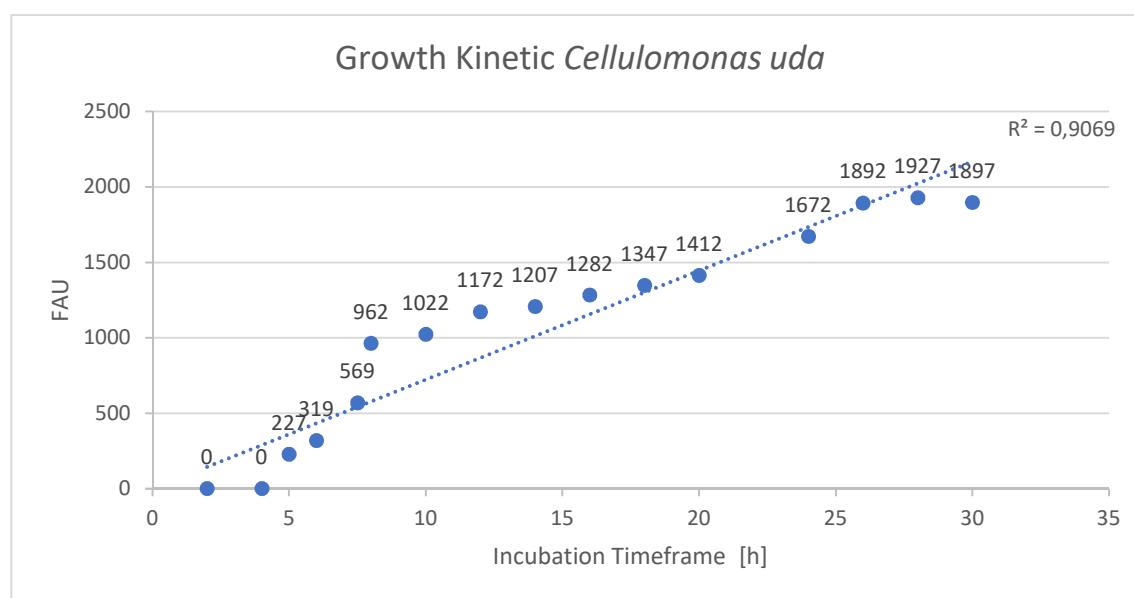


Figure 12 – Kinetic growth curve of *Cellulomonas uda* for a 30-h time frame. The log phase begins at roughly 19 hours.

Since a FAU value of 200 (König 2017) is required for testing, the required volume of *C. uda* can be calculated by first calculating the FAU from the OD<sub>595</sub>:

$$FAU = \frac{OD_{595} - 0.0182}{0.0019}$$

with the constants taken from a calibration measurement done previously in the department of aquatic ecotoxicology.

### 2.8.2. Methodological Background

To determine the activity of  $\beta$ -glucosidase in the test setup, 4-nitrophenyl  $\beta$ -D-glucopyranoside (NOPG) (Sigma-Aldrich/ Merck KGaA, Darmstadt, Germany) is added to the test samples as a substrate. The substrate is then degraded to  $\beta$ -D-Glucose and 4-Nitrophenol through the activity of

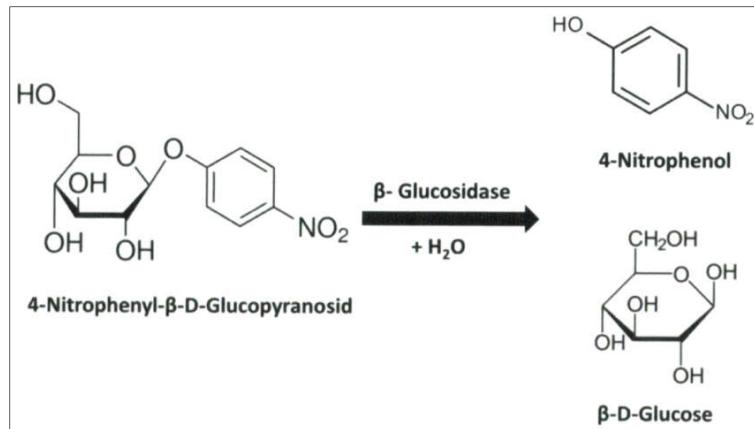


Figure 13 – Hydrolysis of 4-NOPG to 4-Nitrophenol and  $\beta$ -D-Glucose as a result of  $\beta$ -Glucosidase activity (Misovic and Langner 2018).

the  $\beta$ -glucosidase exoenzyme in the water samples (see figure 13). The concentration of the colorant 4-nitrophenol that is released during the enzymatic cleavage can then be measured photometrically at a wave length of 405 nm in a fluorescent.

The mono- and oligomers that are created by this synthesis are then available for uptake through the bacteria and usage in their energy metabolism. In aquatic ecosystems, exoenzymes do not only aid in the degradation of cellulose but also play a vital role in the transformation of organic substances, thereby contributing to the self-purification potential of water systems (Langner 2018). *C. uda* is known to use a wide array of soluble substrates but its ability to grow anaerobically on cellulosic compounds has been rarely studied (Poulsen et al. 2016). As an aerobic, facultative anaerobic bacteria, *C. uda* can splice cellulose by usage of different exoenzymes like  $\beta$ -glucosidase into its basic monomers. This ability can be used to calculate the self-purification potential of aquatic systems. Inhibition of the  $\beta$ -glucosidase exoenzyme would

therefore limit the growth of *C. uda* and reduce the degradation rate of cellulose in cases when the stream is infected by pollutants.

Since no final validity criteria were pre-defined, the validity criteria from König (2017) and Langner (2018) were developed further as part of the test setting. Gluconic acid (CAS 527-07-1, Sigma-Aldrich/ Merck KGaA, Darmstadt, Germany) was used as the PC. Gluconic acid is a carboxylic acid found in plants, honey and wine, which is artificially prepared through fungal fermentation. The carboxylic acid is formed by the oxidation of the first carbon of glucose and has antiseptic and chelating properties (NCBI 2019). It has been shown (Eriksson 1978; Lieser 2006; Cannella et al. 2012) to inhibit the expression of the  $\beta$ -glucosidase exoenzyme, leading to the utilization of gluconic acid as the PC substance. For the SC DMSO was used in the same concentrations as for the test samples, for NCs the bacteria were placed in undiluted test medium. To determine the strength of a pollutant or a compound the REF 50 inhibition rates of the steps of the dilution series (see table 7) are calculated and compared with GraphPad<sup>®</sup> Prism. 5.0 (GraphPad Software Inc., San Diego, USA).

*Table 7 – Dilution Series applied in the Cellulomonas uda test.*

*For SC and PC, the concentrations are mentioned in mmol, for the sample compound the concentration factor. For the SC DMSO was added in the same concentration factors as used for the samples to preclude that effects in the concentrations occurred as a result of the DMSO. For the PC gluconic acid was added with the same concentration steps as for the samples to factor in the possibility that inhibition is weakened in higher dilution rates.*

Row on Multititer Plate	Dilution Factor (Sample dilution in 1.5 steps)	Solvent Control (conc/ well [mmol]) DMSO (78.3g/Mol)	Positive Control (conc/ well [mmol]) Gluconic Acid (218g /Mol)	Extract Concentration of Samples
A	1:12.5	0.00260	9.17	80.0
B	1:18.8	0.00170	6.11	53.3
C	1:28.1	0.00113	4.08	35.5
D	1:42.2	0.000760	2.72	23.7
E	1:63.3	0.000500	1.81	15.8
F	1:95.0	0.000340	1.21	10.5
G	1:142	0.000220	0.800	7.02
H	1:213	0.000150	0.540	4.68

### 2.8.3. Test Design

For each test run (TR) one plate was assigned as a control plate, whereas further plates contained the samples. PCs, SCs and samples were prepared on a composition plate and diluted in 8 steps ranging from an 80-fold concentration to a 4.68-fold concentration (see table 7) of the native sample. The compounds from the composition plate were afterwards transferred into the correspondent row in the bacteria plate. In the bacteria plate that contained the controls the first row was filled with the photometric blank (PB), required to account for inherent colors. The next three rows were filled with NC. Depending on the number of samples that were tested on the bacteria plates containing the samples, a varying number of NC were added. The plates were then measured with “Spark<sup>®</sup> multimode microplate reader” (Tecan Trading AG, Männedorf, Switzerland) at a wave-length of 400 nm to account for inherent colors that may influence the final results before the injection of bacteria and 4-NOPG substrate. In the end the microtiter plates were sealed with sterile breathable sealing (Breath Easy, Carl Roth GmbH & Co. KG, Karlsruhe, Germany), placed on an incubator (Titramax<sup>®</sup> 1000, Heidolph Instruments GmbH & CO. KG, Schwabach, Germany) and incubated for 24 hours at 450 rpm and 30°C. After 24 h the stop reagent di-Sodium carbonate was added to stop bacterial growth. Afterwards the metabolic rate of the substrate was measured with the “Spark<sup>®</sup> multimode microplate reader” at a wavelength of 400 nm. A detailed step by step explanation of the preparation can be found in chapter A4-A7.

### 2.8.4. Analysis and Validity

The test method as well as the test setting is still in the experimental phase and follows the outlines provided by König (2017) and Langner (2018). As proposed by Langner (2018) it shall be analyzed if gluconic acid can be used as a PC. Therefore, the EC50 of gluconic acid shall be regarded as one of three different criteria of validity with the other two being the NC and the SC. Both, the NC and the SC shall demonstrate that no inhibition occurred in the growth phase. In short, the test setting for the inhibition test with the model organism *C. uda* (Misovic and Langner 2018) included the following parameters:

- Test elements included samples, PB, NC, PC and SC
- Two microtiter plates with 96 cavities, one for the controls, one for the extracts

- Each extract was tested with three TRs
- Criteria for validity were not pre-defined
- Test criterion was the metabolic rate of the substrate necessary for bacteria growth, induced through the inhibition of the expression of the  $\beta$ -glucosidase exoenzyme
- For calculations of enrichment, dilution factor and metabolism rate see chapter A7

## 2.9. Calculation of TUs and Toxicity

The calculation of the TUs is based upon the results of the chemical analysis, conducted by the BfG. The toxicity of the chemical compound for each substance was determined by calculating the arithmetic mean pesticide load of a substance during one event and for each stream.

$$TU_{(sum)} = \log \sum_{i=1}^n \frac{C_i}{LC50_i}$$

For calculation of the arithmetic mean, only values above the LOQ were considered. The results of the arithmetic mean were then divided by the LC50 values of *D. magna* (values taken from the "Pesticide Properties DataBase (PPDB)" of the University of Hertfordshire) for each chemical substance. In a final step these values were added together for a single event in a single stream and logarithmized to calculate the BQE specific TUs. In this method, already used and formalized by Ohe et. al (2009),  $n$  is the number of compounds considered,  $C_i$  the arithmetic mean of a measured environmental concentration of compound  $i$  and  $LC50_i$  the acute lethal concentration for *D. magna* in an 48h standard acute toxicity test.

As one aim of this thesis was to evaluate the applicability of *C. uda* as a test organism for in-vitro testing of polluted water samples and to assess differences in the toxicity of each stream, the results of the TUs were then plotted against the 50 percent inhibition rate of *C. uda* with the aim to see if lower, and therefore more toxic, TUs correlate with higher inhibition rates.

## 3. Test Results

Test results for *D. magna* showed no increase in mortality rates, whereas inhibition for *C. uda* could be observed in all TRs. Chemical analysis showed that a constant appearance of PPP could be seen in all streams with May being the month in which the strongest pollutants occurred. The Langder Flutgraben was the most toxic river system with TUs in May being higher than – 2.

### 3.1. Chemical Analysis

The water samples were tested by the BfG for 57 substances, 40 of them pesticides and 17 metabolites (see figure 14). These water samples were taken at five different rainfall events between March and mid of June. Two further rainfall events occurred in July and

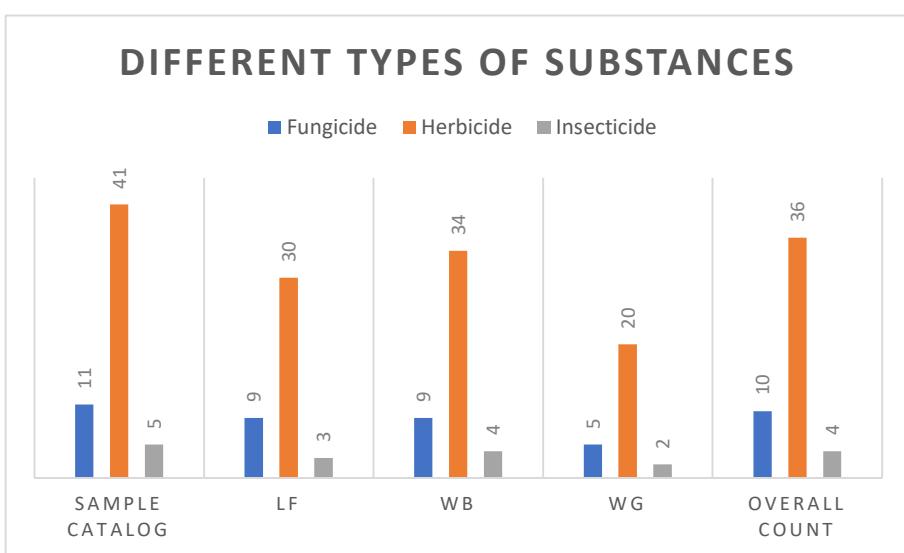


Figure 14 – Types of substances tested through the sample catalog of the BfG. The 57 substances tested are shown on the left. Next, the number of each type of pesticide found in LF, WB and WG, On the right the total numbers found in any stream.

August, but for neither of them a chemical analysis was available until the finalization of this thesis. The distribution of the different pesticides found in the river systems (see figure 14) and the number of chemicals that could be observed at all times in the streams as well as the days at which at least one chemical surpassed its RAC value is shown in table 8:

Table 8 – Diagram of chemicals above threshold limits.

Values above LOQ 100 % of the time and days with at least one chemical surpassing the RAC (regulatory acceptable concentration).

\* 2 Herbicides (*S*-Metolachlor, Terbutylazine) and 3 Metabolites (Metazachlor-ESA, Metolachlor-ESA, Terbutylazin-desethyl)

Parameter	Langder Flutgraben	Waschbach	Weidgraben	All
Number of chemicals that were 100 % of time above LOQ	13	9	10	5 (*)
Number of different days with at least one chemical surpassing RAC (max possible days)	3 (5)	4 (4)	1 (2)	5 (5)

In total, 87.78 % (50 of 57) of all possible chemicals for which the samples were tested could be found at least once in at least one stream. In detail 90 % of the tested fungicides, 88 % of herbicides and 80 % of the insecticides could be found, with most ubiquitous findings of chemicals in the Waschbach (82 % of all possible chemicals) and the lowest in the Weidgraben (57 %).

The concentration of thiacloprid, a neonicotinoid, was more than 176 times higher than the allowed RAC in water sample WB IV 24 May from the Waschbach. This was by far the highest transgression above RAC values found of any chemical during the sampling campaign (second was S-Metolachlor with a value 12 times higher than allowed by the RAC criterion). RAC values were surpassed at all five sampling days in at least one stream. However, RAC values in the Langder Flutgraben and the Weidgraben were only surpassed in May, whereas at the Waschbach a RAC value was surpassed on all 4 sampling days.

With regard to the number of occurrences, a strong seasonality can be seen for May at all sampling sites with 91 of 100 findings of surpassed RAC values occurring during this month.

The events of 15<sup>th</sup> and 24<sup>th</sup> May each led to the transgression of the RAC value of five different substances at the Langder Flutgraben with the highest load in sample LF III 24<sup>th</sup> May. This sample also showed the highest concentrations of S-Metolachlor (14.5 µg/L – 11.89 times above RAC) and Prosulfocarb (12.8 µg/L – 3.36 times above RAC) as well as the second highest concentration for Terbuthylazine (8.69 µg/L – 3.45 times above RAC). These values were sometimes much higher than the RAC, whereas they were lower by several magnitudes than the LC50 values for *D. magna* as can be seen in table 9. The seasonal occurrence of PPP in the Langder Flutgraben, the Waschbach and the Weidgraben is shown in figures 15 to 18.

*Table 9 – PPP that have been above the RAC value at at least one occasion.  
LC50 values for Daphnia magna are divided with the values provided by the chemical analysis.*

Substance	RAC (in µg/ L)	LC50 <i>Daphnia magna</i> (in mg/L)	Chemical Analysis (C) (in µg/ L)	LC50 <i>Daphnia magna</i> / C
Diflufenican	0.0250	> 0.240	0.0470	5.11*10 <sup>3</sup>
S-Metolachlor	1.22	23.5	14.5	1.62*10 <sup>3</sup>
Prosulfocarb	3.80	0.510	12.8	4.00*10 <sup>1</sup>
Terbuthylazine	2.50	21.2	8.69	2.44*10 <sup>3</sup>
Thiacloprid	0.00400	85.1	0.705	2.13*10 <sup>7</sup>
Thiamethoxam	0.0930	> 100	0.0920	1.08*10 <sup>6</sup>

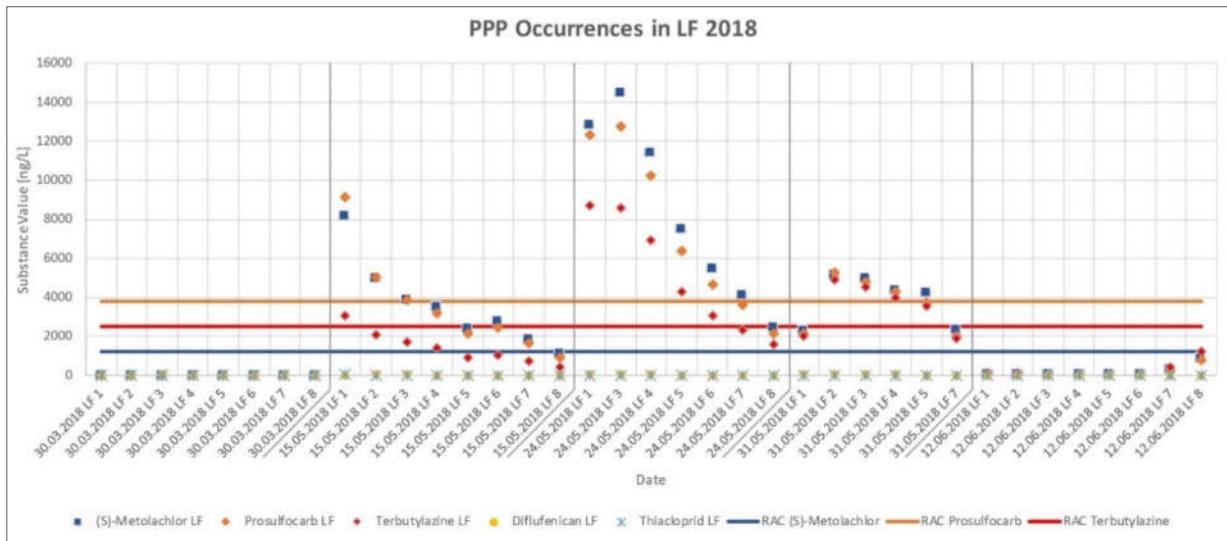


Figure 15 – Diagram [in ng/L] for LF for the 5 PPP that have surpassed RAC.

Highest values are S-Metolachlor with 14.5 µg/L (14502 ng/L), Prosulfocarb with 12.8 µg/L (12762 ng/L) and Terbutylazine with 8.69 µg/L (8692,7 ng/L). RAC Values are shown for S-Metolachlor with 1.22 µg/L (1220.0 ng/L), Prosulfocarb with 3.80 µg/L (3800.0 ng/L) and Terbutylazine with 2.50 µg/L (2500.0 ng/L).

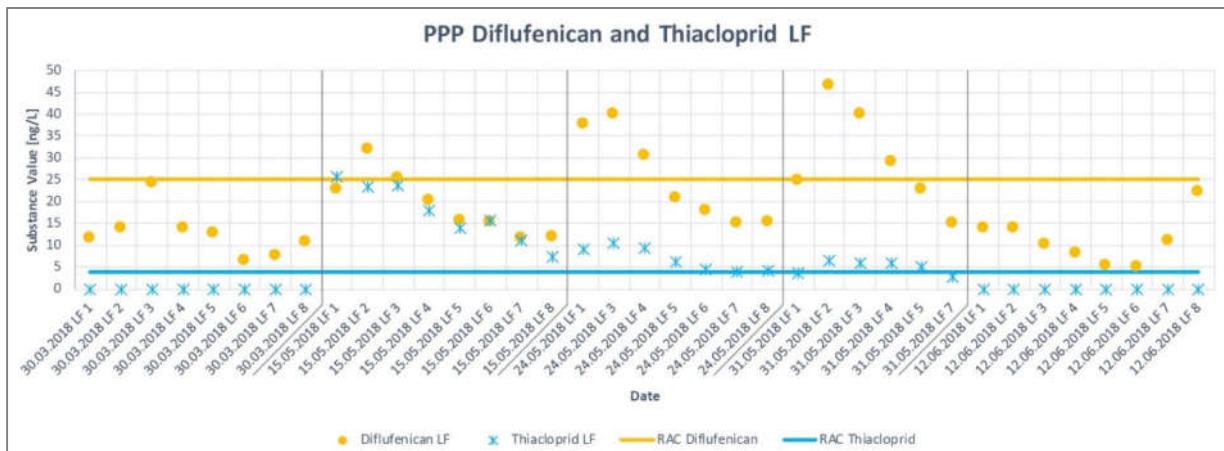


Figure 16 – Diagram [in ng/L] for LF for Diflufenican and Thiacloprid and their RAC values.

Highest value for Diflufenican was 0.0466 µg/L (46.6 ng/L – RAC value 0.0250 µg/L (25 ng/L)) and for Thiacloprid 0.0258 µg/L (25.8 ng/L – 0.00400 µg/L (4.00 ng/L)).

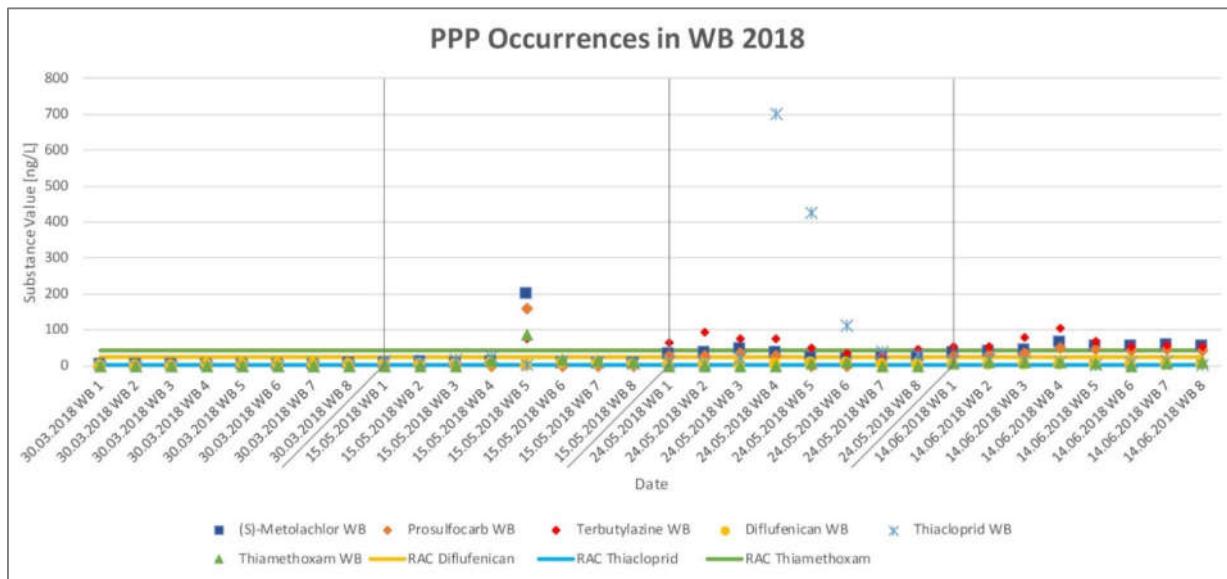


Figure 17 – Diagram [in ng/L] for WB for the 2 PPP that have surpassed RAC. The highest PPP value for Thiocloprid was 0.705 µg/L (705 ng/L – 0.00400 µg/L (4.00 ng/L)) and for Thiamethoxam 0.0850 µg/L (85.5 ng/L – RAC value 0.0430 µg/L (43.0 ng/L)). Lines for RAC values are shown for Diflufenican, Thiocloprid and Thiamethoxam due to scaling issues.

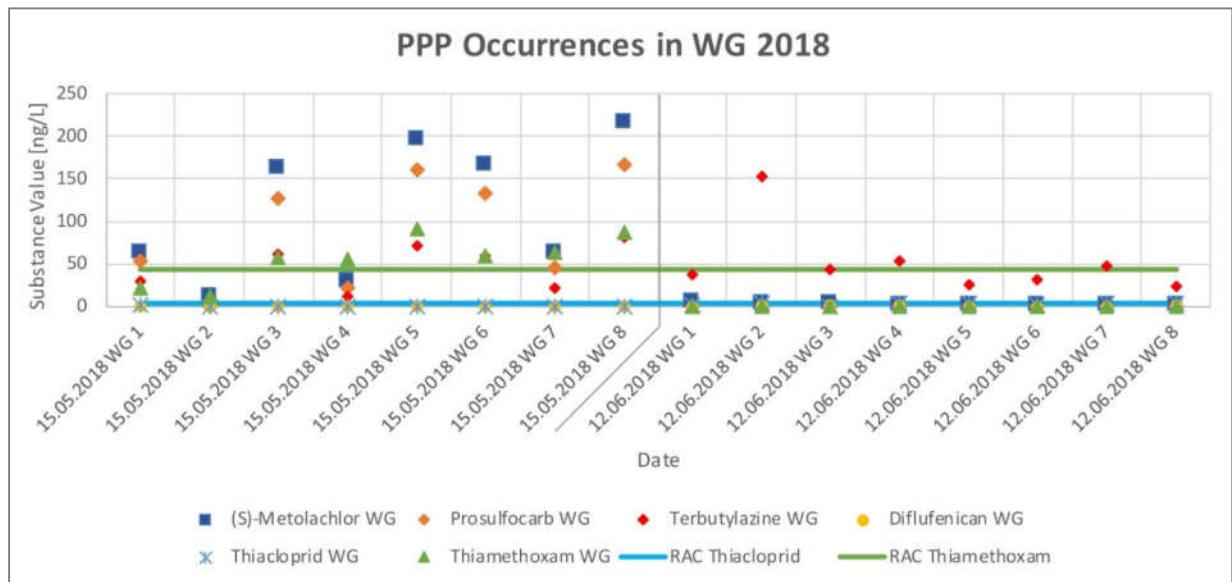


Figure 18 – Diagram [in ng/L] for WG for the 2 PPP that have surpassed RAC. The highest PPP value for Thiocloprid was 0.00420 µg/L (4.20 ng/L – RAC value 0.00400 µg/L (4.00 ng/L)) and for Thiamethoxam 0.0922 µg/L (92.2 ng/L – RAC value 0.0430 µg/L (43.0 ng/L)). Lines for RAC values are only shown for Thiocloprid and Thiamethoxam due to scaling issues.

### 3.2. In-vivo Tests with Native Water Samples and *Daphnia magna*

In-vivo tests with the native water samples were conducted directly after the heavy rainfall event. Test subjects were one day old *D. magna* neonata that were controlled after 24 h and 48 h. The OECD criteria for validity of  $\leq 10\%$  in the NC was met in all tests and the test organism reacted with a 100 % mortality rate after 24 h in the PC with 3.5 M potassium dichromate, demonstrating the susceptibility of the organism. Of the 132 tests conducted with native samples that were taken after heavy rainfall events, only six (see figure 19) showed increased mortality rates. The water sample WB VIII 30<sup>th</sup> March was the only one that showed a strong statistical significance (\*\* p < 0.001). Five samples (LF VI – 30<sup>th</sup> March, LF VII – 14<sup>th</sup> June, WB VI 15<sup>th</sup> May, WB IV 24<sup>th</sup> July, WG I 24<sup>th</sup> August) showed a low statistical significance (\* p < 0.05).

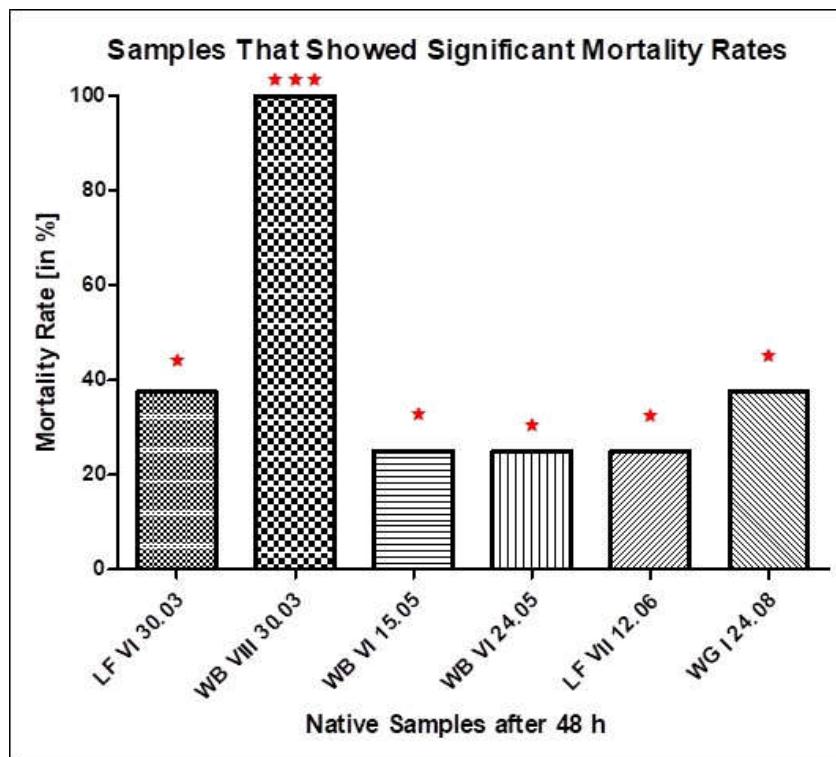


Figure 19 – Native water samples that showed increased immobilization rates of daphnids.  
Data analyzed with Fisher's exact test. P-values are two-tailed with confidence interval of 95 %.  
★ p < 0.05, ★★ p < 0.01, ★★★ p < 0.001

### 3.3. In-vivo Tests with Extracts and *Daphnia magna*

In vivo-tests with the extracts that included enriched concentrations of the native samples showed no significant mortality rate in the four extracts (LF V and LF VIII 24<sup>th</sup> May, WB V and WB VIII 15<sup>th</sup> May). The OECD criteria for validity of  $\leq 10\%$  in the NC was met in all 4 tests and the test organism reacted with a 100 % mortality rate after 24 h in the PC with 3.5 M potassium dichromate, demonstrating the susceptibility of the organism.

### 3.4. In-vitro Tests with *Cellulomonas uda*

Three TRs with all extracts were conducted with *C. uda*. Tests included different steps of concentration reaching of 80 times down to 4.68 times of the native samples. An exemplary result of one TR is shown in figure 20. As the test method is still in the experimental phase no values for toxicity and no criteria for validity had been defined before testing.



Figure 20 – Results of *Cellulomonas uda* test of 15th December 2018 showing TR2 with the extracts of 24<sup>th</sup> May and 31<sup>st</sup> May 2018 after application of stop reagent. As can be seen the first two rows show generally higher coloration and were therefore excluded from calculation.

#### 3.4.1. Negative Control

To assess the possible range of NCs all 1072 negative values from all tests were pooled in one histogram (see figure 21) and divided into different ranges. Of these 1072 values 934 (87.13 %) were in the range of 1.1 to 1.7 and 138 (12.87 %) above or below them. If all samples were considered, the mean was 1.33. Figure 21 shows the mean NC values received during one TR, indicating that two TRs (07.12 and 14.12) were below this threshold.

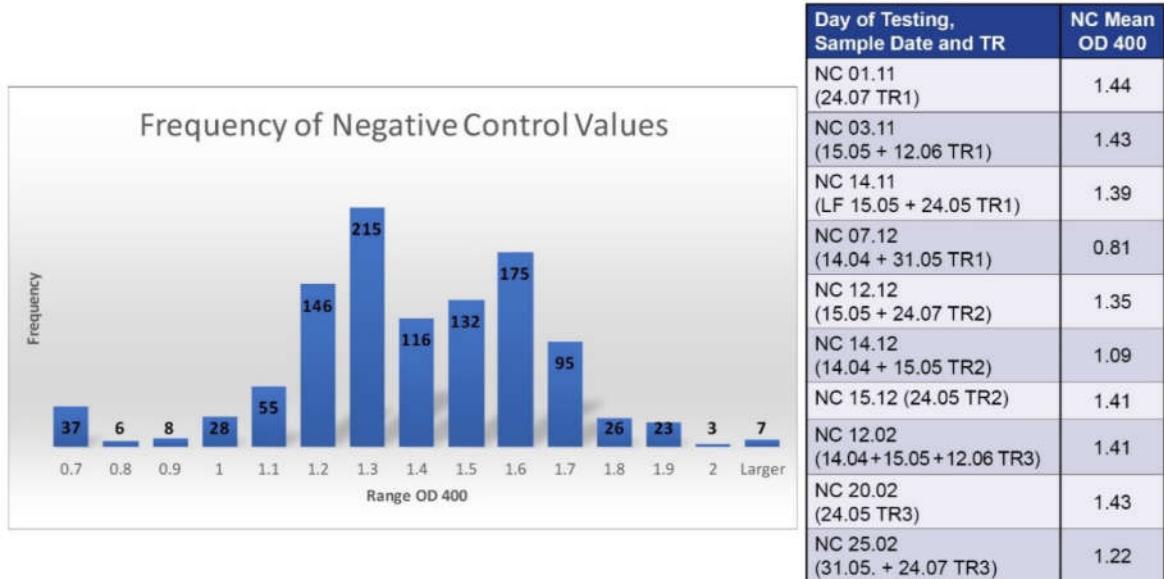


Figure 21 – Histogram of all NCs that occurred during testing.

$n = 24$  for each control plate, varying numbers depending on available wells on test plate. The TR at 07.12 was  $< 1$  and therefore considered not valid.

### 3.4.2. Positive Control

The range of valid values for the PC was calculated through the EC50 values of the 10 plates containing the controls that were created during testing, providing a preliminary basis to assess the validity. Figure 22 shows the distribution of the 10 EC50 values for the PC.

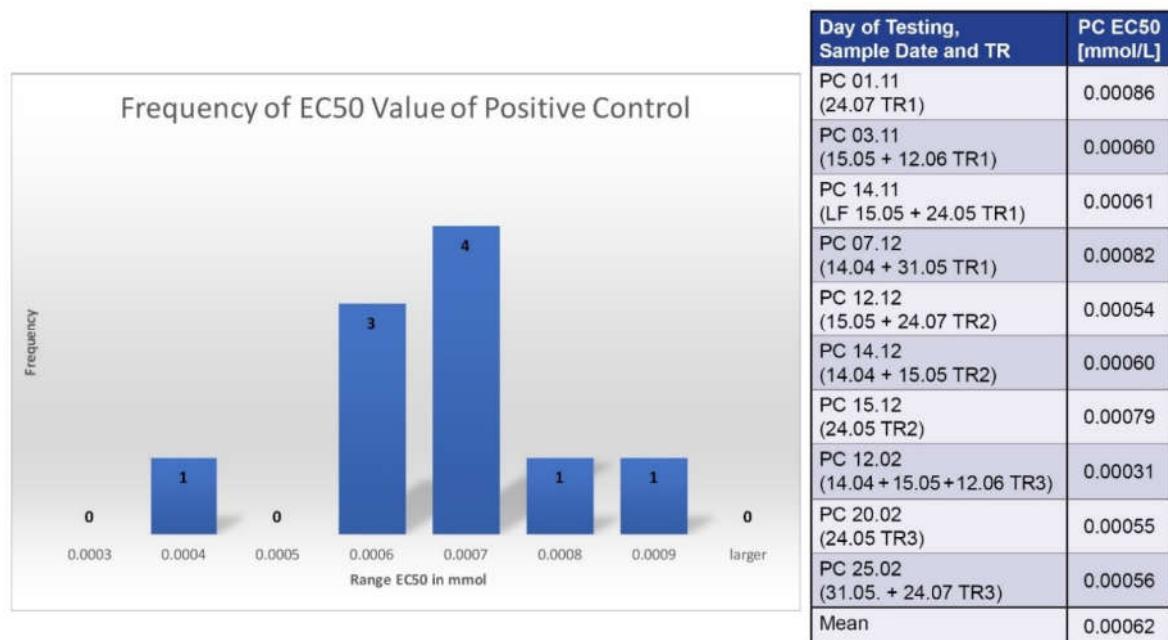


Figure 22 – Histogram of EC50 values for the PC for all TRs with *Cellulomonas uda*.

The distribution shows that 70 % of the TRs were in the range of 0.0006 and 0.0007 mol. The result of 12.02.2018 was the only that strayed from the other results.

Of these 10 values 7 were in the range of 0.0006 or 0.0007 mmol, whereas the mean was 0.000625 mmol. Only the result of 12.02.2019 was below a value of 0.0005 mmol, whereas the results of 01.11 and 07.12 were above 0.0008 mmol.

### 3.4.3. Solvent Control

Figure 23 shows the inhibition of the SC in all control plates. Criterion for validity has been designated as no inhibition of bacteria growth is initiated through DMSO. However, as can be seen in figure 23, inhibition generally occurred at the highest concentration of 80 as well as in the second highest with 53.33. The two highest concentrations in the sample plates were therefore discarded from analyzation.

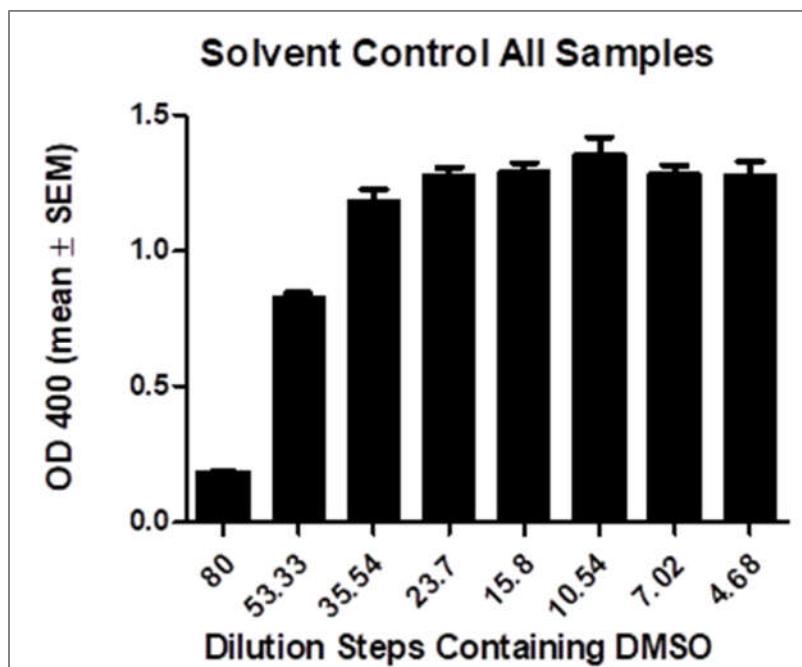


Figure 23 – Dilution series for all SC, showing that inhibition occurred in the rows containing the highest concentrations. N=3 per dilution row per control plate.

### 3.4.4. REF50 Values

The REF50 values for *C. uda* were calculated by combining the results of the three TRs conducted with each sample for which an extract was available. As can be seen in figure 24 the inhibition often occurred between extract concentrations of 10.54 and 23.7. Values in the two highest concentrations of 80 and 53.3 were excluded from the calculation as the SC suggested that DMSO levels were responsible for inhibition at these levels. Figure 24 also shows that the inhibition occurred abruptly between two values and that no TR was identical. Results show a high variance

in values between the different TRs, whereas the trend during one TR is generally reflected in the other TRs, excluded a few outliers. However, a few examples (i.e. LF VI and VII 15<sup>th</sup> May, WB II 15<sup>th</sup> May, LF V 24<sup>th</sup> May and WB II 24<sup>th</sup> May) show very little variance.

The data offers no indication to why there is consistency in some values whereas in others a wide spread can be observed. As the number of TRs was 3 for all samples, the spread in the 95 % CI

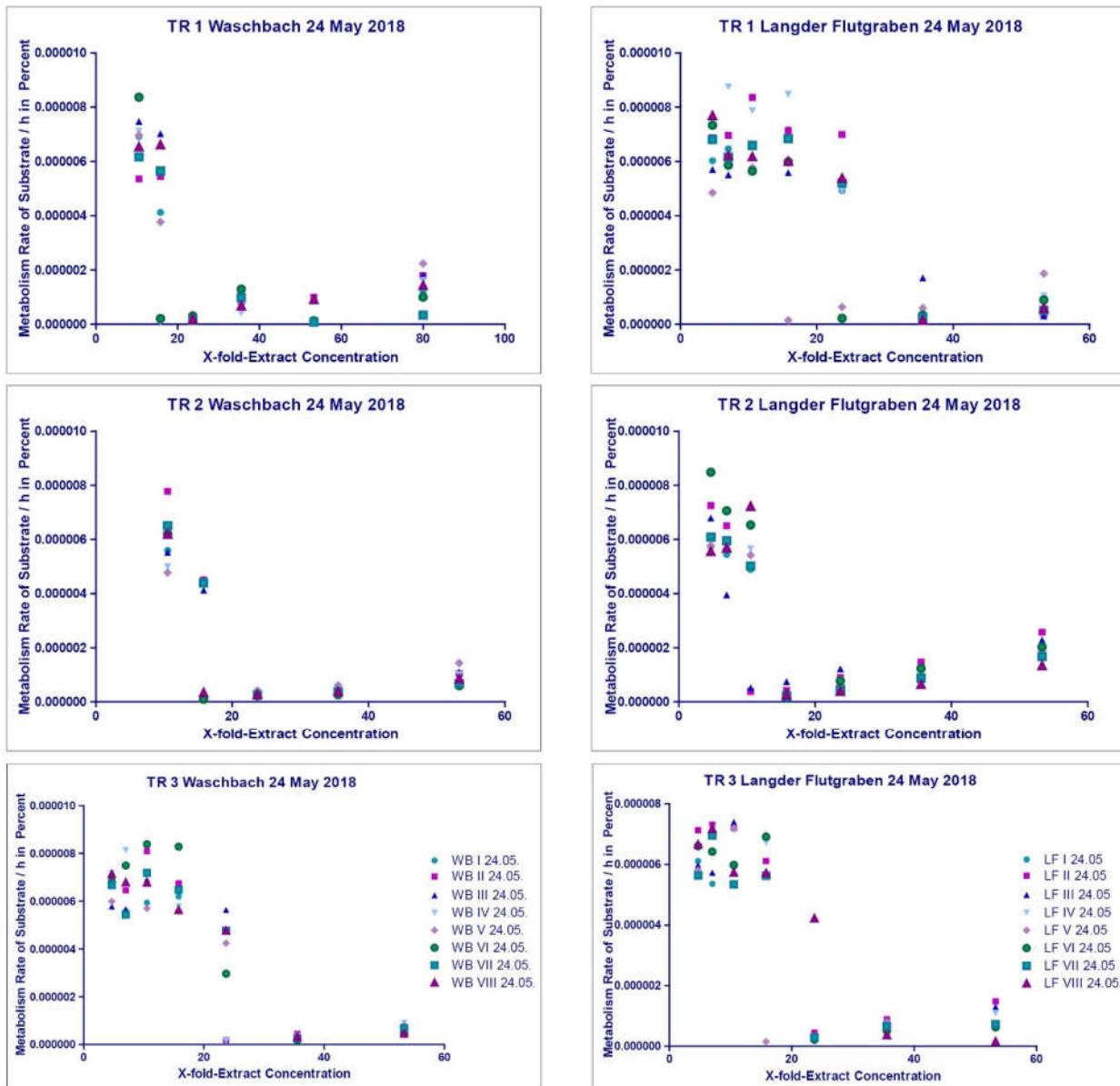


Figure 24 – Comparison of three TRs for WB and LF on 24<sup>th</sup> May 2018. Shown are the 8 samples that represent one day of sampling (LF I to LF VIII) in the graph. The x-axis shows the concentration in the extracts to demonstrate the point when inhibition of bacterial growth set in, whereas the y-axis presents the metabolism rate of substrate per hour in percent. As can be seen inhibition always set in around a 20-fold concentration. n=1 per extract per dilution row. Per sample 8 dilution rows were analyzed.

(confidence interval) is wide, leading to no significances between samples taken on the same location and on the same day. The comparison of the REF50 values for the streams show that the Langder Flutgraben is more toxic than the Weidgraben and the Waschbach. However, only 13 samples for testing were available for the Weidgraben, whereas 22 were available for the Waschbach and 26 for the Langder Flutgraben. The REF50 mean values of the three TRs calculated for each sampling event are shown in table 10. The calculation for the means was conducted with all values as well as without the two TRs (07.12.2018 and 12.02.2019) that were considered invalid after analyzing the controls, meaning that not three but only two TRs were considered for calculating the mean for the affected events (TRs left out for 07.12.2018: TR1 31.05 and for 20.02.2019: TR3 15.05 and 12.06). These values were later used for calculating the toxicity in all streams by plotting them against the TU sums.

*Table 10 – Mean REF50 values for all TRs conducted with all samples.*

*Results are separated by stream (Langder Flutgraben, Waschbach and Weidgraben), day of event and for all values for one stream. The mean was calculated for all values from all TRs as well as for all values except the two TRs (07.12.2018 and 12.02.2019) considered invalid. A complete list showing the results of each sample for each day can be found in the annex (tables TA 6 – TA 8).*

	All Values	Without Invalid		All Values	Without Invalid		All Values	Without Invalid
<b>Mean LF 15.05</b>	17.0	15.1	<b>Mean WB 15.05</b>	19.6	19.5	<b>Mean WG 15.05</b>	12.1	12.1
<b>Mean LF 24.05</b>	16.8	11.0	<b>Mean WB 24.05</b>	16.5	16.5	<b>Mean WG 24.05</b>	18.1	16.3
<b>Mean LF 31.05</b>	10.7	12.1	<b>Mean WB 14.06</b>	17.5	15.0	<b>Mean WG 31.05</b>	16.2	16.0
<b>Mean LF 12.06</b>	19.7	15.3	<b>Mean WB 24.07</b>	20.5	20.5	<b>Mean WG 12.06</b>	17.6	13.1
<b>Mean of all Values</b>	<b>16.1</b>	<b>13.4</b>	<b>Mean of all Values</b>	<b>18.5</b>	<b>17.9</b>	<b>Mean WG 24.07</b>	18.3	18.3
						<b>Mean of all Values</b>	<b>16.5</b>	<b>15.1</b>

### 3.5. Toxic Units and Calculation of Toxicity

Calculation of TUs are based on the combination of 48 h LC50 values of *D. magna* with the average toxicity of each chemical for all rainfall events. The TU values were then plotted against the results of the *C. uda* testing. Results were calculated for a general trend for all streams and events combined, as well as for a trend for each river. Graphs could only be calculated if TUs were available from the chemical analysis as well as extracts for *C. uda* testing. Since the TRs of 07.12.2018 and 12.02.2019 showed some inconsistencies in either the PC or the NC, two data sets were calculated, one that included all values and another that left these two TRs out. The

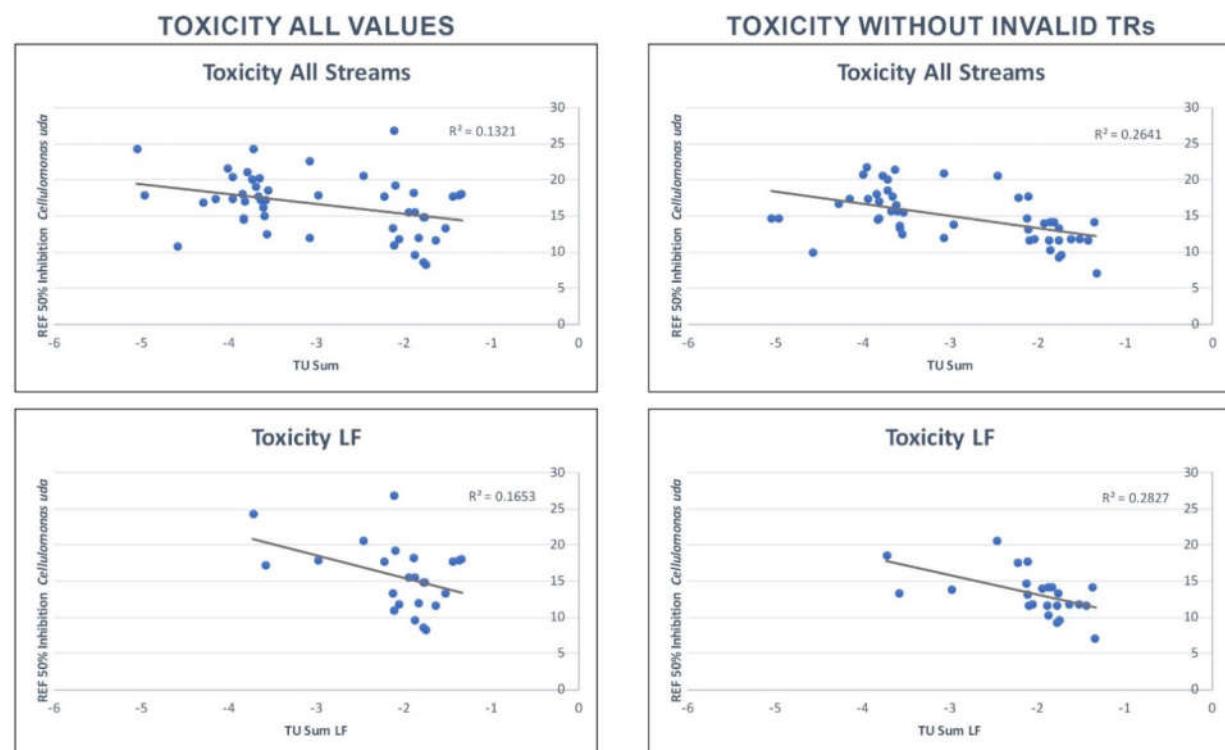
calculated values are shown in table 11. TU sum values of 30<sup>th</sup> March and 15<sup>th</sup> May show a highly significant p-value of < 0.001 on the Langder Flutgraben between these two events.

*Table 11 – Combined TU sum values for every heavy rainfall event.*

The most toxic values were LF 15.05, LF 24.05 and LF 31.05. (Significant differences between a TU < -4 compared to sites with a TU in the range of <- 3 to <- 2, <- 2 to <- 1, and <- 1 to 0 are indicated by p-values of < 0.01, < 0.05 and < 0.001, respectively. All TU sum values for all substances and all events can be found in tables TA 6 – TA 8.

Heavy Rainfall Event	TU Sum
TU_LF 30.03.	-4.23
TU_LF 15.05.	-1.91
TU_LF 24.05.	-1.57
TU_LF 31.05.	-1.88
TU_LF 12.06.	-3.17
TU_WB 30.03.	-4.44
TU_WB 15.05.	-3.29
TU_WB 24.05.	-3.67
TU_WB 14.06.	-3.61
TU_WG 15.05.	-3.29
TU_WG 12.06.	-4.68

REF50 values that showed significant correlations are shown in figure 25 and include values for all streams and the Langder Flutgraben. No correlation could be calculated for WB since the toxic units were not sufficiently toxic (see figure A 11). For WG the available number of tested extracts was too low to calculate any correlation (figure A 11 and table 12). Table 12 shows that the strongest correlation could be observed for “all streams without toxic values” with a highly significant p-value of < 0.001, whereas the p-value of < 0.01 for “all streams with all values” was less significant. For “LF without toxic values” the p-value showed a strong significance of < 0.005 and for “all values” < 0.05.



*Figure 25 – Comparison of the toxicity for all streams and Langder Flutgraben. Distinguished by toxicity accounting for all values and toxicity not accounting invalid TRs. Figures for WB and WG in annex (figure A11). The figure for all streams shows the huge spread between the values of LF and of the other streams. For n see table 12.*

Table 12 – Correlation of the toxicity of all streams.

Correlation indicated by p-values of  $< 0.01$ ,  $< 0.05$ ,  $< 0.001$  and  $< 0.005$ . The strongest significance can be seen for all streams without, whereas a strong significance can be observed for LF without invalid. As can be seen the n for Weidgraben was too low to provide meaningful data.

Parameter	All Streams all values	All Streams w/o invalid	LF all values	LF w/o invalid	WB all values	WB w/o invalid	WG all values	WG w/o invalid
$r^2$	0.132	0.264	0.165	0.283	0.0241	0.0003	0.328	0.0107
r	0.363	0.514	0.406	0.532	0.155	0.0173	0.573	0.103
n-2	46	46	22	22	17	17	3	3
p-value	< 0.01	< 0.001	< 0.05	< 0.005	ns	ns	ns	ns

## 4. Discussion

The requirement for good ecological and chemical status, stated in the WFD, has been the background for the establishment of the NiddaMan project. As an exemplary region the Wetterau has been chosen for its agricultural structure that is mainly based on intensive farming. Yet, the Wetterau region is also an important bird migration area crossed by many small and medium river systems that debark into the Main river and ultimately the Rhine river. This gives the Wetterau region a perfect characteristic to study the current state of improvements made since the introduction of the WFD as well as the situation of PPP and their implications on aquatic organisms and through the food chain to animals ranked higher in the trophic level. The combined effects of heavy rainfall events and subsequent pesticide loads in SWBs remain a less studied area, despite its consequences for biodiversity and human health (Brinck et al. 2017; Beketov et al. 2013; Kienzler et al. 2016; Hering et al. 2010) - either directly through the provision of drinking water or indirectly as residues in food or through the degeneration of ecosystem services. The aim of this thesis was to develop a multi-level system to assess the toxicity of SWBs using chemical analysis and in-vivo and in-vitro test assays. In a final step the results of these methods were combined with the calculated TUs (Liess and Ohe 2005) to determine the toxicity of the different river systems. The results of this approach show highly promising outcomes. The samples taken after heavy rainfall events showed increased toxic levels that were often detectable at the beginning of a sampling period (see figures 15 to 18) and subsided through the course of a day demonstrating that influxes of toxic compounds occur after heavy rainfall events. These results confirmed earlier studies conducted in other parts of the world in regard to surface run-off after heavy rainfall events (Meite et al. 2018; Corada-Fernández et al. 2017). Since the approach for this thesis was considered a pilot study, *D. magna* was used as an already established and reliable test organism that is continuously used for in-vivo test assays with chemical pollutants (Köhler and Triebeskorn 2013). The in-vivo tests with *D. magna* did not show an increase in mortality, neither in the tests with native samples, conducted parallel to the chemical analysis, nor in the tests with the extracts that used increased concentrations. Based on the findings of the chemical analysis these results were to be expected as the LC50 values are several magnitudes higher than the values found during the sampling period (see table 9). To test a further dimension to determine the toxicity, the bacteria *C. uda* was used as the test organism for in-vitro assays. Test

results showed an inhibition of growth induced by the pollutants, leading to the conclusion that *C. uda* can be used as a test organism for the determination of pollutants, despite the test setting still being in an experimental phase (Köhler and Triebeskorn 2013; König 2017; Köhler and Triebeskorn 2013; Langner 2018). In a final step, the EC50 values of the in-vitro tests were plotted against the TUs derived from the LC50 value of *D. magna* and the results of the chemical analysis. Through this method it was possible to distinguish the toxicity between the different river systems, showing that the Langder Flutgraben possessed the most toxic values during the sampling campaign.

#### 4.1. Temporal Variations in Surface Water Bodies (Hypothesis 1)

Rain is an important cause for the influx of pesticides into surface waters (Topaz et al. 2018). It is therefore vital for the health of the river system to understand which effects on the macrobenthos will occur when spraying of pesticides is followed by heavy rainfall events. The period of investigation lasted from 30<sup>th</sup> March to 15<sup>th</sup> August and was marked by a dry spring that was at several points interrupted by thunderstorms and heavy rainfall events (see chapters 2.1 and 2.3.4.), followed by an extremely dry summer that led to early harvests cutting short the spraying period in that year. The absence of steady rain during long periods in spring and summer led to falling water levels in streams that were not replenished by those, often local, events (HLNUG 2018b). This situation led to low water levels or water levels that rapidly fell after a rainfall event (see figure 8), especially in the later parts of spring and early summer leading to fewer collected samples than anticipated. Nevertheless, in the region of Hesse 20 heavy rainfall events occurred in 2018, the second highest number in records (HLNUG 2018b). Of these 20 heavy rainfall events, 8 led to a triggering of the event-driven samplers in the investigation area. As can be seen in figure 1, May was marked by local heavy rainfall events between the mid and end of that month leading to three samples taken during that timeframe. Since this is also the main spraying season, the chemical analysis showed that the most toxic samples were taken during this period, among them all but one (Thiacloprid Waschbach 14<sup>th</sup> June) with transgressions of the RAC values. As no water samples were taken on days that did not follow a heavy-rainfall event and no chemical analysis was available for the events of July 24<sup>th</sup> and August 15<sup>th</sup>, the samples taken on March 30<sup>th</sup> and June 12<sup>th</sup> must serve as the baseline for dates when spraying did not occur on

elevated levels and therefore did not lead to such drastic influxes as can be seen in the samples from May 15<sup>th</sup> and especially 24<sup>th</sup>. Since samples were only taken during one season, it cannot be concluded how severe the weather situation has influenced the sampling process or if in wetter years more pesticides would be discharged into the SWBs.

As the area of investigation, the Horloff catchment area was chosen due to its strong focus on conventional farming that uses huge amounts of PPP on the one hand, and its diverse river systems that often border directly on fields on the other hand. It is also an important bird migration area with birds visiting this area especially in the main spraying season between March and May (BZL 2019; RP Gießen 2016). These different aspects show that the aim of the WFD (EP 2000) to consider river systems as entities intertwined at different sections with its surroundings is correct.

As can be seen in table 1, more than 50 percent of the surface area in the Nidda region is used for agriculture. As there exists no precise data for the Horloff, it can be assumed that a similar percentage of the area in the Horloff region is used for agriculture (see table 1 and the map in figure 2) as well, demonstrating the huge economic importance of the agricultural sector for the region. This picture can also be observed in the surroundings of the river systems chosen for sampling with the Langder Flutgraben and the Waschbach running through fields for most of their course. The Weidgraben presents a slightly different aspect as it runs through forests from the middle section onwards. All three streams are considered to be small river systems that are, until now, underrepresented in the monitoring system of the WFD (Brinck et al. 2017). Yet, these small river systems often bear the main load of sudden peaks of pesticides due to their vicinity to fields and lower water discharges (EEA 2018). The relevance of this aspect could also be seen in the analyzed river systems, in which high loads of discharges from diffuse sources were detected during the sampling campaign. The results of the chemical analysis showed that more than 87 % of the PPP in the substance catalog used by the BfG were found in at least one river, among them eight substances (table TA 2 – TA 5) which were no longer approved for use in agriculture. Five substances (see table 8) were present in all samples collected in all streams, whereas 13 were present in the Langder Flutgraben at all times, showing a higher basic pollution in this stream. This demonstrates that a wide range of PPP that are applied in agriculture, end up in SWBs and that farmers usually do not discard substances when the license expires, defying the aim of the

NAP to improve the sustainable usage of PPP (Brinke et al. 2017). Of the three river systems analyzed, those that directly border on fields (Langder Flutgraben and Waschbach) for most of their course showed the highest number of PPP in the water stream. The findings in the Weidgraben were much lower, as only 57 % of the chemicals found in the Waschbach were also found in the Weidgraben. Several factors may contribute to this difference – the sampler at the Weidgraben was placed in a small stretch of riparian forest that followed a longer stretch of mixed coniferous forest, indicating that some self-purification had already happened at that point. Intensive farming was mostly found in the upper parts of the river, a factor that distinguished it from both other systems. Also, the morphological characteristic of the river type differentiates the Weidgraben from the two other streams as it possesses finer material like sand and flows slower, offering the possibility that more pesticides are deposited in the sediments instead of floating in the water column. However, the malfunction of the sampler at the Weidgraben (see chapter 4.5) may also have played a significant role since fewer water samples were collected and only 16 were analyzed by the BfG, since the samples were not distributed correctly by the sampler in the glass bottles in the bottle compartment. The highest number of different substances above the RAC value were found in the Langder Flutgraben on 24<sup>th</sup> May during the sampling campaign. Several factors could be cited to explain this situation, among them the absence of buffer strips at this stream, offering the possibility that the farms did spray pollutants directly into the stream when reversing the tractor, a factor supported by the scything of the swimmer on 12<sup>th</sup> June. Also, the gradient of the slope was higher at the Langder Flutgraben than at the Waschbach and the Weidgraben. This factor, combined with strongly shifting water levels at this SWB, could have led to an increased accumulation of substances during drier periods. PPP could have been deposited on the dry banks and were washed into the creek through surface run-off once it started to rain. Since the plant cover on the fields in the vicinity was not surveyed, the regard of how much this aspect had influenced the sampling process should not be speculated on.

The sample WB IV 24 May contained 705 ng/L of Thiacloprid, exceeding the RAC value of 4 ng/L for this sample by a factor of 176. This was the highest exceedance of any substance in any sample collected. This sample was collected more than one hour (see sampling program in table 4 and figure 17) after the start of the sampling process, indicating that the leaching potential for Thiacloprid is lower than those of other substances or that it was washed into the river at a point

that was more upstream. Figure 8 shows the shifting water levels during the period of investigation and the rise and fall of those levels after heavy rainfall events. The successional influx of PPP that followed these heavy rainfall events can be seen in figures 15 to 18. The most severe influx of PPP could be observed in May, a highly important time for nature, as it is the month when many birds breed, and many flowers are in bloom. Since the blooming flowers attract many insects, their flight and occurrence may be heavily influenced by pesticides (Liess and Ohe 2005). In a further downward spiral, the number of insects may then dwindle leading to insufficient food sources for breeding birds.

To summarize the results of the sampling period, it can be concluded that hypothesis one:

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**H1: Temporal variations of peak pesticide loads with varying compounds can  
be identified in surface water bodies after heavy rainfall events**

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can be considered correct.

#### **4.2. Assessment of Toxicity with In-vivo Tests using *Daphnia magna* (Hypothesis 2)**

Parallel to the chemical analysis all samples were tested with filtered water (see chapter 2.4) with *D. magna*, *H. azteca* and *C. riparius* (Ratz 2019) shortly after sampling to analyze the possibility that compounds made of a variation of substances show stronger effects than a test with only one or more substances as is usually done in a laboratory setting (Köhler and Triebeskorn 2013). Test subjects in all tests with *D. magna* were neonata that were not fed during testing. All subjects were placed in a conical container that contained 2 ml of the native sample, according to OECD guideline 202 for acute toxicity. Except for six samples, results from these acute toxicity tests generally did not show increased mortality (see figure 19). However, five of these six samples only show a low significance and may also result from issues with the handling of the daphnids in the conical container, like shaking or tensions. The only strongly significant sample of WB VIII March 30 could not be connected to findings in the chemical analysis. This test was conducted belatedly with frozen water that was not filtered, offering the possibilities that either the filtrate was the reason for the mortality or that a highly toxic substance had remained in the brown glass bottle. Retrospectively, no attempt for explanation could be further analyzed since the glass bottle had already been cleaned and the remaining sample water filtrated.

As the filtrate was not analyzed after filtration of the samples it may be possible that some substances like suspended particles were missed so that the full force of the pesticide load was not retrieved. Also, 36 hours often frequently elapsed between the point at which the sampling process was initiated and the time at which the samples were retrieved, making it possible that highly volatile substances were already degraded at this point, thereby offering the possibility that the effects in the natural environment may be more severe than judged from the in-vivo laboratory testing.

Following the collection of the water samples, 500 ml of the samples were transferred on a Telos<sup>©</sup> C18 column and eluted to receive an extract enriched by the factor 1000. In-vivo testing with the extracts as well as in-vitro testing was conducted with extracts from the water sample that were prepared by inspissating the substances in the water onto a Telos™ C18 column. Langner (2018), albeit using a different type of column (Oasis<sup>©</sup> HLB 6 cc Vac Column), described that many of the silica columns that can theoretically be used for SPE were not suitable as they did not retain the hydrophilic substances found in the native samples. Whereas a variety of other columns had been tested for their ability to enrich hydrophilic substances by the department of aquatic ecotoxicology in the autumn of 2017 (Langner 2018), the C18 columns were not among them. This analysis is currently conducted by the BfG to assess if and which substances are not retained by the C18 columns.

Based upon the findings of the chemical analysis four extracts (see chapter 2.7.3.) containing substances with high and low concentrations were chosen to contain a wide range of possible toxic rates to account for the possibility that effects may occur in higher concentrations that are not unlikely in the natural environment. The OECD guideline 202 for acute toxicity tests with *D. magna* recommends the usage of at least 2 mL test substance per daphnid. Due to the limitations imposed by the available test extract and the required dilution series a new test scenario was developed: one daphnid was placed in one well containing only 1/10 (200 µL) of the recommended amount of test substance on a 96-microtiter plate that also included the SCs and NCs but not the PCs which were placed on a different plate. This test setting was conducted with a 20-fold increase as the highest concentration as a pre-test with DMSO suggested that daphnids cannot withstand higher concentrations of DMSO.

The test setup worked well albeit being used rarely by other researchers (Baumann et al. 2014). The functionality could be indirectly demonstrated by the results of the test, since these samples did not show any increase in mortality as well.

Considering the magnitudes between the LC50 values and the values found in the samples “no observed effects” was the most likely scenario for the 48 h acute toxicity tests. It can be debated that a chronic test may have also been desirable when considering that NOECs of these substances are usually much lower, but even these values were beneath the highest concentration found in the water samples. Furthermore, the test frame of a heavy rainfall event clearly indicates a short duration scenario that leads to a one-time pulse exposure of pesticides. In rare cases these pulse exposures may be repeated events caused by a series of several heavy rainfall events happening in a short duration of time. Yet, the findings from May, the only occasion at which a chain of heavy rainfall events occurred, did not show a drastic increase in pesticide load after repeated rainfalls. It seems more plausible that spraying was conducted more often and with more severity during that period, leading to the high pesticide loads. Nevertheless, the scenario of repeated pulse effects would deserve some further analyzation since repeated exposures might lead to more chronic effects that cannot be excluded by the findings in this thesis. To put it in a nutshell, there is a negative answer to the question of whether a chemical compound that is similar to the ones found during the spring and summer campaigns would lead to a toxic compound with adverse effects for *D. magna*. One must, however, keep in mind that *D. magna* has been branded as little susceptible to substances, compared to species like mayflies (*Ephemeroptera sp.*) (Köhler and Triebeskorn 2013), indicating that research with more susceptible species from the EPT taxa may show more severe results than with *D. magna*, which has been used in this test setting due to its role as the standard test organism for tests with pollutants.

#### **4.3. Assessment of Toxicity with In-vitro Tests with *Cellulomonas uda* (Hypothesis 2)**

Xenobiotic stress factors are common to SWBs and can lead to severe consequences for aquatic biodiversity and ecosystem health (Malaj et al. 2014) but their effects are often poorly understood. As could be seen in chapter 4.1 the question whether heavy rainfall events lead to adverse effects on aquatic organisms in SWBs due to the chemical compound flushed into these river systems, is therefore of great interest. To circumnavigate the issue of using highly sensitive

organisms like mayflies for tests for toxicity, it was decided to use *C. uda* as a proxy that is easier to handle in laboratory settings to determine the severity of toxic effects.

*C. uda* was first considered in the 1980s for tests in different fields that required a measurable biological indicator for the degradation rate of cellulose (Chosson 1987; Coninck-Chosson 1988; Dermoun and Belaich 1985; Dermoun et al. 1988). Since then, this bacterium, found in many soils and aquatic systems in which cellulose degradation occur, has been analyzed on and off in a multitude of applications. Renewed interest started in the early 2010s with a (Obst et al. 2012; Blankinship et al. 2014; Chae et al. 2017) focus on using the bacterium for detection of contaminants in soils and aquatic systems. In two master theses conducted in the department of aquatic ecotoxicology of Goethe-University, Frankfurt (König 2017; Langner 2018) *C. uda* has been studied with regard to the toxicity of pollutants that resulted from the influx of wastewater from sewage treatment plants.

Since the *C. uda* test is still in its experimental phase it may be too early to draw final conclusions. Yet, it has been shown that an increased toxicity results in the inhibition of exoenzymatic activity of the  $\beta$ -glucose enzyme. The calculated REF50 values for *C. uda* showed that the  $\beta$ -glucose enzyme activity is in all cases inhibited through the addition of a pollutant and that in most cases this inhibition takes place between a concentration of 10.54 and 23.8. This narrow range as well as the high variations of results in different TRs for the same sample make it difficult to determine a precise point for toxic values that can be mirrored in the results of the chemical analysis at the current state of this test method. Figures 26 and 27 show the spread of the REF EC50 values for LF 24 May that were calculated from the TRs. Some singular TRs (i.e. TR 3, conducted 25<sup>th</sup> February 2019, with samples from LF and WG 31 May, see data CD) showed a perfect mirror to the results of the chemical analysis, some nearly perfect consistency between different TRs (i.e. LF V 24 May), whereas others strayed more strongly. Ideally the outcomes would have represented the results of the chemical analysis and it therefore have been possible to establish values for toxic thresholds. Nevertheless, all samples tested reacted to the PPP through growth inhibition, suggesting that the exoenzymatic activity had been blocked. As can be seen in the range of the CI the REF50 values of the different TRs did not stray significantly, so that it was not possible to define toxic values at this point.

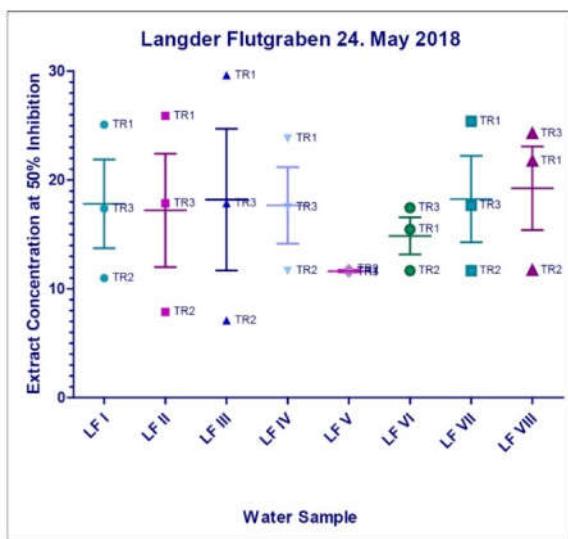


Figure 26 – Graphs and tables for REF50 values for Langder Flutgraben 24<sup>th</sup> May.

Figure shows the distribution of REF EC50 values and CI for the three test runs (TR1 to TR3) conducted with *Cellulomonas uda*. The high toxicity of Prosulfocarb, S-Metolachlor and Terbutylazine is only represented in TR2 of LF II and LF III.

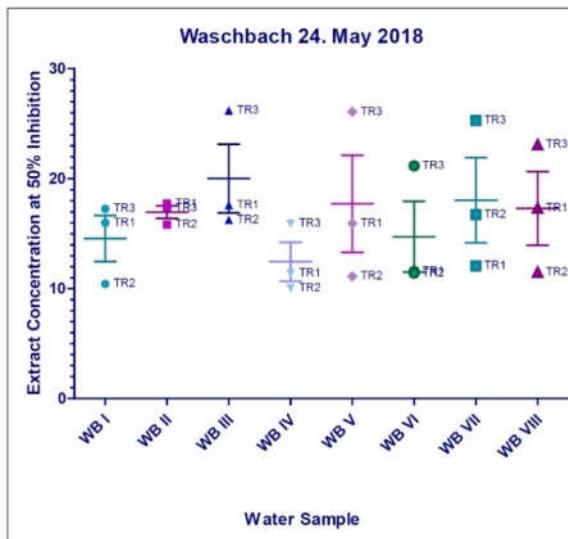


Figure 27 – Graphs and tables for REF50 values for Waschbach 24<sup>th</sup> May.

Figure shows the distribution of REF EC50 values and CI for the three test runs (TR1 to TR3) conducted with *Cellulomonas uda*. The high toxic load with Thiacloprid can be seen in sample WB IV.

Still, the results suggest that it is possible to utilize *C. uda* for assessing the pollution and the self-purification potential of SWBs since at the same time most TRs demonstrated that the trend for the water samples follows the pesticide load, which can i.e. be seen in WB IV 24.05. More research would be necessary to determine toxic values through tests that are conducted with the aim to develop precise thresholds.

The combined results from the in-vivo and in-vitro tests therefore lead to the conclusion that hypothesis 2:

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**H2: It is possible to assess the toxicity of high pesticide loads consisting of varying compounds through the utilization of *Daphnia magna* and *Cellulomonas uda***

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can be considered accurate since *D. magna* did not show increased mortality in the acute toxicity tests, whereas *C. uda* responded in the in-vitro assays to the compounds with growth inhibition. The results for *D. magna* were in conclusion with the LC50 values found in literature, whereas the REF50 values for *C. uda* showed encouraging results despite showing varying values.

#### 4.4. Correspondence of Biological Response to Toxic Units (Hypothesis 3)

Assessing the chemical status of a river system depends on a multitude of often unknown factors like the season (Brinke et al. 2017) during which the water samples are taken, the morphological degradation and the eutrophication level (Ohe et al. 2009b). Heavy rainfall events are natural but not common events that lead to very specific stress situations in SWBs (Meite et al. 2018; Nolan et al. 2008; Brinke et al. 2017) due to the sudden influx of large quantities of pollutants. These pulse situations can lead to very different effects on SWBs compared to chronological long-time exposure due to sudden spikes in pesticide loads. The WFD uses five status classes in its classification scheme, ranging from high to bad, to assess the water quality of a SWB and has the binding aim that the ecological and chemical status for all SWBs must reach the second highest status class “good” by 2027 (EP 2000). This status is preliminarily assessed through the measuring of the most affected BQE (Ohe et al. 2009a). The usage of BQE specific toxic units for quantifying toxic stress related to organic pollutants is recommended (Peterson 1994; Ohe et al. 2009a; Liess and Ohe 2005) since specific toxicants like herbicides or insecticides usually cause different effects on different trophic levels (Ohe et al. 2009a). Since the in-vivo testing was conducted with *D. magna* the BQE specific TUs were also calculated for this standard organism.

Figures 25 and A 11 demonstrate that it is possible to assess the toxicity of river systems through the plotting of TUs against REF50 values derived from the in-vitro test assays. An increase of toxicity shows that higher inhibitions of REF50 values for *C. uda* correspond well with the picture

seen in the chemical analysis, especially if the two TRs (07.12.2018 and 12.02.2019) that showed inconsistencies in the presumed validity criteria are excluded. In cases where only low levels of toxicity are observed in the TUs, the trend is weak (Waschbach), whereas in higher toxic settings a significantly strong trend can be observed (Langder Flutgraben).

The most toxic events occurred at the Langder Flutgraben on 15<sup>th</sup>, 24<sup>th</sup> and 31<sup>st</sup> May with negative values higher than – 2, demonstrating that that month is the most toxic and that the Langder Flutgraben is the most toxic river of the three sampled. The results from June then showed abating values due to lower spraying rates. These findings confirmed the earlier results of Liess and Ohe (2005), who demonstrated that May is the most toxic month and that TUs are lower in streams that passed through a stretch of riparian forest, like the Weidgraben. The Waschbach also showed low toxic values, most likely since the location of the sampler was close to a bird preservation area. However, differentiating from Liess and Ohe (2005) no analysis of the macrobenthos was conducted as part of this thesis, therefore no calculation for the SPEAR is available. Liess and Ohe (2005) described a reduction of 60 % in SPEAR between the month of April and May, if the TUs were between – 1 and – 2. Since the shift in TUs between 30<sup>th</sup> March and 15<sup>th</sup> May was similar in the current sampling campaign (– 4.23 to – 1.91), it can be assumed that similar reductions appeared in the Langder Flutgraben as well.

Liess and Ohe (2005) also raised the point that pesticides from surface run-off might adversely affect the mortality rate of other benthic invertebrates when it reaches the 48h acute toxicity LC50 value for *D. magna* and cite the mortality rate of *H. azteca* to ES-Fenvalerate as an example. Studies for Thiacloprid suggest that other aquatic invertebrates like *H. azteca* or *C. riparius* (Barden 2001) react 1000 times more sensitive to this substance than *D. magna*. Tests with these organisms that use higher concentrations of the extract are part of an ongoing study conducted by Ratz (2019) and shall provide more insight in the toxicity of the sampled compounds.

To sum up the results of the tests with native samples and extracts for in-vivo testing on the one hand, and the REF50 values of the in-vitro assays on the other hand, hypothesis three:

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**H3: Toxicity levels in the compounds found in water samples after heavy rainfall events correspond with the biological response**

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can be confirmed.

In the native samples, conducted with *D. magna*, *H. azteca* and *C. riparius*, no effects occurred as suggested by literature. As can be seen in table 9, the laboratory derived LC50 values for *D. magna* exceed the values found in the chemical analysis as well as the RAC values by several magnitudes. It was therefore highly unlikely to observe any reaction to the PPP in the native testing as well as with the even higher concentrations in the extracts. At this point it can therefore be concluded that the present method of spraying pesticides does not affect these organisms directly. However, the natural setting contains a multitude of different possibilities that cannot be re-enacted in a laboratory setting (Liess and Ohe 2005). Therefore, the possibility of toxic effects on other trophic levels or more severe reactions to the substances in higher concentrations cannot be totally excluded. Yet, it is a clear indicator that the concentrations of the substances in the compounds taken after heavy rainfall events did not lead to elevated effects on the organisms tested with in-vivo assays. Still, it needs to be considered that the vast majority of the substances tested and found in the chemical analysis were herbicides and not insecticides. Herbicides might show more dramatic effects on plant organisms like algae, thereby harming the nutrient basis for other organisms, leading to indirect effects.

The effects of the compounds on plant organisms have not been tested as part of this thesis but are part of an ongoing test series in the department of aquatic ecotoxicology. At this point it can be concluded that heavy rainfall effects lead to compounds with increased toxicity values as can be seen in the chemical analysis and to some degree in an in-vitro test method using bacteria. The test results from in-vitro tests with *C. uda* as a proxy suggests that more testing is required before the current agricultural practice can be deemed not hazardous for the environment. To finalize, it can be concluded that the method conducted in this thesis works well and could be used to assess toxic risks through compounds found in aquatic ecosystems.

#### 4.5. Assessment of Methodology and Materials and Methods

Conducting field studies is dependent on a multitude of factors, many of which cannot be controlled in all circumstances (Kienzler et al. 2016; Liess and Ohe 2005). The methodology discussion will name several of them as they sometimes interfered with the research process.

2018 was among the driest years in recorded history in Germany (HLNUG 2018b), especially from June onwards (see chapter 2.1). This situation led to fewer collected water samples than expected, a situation that was further complicated by difficulties in handling the water samplers. Generally, the samplers worked well, but several issues occurred:

The re-activation mechanism can only be operated in cases when the swimmer is above the water surface, meaning that one has to wait for the water level to fall. This is problematic in cases in which two or more heavy rainfall events take place in a short period of time. At times the battery connection showed some strains, when freshly charged batteries were not recognized as full or were first shown as full but then shown as empty after a heavy rainfall event without sampling taking place. The heavy rainfall event on 14<sup>th</sup> April led to flooding at the Waschbach and uprooted the sampler from its location and blockaded the swimmers at the Waschbach and the Langder Flutgraben with debris, so that they were not activated. As a result, no water samples were taken by the samplers, even though this event led to the most severe flooding situation observed during the campaign. At the end of May, the mechanism to send an SMS failed at the Waschbach, making it difficult to assess the exact point at which to collect the samples from the bottle compartment. As a result of a technical issue, the sampler at the Weidgraben did not work regularly throughout the whole campaign, collecting samples randomly or filling the bottle compartment itself instead of the glasses. Samples from the Weidgraben are therefore lower in number as well as in quantity of available sample water. This led to fewer extracts as often the amount of water was not sufficient for inspissating and transfer of the substances onto the columns.

A further aim during this thesis was to enhance the knowledge of handling the *C. uda* bacteria and deepen the findings of the aforementioned theses by applying them to the area of pesticide testing. Langner (2018) demonstrated that inhibitions in the growth of *C. uda* occurred as a result of different rates of toxicity, but also stated several issues like the age of the bacteria strain, the inhibition factor of the PC or the weak retention rate of hydrophilic substances in the used columns. Since the retention rate of hydrophilic substances especially affects the transfer of

herbicidal substances on the columns, it was decided to use Telos™ C18 columns instead of Oasis® columns in the tests for this thesis.

Since the test method is still in the experimental phase, results showed a multifaceted picture with regard to handling as well as for the meaning of the data. First pre-tests showed that the initial test setting used by Langner (2018) with NC, PB, PC, SC and the test substances on the same microtiter plate was very difficult to handle. In the test setup used for this thesis PB, PC, SC and three additional NCs were therefore placed on a control plate and separated from the plates containing the samples. This allowed testing of all samples of one heavy rainfall event of one stream in one plate. At the same time, it required the preparation of only one plate with controls at each TR, making handling easier. The dilution series was prepared on an extra composition plate whereas the bacteria and the substrate were applied on the test plate in the end. This improved the manageability of the test method. Considering the validity criteria, the test plates containing the PB, PC and SC usually worked well, as can be seen in chapters 3.4.1 to 3.4.3. However, some constraints remained. In the SC the inhibition induced by DMSO was too strong in the highest or even the two highest concentrations, leading to the exclusion of these values from the data. The NC on these plates seemed not to be influenced by the other substances on these plates, whereas the question if gluconic acid is sufficiently strong for a PC, first raised by Langner (2018), is still open to debate. In the test setting used for the thesis the PC with gluconic acid worked sufficiently well to establish a validity criterion, despite showing lower inhibition rates in the lowest concentrations. Regarding the EC50 values of the PC, it can be concluded that only values of 0.0006 to 0.0007 mmol should be considered as valid. The objection that it is not adequate to consider a test valid or not valid based only on the results from the plates containing the controls alone must be regarded and should be considered in further tests as handling failures may influence the validity of the control plate but not of the sample plate or vice versa. However, this issue is common to all types of in-vitro testing where the control is separated from the substance. Since NCs are also placed on the sample plates, the growth of the NCs should be part of the validity assessment and, instead of combining the results of one TR, should be assessed singularly for one plate. As can be seen in figure 21 values beneath one or even beneath 1.1 and above 1.7 should be discarded as not valid. A recommendation would therefore be to discard all plates in which the mean of all NC samples does not cross the value of 1.1 or stays above 1.7 (a

mean of > 1.7 did not occur in any test). This validity criterion would then have to be combined with the PC to achieve a solid validity criterion. Since the DMSO inhibited the  $\beta$ -glucosidase activity in the highest and second highest concentration level the further usage of DMSO as a SC is questionable, although the columns work well with DMSO. It is therefore recommended to test other substances as solvents to bypass issues connected to DMSO.

#### 4.6. Outlook

Several aspects occurred through the course of this thesis that could be further enhanced in future research and shall therefore be discussed here. In a further step a number of recommendations for policy makers and farmers will be presented that could be utilized to improve the water quality.

The test campaign was conducted in the spring and summer 2018, a dry year with occasional heavy rainfall events. To factor in that some effects may have been the result of this highly unusual year a campaign that lasts several more years would be recommended. This would also offer the possibility to account for seasonal changes, an issue cited by the NAP (Brinke et al. 2017), since in this thesis temporal variations between early spring and late spring were already observed. Regarding the chemical analysis it is highly recommended to include water parameters like pH-value, ammonium and oxygen levels in the sampling process as well as to collect water samples at days without heavy rainfall events continuously to establish a pollutant baseline for the river systems. This method could be further enhanced through an active (Langer et al. 2017) and passive biomonitoring (i.e. for calculating the SPEAR) throughout the campaign.

The test setup for *D. magna* using microtiter plates worked generally well and could be utilized further without much adaptation for all kinds of acute toxicity tests that include this organism. It would be helpful to assess if a different composition of the controls in the microtiter plates could be established forestalling having to utilize an extra plate for PC or SC.

For future test settings several recommendations can be drawn based on the findings presented in this thesis. As *D. magna* is not overly sensitive to pollutants it may be more expedient to include more sensitive organisms from the EPT (*Ephemeroptera* (mayflies), *Plecoptera* (stoneflies) and *Tricoptera* (caddisflies)) taxa. This point is underlined by the surpassing of the RAC values in several cases, demonstrating that more sensitive organisms will most likely show stronger

reactions to the compounds found in the water samples. First results in this regard can be expected shortly as extract tests with *H. azteca* as a proxy for the native Gammaridae family and with *C. riparius* from the diptera order are conducted in the department of aquatic ecotoxicology. The high values of Thiacloprid found in the sample of WB IV 24.05 suggest that tests with the extracts shall provide increased mortality rates for *C. riparius* if applied in higher concentrations. The chemical analysis showed that, apart from Thiacloprid and Thiamethoxam, mostly herbicides were found in the water samples. Future tests should therefore include plants like algae or lemna. These were not part of this thesis but will be tested with the extracts in the department of aquatic ecotoxicology in the coming months.

For the in-vitro test setting using *C. uda*, several amendments could be applied. In the last three theses (König 2017, Langner 2018 and the present one) that utilized *C. uda*, testing was always conducted with a compound of substances. As a result, it is difficult to distinguish clear threshold values of toxicity, since interferences between the substances in these compounds could cloud the real effects of singular substances. It would therefore be recommended to test singular substances with known toxicities first to establish REF50 values for this bacterium.

Since the inhibition of  $\beta$ -glucosidase exoenzyme activity mostly occurred between concentration levels of 10.54 to 23.7, a multilevel approach that first tests wider range of substances and then more detailed levels could be applied to determine the exact toxic thresholds.

A further effect that seems not to have been tested so far, is the influence of light on the growth of the bacterium. Samples tested in the test runs on 12<sup>th</sup> February and 20<sup>th</sup> February 2019 provided unsatisfactory results, possibly as a result of being placed on an incubator in a room not lit by daylight. The TR conducted on 25<sup>th</sup> February 2019 used the same settings and same bacteria strain (05<sup>th</sup> September 2018) but was placed on a shaker in a room with daylight and produced results that nearly perfectly resembled the findings in the chemical analysis. As Langner (2018) already suggested some fault with the bacteria strains used, it may be necessary to analyze the strains, while considering the hereby mentioned factors.

It can further be contested that the finding of Langner (2018) that the  $\beta$ -glucosidase exoenzyme is inhibited at different dilution levels is correct and that this inhibition is also affected by the compound of pesticides. The functionality of the used Telos™ C18 columns seemed well but the results of the chemical analysis for these columns have to be awaited before a conclusion should

be made about further usage. Regarding the controls, it was shown that the validity criteria are sound, yet further tests are required to make the criteria more compelling.

The final step was the calculation of the TUs and the plotting of these values against the mean REF50 values from the *C. uda* TRs. This method worked well, however, more fine tuning for the REF50 values is required to receive values that vary less and can provide more precise results for the toxicity of a single sample in an event. So far samples cannot be distinguished in their toxicity through the REF50 values alone.

Policy makers are often facing substantial issues when trying to balance the interests of nature and of agriculture. This aspect could again be seen during this study, as farmers often cling to their traditional way of farming. The usage of pesticides in an area like the Wetterau, where intensive agricultural farming is common, is still high. Residues of this pesticide usage often end up in aquatic ecosystems, especially in May, a time when many birds breed, plants are in full bloom and insect activity is high. This natural setting is directly or indirectly influenced through the usage of pesticides, either by negatively affecting food sources (i.e. plants for insects and then insects for birds) or reducing the number of organisms due to the toxicity of the PPP. To receive a more comprehensive picture of the situation, monitoring of more small river systems would have to be established as a first step, a proposal already made by the NAP (Brinke et al. 2017).

As could be seen in the chemical analysis, PPP which are no longer approved for use are used by farmers who most likely have these substances stockpiled. To avoid usage of these substances, farmers should be offered a possibility to hand-in these substances with a cash pay-back. To lessen the pressure on aquatic organisms it would be recommended to pay farmers again for cultivating buffer strips near streams, a point underlined by the situation at the Langder Flutgraben (see chapter 4.5). At this point it seems unlikely that farmers would use fewer pesticides or change to organic farming, yet the situation could be improved if farmers were to receive more support in how to apply pesticides more precisely and avoid spraying when heavy rainfall events are forecast.

## 5. Conclusion

Heavy rainfall events lead to an increased influx of pollutants into surface water bodies. These influxes are highest during the spring months when the agricultural crop is in the main growth phase, spraying is at its peak and heavy rainfall events are very common as well. The combination of these factors can lead to severe influxes of PPP, when the spraying occurs shortly before a rainfall and the PPP had no time to be absorbed by the soil. In these cases, small water bodies directly neighboring fields bear the brunt of the pollutants as they often do not possess sufficient buffer zones and water loads to dilute the influxes and are the first port of entry. Yet, they are often underrepresented in the analysis of water bodies.

The main focus of this thesis was therefore to assess the effects of PPP compounds that are discharged into SWBs after heavy rainfall events. The method was to collect water samples directly after a heavy rainfall event and analyze the samples chemically as well as with aquatic organisms in the laboratory to study possible impacts on aquatic organisms.

The applied test setting, starting with the automated sampling of water probes after heavy rainfall events, followed by laboratory research including filtering, in-vivo testing with native samples and extracts and in-vitro testing using GC Gram positive bacteria, worked very well, despite some hindrances and issues in all steps of this setting. It is highly recommended to accompany the biological test setting with a chemical analysis to have further data available that offers more precise conclusions about possible causes for toxic effects and to calculate the TUs, so that the toxicity of SWBs could be distinguished. Through the chemical analysis it could be shown that higher than usual influxes of pollutants happen after heavy rainfall events and that their severity depends on human activities and temporal variations. It could further be shown that *D. magna* does not react strongly to the compounds found after these events, whereas *C. uda* offers the possibility to determine if a sample is toxic, despite the varying values throughout the research. These values are also the main point of criticism as they do not present a clear indication for the toxicity of a sample alone. It remains open if stable toxic values can be established for *C. uda*, but it seems quite viable if further tests provide more data. In combination with the chemical analysis a conclusion can be drawn upon the toxicity of a sample, showing that a toolbox of several methods offers an expedient strategy for drawing conclusions about the severity of a heavy rainfall effect and the stress factor induced by pollutants.

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## A1 Function of Event-Driven Samplers

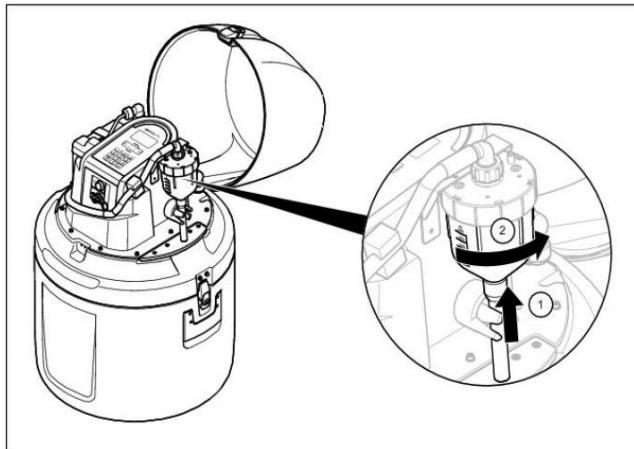


Figure A 1 – Portable Sampler P6 L with the open top part including the keypad and the dosing vessel (right) (MAXX Mess- und Probenahmetechnik GmbH 2015).

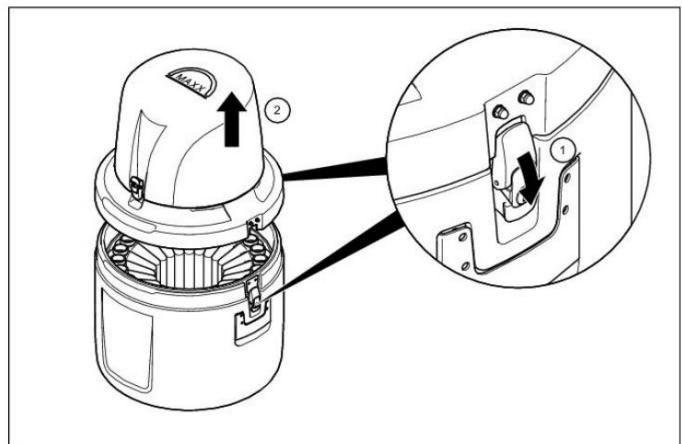


Figure A 2 – Portable Sampler P6 L with the closed top part and the bottle compartment in the lower section (MAXX Mess- und Probenahmetechnik GmbH 2015).

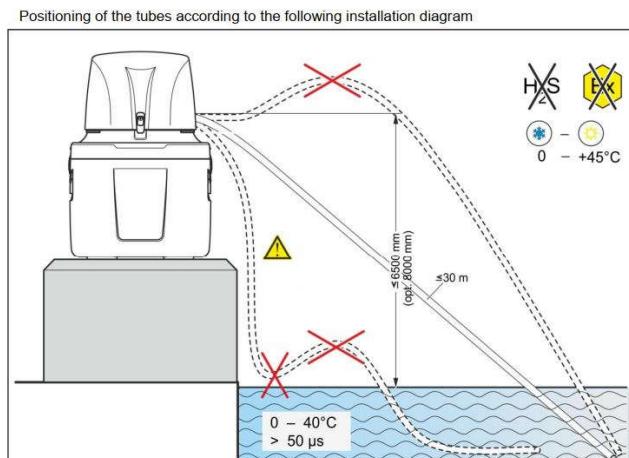


Figure A 3 – Positioning of the intake tube that connects the sampler with the water body (MAXX Mess- und Probenahmetechnik GmbH 2015).



Figure A 4 – Event-Driven Sampler at Langder Flutgraben. Picture taken after the heavy rainfall event on 30th March 2018.

## A2 Microtiter Setup for Extract Test with *Daphnia magna*

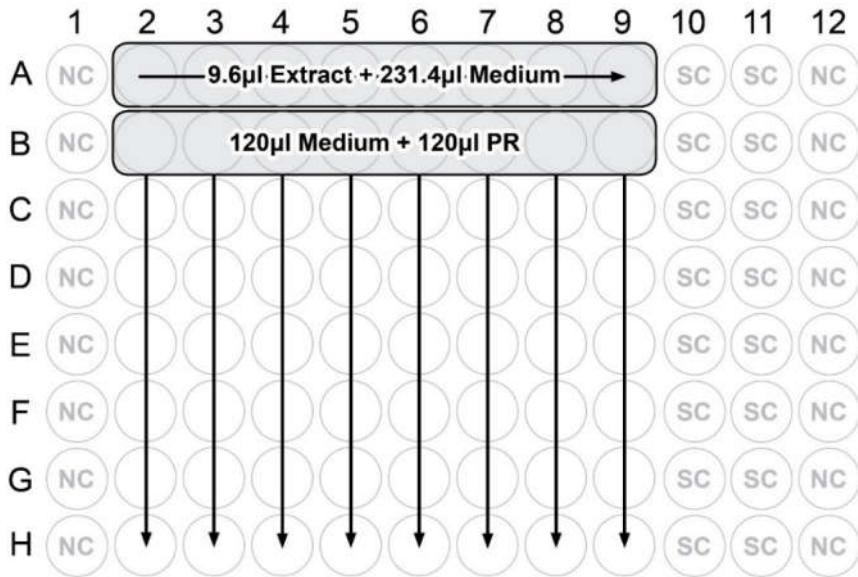


Figure A 5 – Schematic display of test setup for in-vivo tests with extracts. Extract was filled in row "A" and diluted from "A" to "H" by factor 1.5. NC were placed in columns 1 and 12, SCs in 10 and 11. No PC was placed on the microtiter plate.

## A3 Statistical Analysis *Daphnia magna*

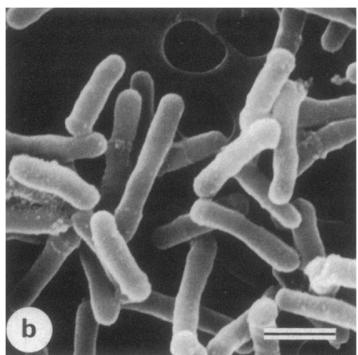
Results of the in-vivo testing with the native water samples were based upon the following aspects:

- Test elements included samples, negative-, positive- and solvent-control
- Test criterion was the immobilization rate, controlled after 24 h and 48 h
- To evaluate this test criterion a two-tailed Fisher's exact test was used with a constant level of significance ( $\alpha = 0.05$ ) and the:
  - null-hypothesis  $H_0$  that the samples have no effect compared to the control
  - alternative hypothesis  $H_1$ , mortality rates rise significantly
- According to this test criterion the values were compared by usage of a  $k \times 2$ -field- $\chi^2$ -test between the values of the NC and the values of the sample water leading to:

$$\chi^2 = \sum \frac{(Observed\ Mortality - Survival\ Rate\ Negative\ Control)^2}{Observed\ Mortality}$$

- Levels of significance have been highlighted by stars on the graphs  
( $\star = p < 0.05$ ;  $\star\star = p < 0.01$ ;  $\star\star\star = p < 0.001$ )

## A4 SOP for the Inhibition Test with *Cellulomonas uda*



For testing, SPE columns with a 1000-fold concentration of the collected water samples (see chapter 2.5) were used. Testing was conducted in a sterile environment in microtiter plates with 96 cavities. In a first step, stock solutions for the different steps of the test had to be created (see table TA 1).

Figure A 6 – *Cellulomonas* sp. (Lamed et al. 1987)

## A5 Preparation of Test Substances

Table TA 1 – Required stock solutions for the *Cellulomonas uda* test with production and storage recommendation (Misovic and Langner 2018).

Stock Solution	Making	Sterilization and Storage
Growth Medium 53	Solve: <ul style="list-style-type: none"><li>• 10g Casein-Pepton</li><li>• 5g Yeast Extract</li><li>• 5g Glucose</li><li>• 5g NaCl</li></ul> in 1L deionized water	Calibrate pH to levels between 7,2-7.4, autoclave by 121°C, store cool
Agar Plates	Autoclave 200mL growth medium with 5g agar and cast into plates	Seal with Parafilm® and store cool
Test Medium	Spike 1L dionized water with TropicMarin salt until a conductivity of 400 µS/cm is reached	Calibrate pH to levels between 7.2-7.4, autoclave by 121°C, store cool
Positive Control	Use gluconic acid 10g/L (CAS 527-07-1)	Autoclave by 121°C
Stop - Reagent Di-Sodium carbonate	Solve 10.6g Sodium Carbonate / 100mL in dionized water	Store in darkness
Sodiumchloride 0.14 mol/l	Solve 8,18g NaCl in 1L deionized water	Autoclave by 121°C
Substrate: 4-NOPG*	Solve 8mg NOPG in 8mL NaCl 0.14M *NOPG = 4-Nitrophenyl-β-D-glucopyranoside, (CAS 2492-87-7)	Store solution cold for a maximum of 24h

If not stated otherwise, all steps were done in a sterile environment. The chromogenic 4-NOPG substrate was prepared freshly for each TR by solving 1 mL NaCl (0.14 mol/L) per 1 mg 4-NOPG. All other substances required for testing were pre-prepared as stock solutions (see table TA 1).

## A6 Preparation of Test Plates

Preparation for testing begins with the incubation of a batch of *C. uda* 19 hours prior to the actual test. 50 µL *C. uda* are grown in 50 mL growth medium 53 and are then shaken at 30°C and 150 orbital rpm for 19 h +/- 1h on a standard analog mini shaker. Control and sample plates were prepared in a similar way with the process shown schematically in figures A7 to A10. Figure A7 shows the distribution of the wells on the composition plate, Figure A8 on the test plate for the controls. Column 1 was left empty as this column was used for the photometer blank on the test plate. Columns 2 to 4 were used for the first 3 NCs but were left empty as well as the NCs were also prepared on the test plate and not diluted. Columns 5 to 7 contained the SC, DMSO and

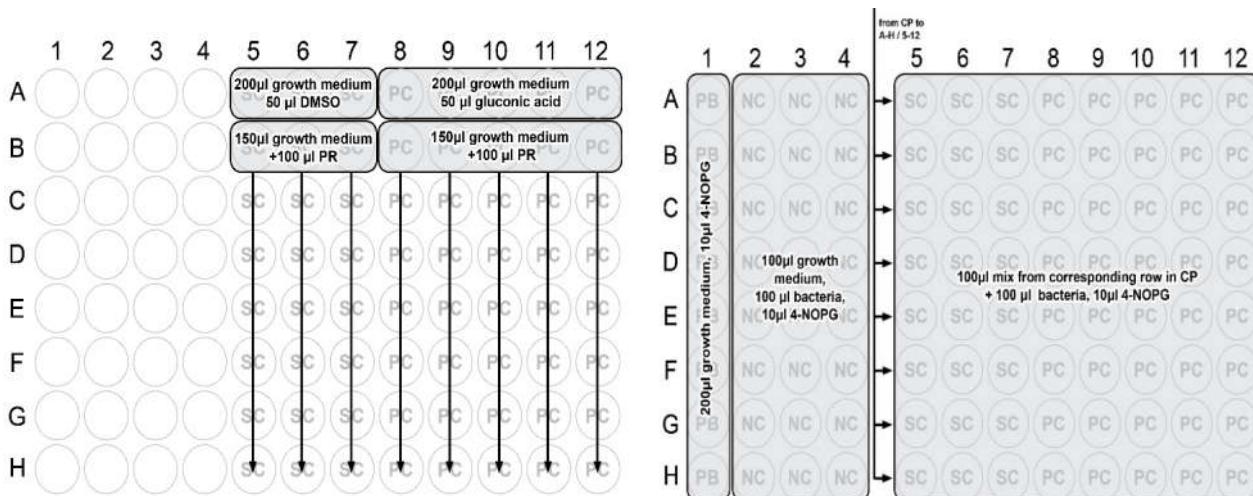


Figure A 7 – Composition plate for controls containing SC and PC. Columns 1 to 4 are left empty. All other columns in row "A" are filled with 200 µL medium and 50 µL DMSO (SC) resp. gluconic acid (PC). Samples are then diluted by mixing 100 µL substance from the previous row.

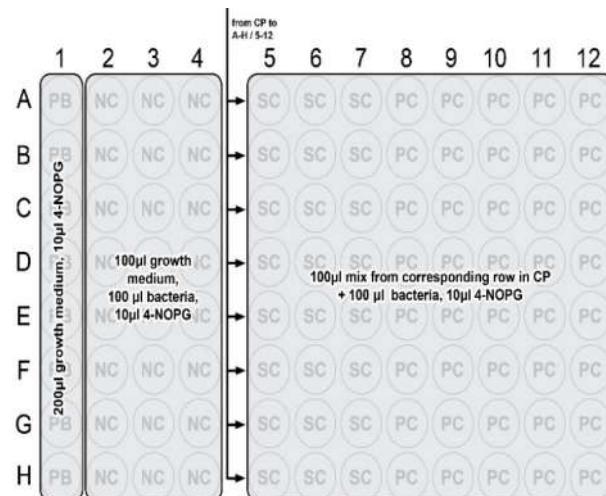


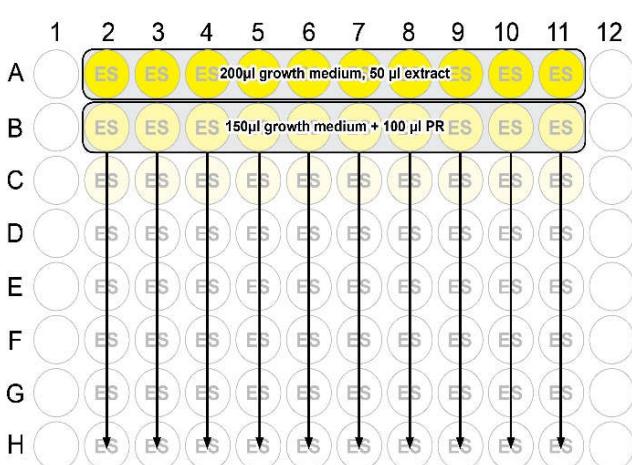
Figure A 8 – Test plate for controls containing PB (column 1), NC (column 2 to 4) and the transferred substances with the SC (columns 5 to 7) and the PC (8 to 12). The PB does only contain growth medium and 4-NOPG, whereas all other wells are filled with 100 µL *Cellulomonas uda* and 10 µL 4-NOPG.

columns 8 to 12 the PC with gluconic acid. At the beginning of the test 200 µL test medium containing tropic marine salt were applied to columns 5 to 12 in row "A", whereas rows "B" until "H" contained 150 µL test medium each. In the next step 50 µL DMSO was applied to the columns 5 to 7 in row "A" as well as 50 µL gluconic acid to columns 8 to 12. To mix the medium and the substances a multichannel pipette (Eppendorf Xplorer plus® 12-channel, 15-300 µL, Eppendorf AG, Hamburg, Germany) was used to dilute the substances in steps of 1:1,5 (the dilution series of DMSO and gluconic acid can be seen in table 7). The required dilution was achieved by pipetting

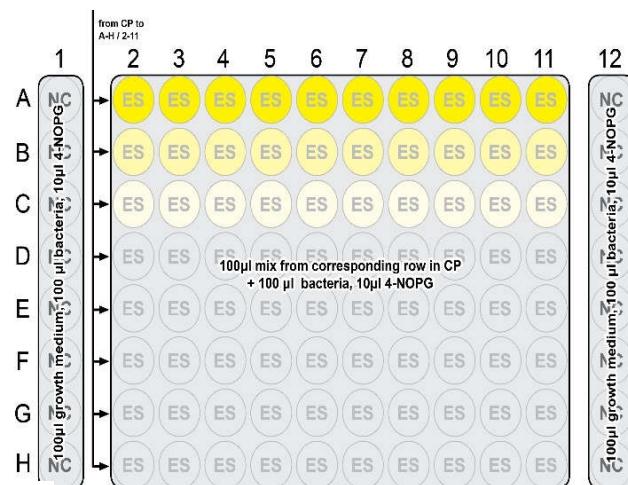
100 µL from the prior row into the next and mixing it twice. As described in chapter 2.5 the concentration of the SPE columns was 1000 times stronger than the native sample. For tests with *C. uda* the highest concentration in row "A" was 80 times the concentration of the native sample whereas the lowest concentration in row "B" contained a concentration with the factor 4.68.

The structure of the test plate followed the structure of the composition plate with the difference that column 1, containing the PB was filled with 200 µL test medium and columns 2 to 4, containing the NC, with 100 µL test medium. Columns 5 to 12 were filled with 100 µL of the compound from the composition plate. To avoid the contamination of the compound with higher dilutions, the dilution with the lowest concentration was transferred first (i.e. row "H" from the composition plate in row "H" of the test plate).

On the composition plate for the samples columns 1 and 12 were always left blank as they would contain the NC on the test plate. Columns 2 to 11 could be either filled with test samples, more



*Figure A 9 – Composition plate for the extract samples (ES) in which the dilution series is conducted. Columns 1 and 12 are reserved for the NC, whereas columns 2 to 11 can be filled with extracts or additional NC. Highest concentrations are marked yellow since the extracts were pigmented. Otherwise the method follows the one described for the SC and PC plate.*



*Figure A 10 – Test plate for ES containing the NC. As in the PC and SC plate 100 µL substance is transferred from the CP to the test plate and mixed with 100 µL bacteria and 10 µL 4-NOPG.*

NCs or the SPE blanket NCs. The SPE NC followed the same dilution pattern as the samples and were required to test the functionality of the Telos™ C18 columns. As in the plates with the controls, row "A" was filled with 200 µL test medium to achieve the required concentration, whereas rows "B" to "H" were prepared with 150 µL test medium. 50 µL of the extract was then pipetted into the appropriate well in row "A" leading to an amount of 250 µL of extract/ medium solution. This solution was mixed with a multi-channel pipette and pipetted in the same way as

in the control plates. In concordance with the method used for the control plates 100 µL of the compound (see figure A9) were transferred from the responsive row in the composition plate to the test plate. Columns 1 and 12 as well as all other columns that did not contain extract in the composition plates were filled with 100 µL test medium in the test plate.

Measuring of the fluorescence of inherent colors was conducted with the “Spark<sup>®</sup> multimode microplate reader” before adding the bacteria and the 4-NOPG substrate to the solution in the test plates, to factor in the possibility that the FAU was not measured precisely.

After measuring the plates, the required concentration of the bacteria culture had to be calculated. The calculation of the required bacteria volume was dependent on the point at which the bacteria were taken from the suspension. The value was then calculated by measuring the OD 595 and converting it into the required FAU 200.

Since the bacteria were cultured in growth medium 53, the extracted volume was filled into a centrifuge tube and centrifuged at 4.400 rpm for five minutes (Eppendorf Centrifuge 5702, Eppendorf AG, Hamburg, Germany). Afterwards, the growth medium was disposed, the bacteria placed in the required amount of test medium and 100 µL bacteria culture dispensed in every well except the PB on the control plate. As the final step, the pre-prepared solution containing the growth substrate with the 4-NOPG was applied. In each well 10 µL growth substrate were pipetted by utilization of a multichannel pipette (Eppendorf Xplorer plus<sup>®</sup> 12-channel, 5-100 µL, Eppendorf AG, Hamburg, Germany). The application was carried out from the left to the right of the plates due to the nature of the basin used to house the solution. Finally, the microtiter plates were sealed with sterile breathable sealing (Breath Easy, Carl Roth GmbH & Co. KG, Karlsruhe, Germany), placed on an incubator (Titramax<sup>®</sup> 1000, Heidolph Instruments GmbH & CO. KG, Schwabach, Germany) and incubated for 24 hours at 450 rpm and 30°C.

At the end of the 24h time frame 40 µL of the stop reagent Di-Sodium carbonate were pipetted into each well. Afterwards, the metabolic rate of the substrate was measured with the “Spark<sup>®</sup> multimode microplate reader” at a wave-length of 400 nm.

## A7 Calculation of Enrichment and Metabolism Rate

The enrichment of the test volume in the test can be calculated by the formulas:

$$\text{Enrichment in Test} = \frac{\text{Enrichment of Extract}}{\text{Dilution Factor}}$$

and

$$\text{Dilution Factor} = \frac{V_{\text{Extract+Medium}} * V_{\text{cavity}}}{V_{\text{Extract}} * V_{\text{Medium-Extract-Compound}}}$$

Whereas the dilution factor can be seen in table 9, the following terms are considered constants:

- Enrichment of Extract: 1000
- $V_{\text{Extract+Medium}}$  (Composition Plate): 250 µL
- $V_{\text{Extract}}$  (Test Plate): 50 µL
- $V_{\text{cavity}}$  (Test Plate): 250 µL
- $V_{\text{Medium - Extract - Compound}}$ : 100 µL

The Calculation of the metabolic rate included the following steps

- Measuring of the OD rate at 0 h and 24 h
- Adjustment of all wells by subtraction of the PB
- Adjustment of the inherent colors for all wells by subtraction of sample blank (only necessary for highest concentration factors)

Calculation of metabolic rate [mol/h]

$$\frac{SMR}{h} = \frac{\rho * F * k}{t}$$

Calculation of metabolic rate [%/h]

$$\% \frac{SMR}{h} = \frac{\rho * F * DF * k}{S * t} * 100$$

SMR = Substrate Metabolic Rate
h = hour
$\rho$ = extinction factor, adjusted by subtraction of PB
F = photometric factor of calibration for the final product (colorant) with 1/ increase of the colorant (mol)
DF = dilution factor
k = adjusted factor, in cases of chromogen substrates like 4-NOPDa equals 1 (substrate consists of equal parts of colorant and substrate)
t = 24 h
S = applied amount of substrate

Calculation of REF EC50 values including 95% Confidence Interval (CI) was conducted by combining the values of the three TRs with GraphPad Prism<sup>©</sup> 5.0.

## A8 Key Information of Chemical Analysis

All tables shown in this chapter are taken from the MS-Excel<sup>©</sup> table that was provided by the BfG and included the results of the chemical analysis. Shown are the key information for all 57 pesticides (fungicides, herbicides and herbicide metabolites, insecticides) that were tested by the BfG, with their end of license, current RAC value [in µg/L] in Germany and the maximum concentration found in the samples [µg/L]. Samples that have a zero value in the column "max concentration" were not found during testing. Substances that do not have an entry in "End of License" are not approved for use in Germany.

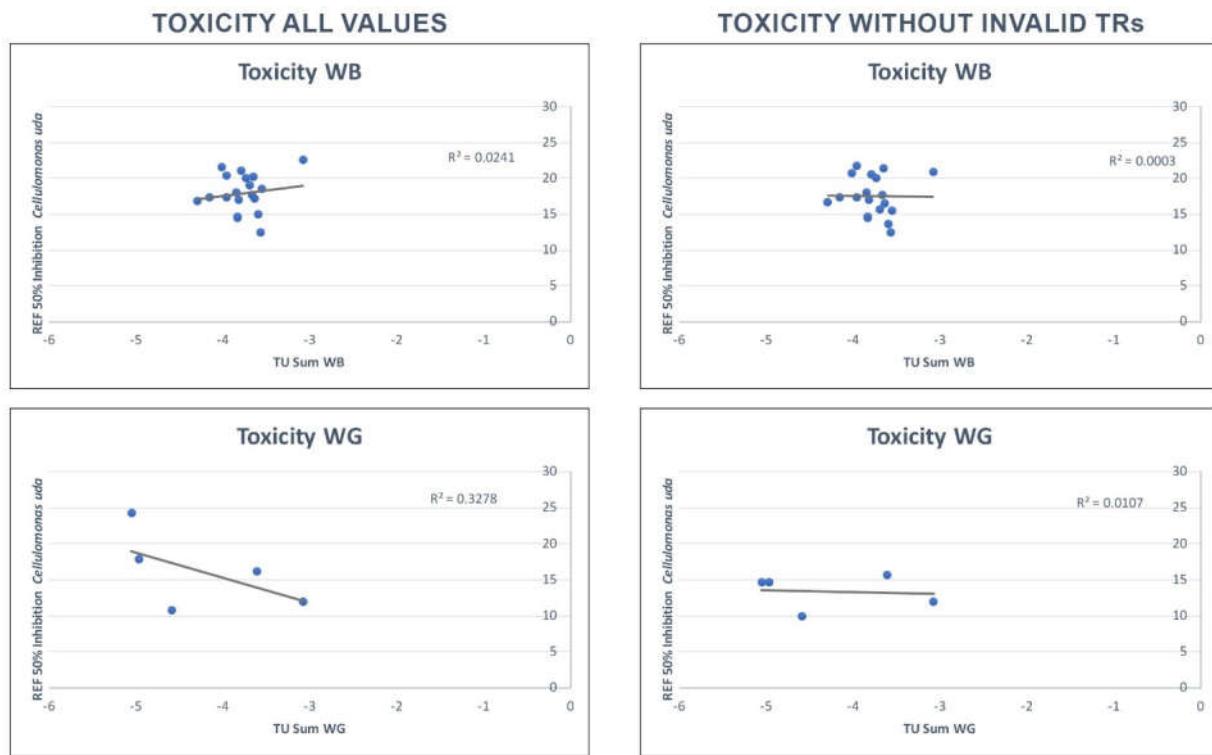


Figure A 11 – Comparison of the toxicity for Waschbach and Weidgraben.

Distinguished by toxicity accounting for all values and toxicity not accounting invalid TRs.

Figures for all streams and LF in chapter 3.5 (figure 25). No correlation could be observed between the values. For n see table 12.

*Table TA 2 – List of 11 fungicides analyzed by the BfG.*

*The only fungicide not found in any stream was Prochloraz. Carbendazim and Cybutryn are not approved for use anymore but were still traceable in the samples.*

Substance	End of Licence	RAC Value Germany (in µg/L)	Max Concentration in [in µg/L]
<b>Carbendazim</b>	-	0.15	0.0144
<b>Difenoconazol</b>	Dec-21	0.36	0.0056
<b>Dimethomorph</b>	Dec-24	5.600	0.0174
<b>Epoxiconazol</b>	Dec-25	0.460	0.1376
<b>Fenpropimorph</b>	Apr-20	0.195	0.0865
<b>Cybutryn (Irgarol)</b>	-	0.000	0.0021
<b>Prochloraz</b>	Dec-25	5.000	0.0000
<b>Propiconazol</b>	Dec-23	2.000	0.1751
<b>Prothioconazol-desthio</b>	Dec-26	0.334	0.2600
<b>Tebuconazol</b>	Dec-25	0.625	0.2480
<b>Triadimenol</b>	Dec-26	3.400	0.0589

*Table TA 3 – List of 26 herbicides parent substances analyzed by the BfG.*

*Four substances were not found during sampling, whereas five substances that are not (anymore) approved for use were found in the samples*

Substance	End of Licence	RAC Value Germany (in µg/L)	Max Concentration in [in µg/L]
Aclonifen	Dec-24	0.500	0.000
Chloridazon	Dec-22	56.000	0.037
Chlortoluron	Oct-20	2.300	0.007
Chlomazon	Dec-25	5.660	0.005
Desamino-Metamitron	-	-	0.158
Diflufenican	Dec-27	0.025	0.047
Dimethachlor	Oct-19	3.500	0.021
Dimethenamid	Dec-27	1.520	0.086
Fluazifop	Dec-22	146.000	0.012
Flufenacet	Dec-24	12.000	0.143
Flurtamone	Dec-24	1.500	0.096
Isoproturon	-	1.300	0.010
Metamitron	Dec-24	38.000	1.354
Metazachlor	Dec-22	1.670	0.023
(S)-Metolachlor	Jul-19	1.220	14.502
Napropamide	Dec-20	23.700	0.000
Prosulfocarb	Dec-26	3.800	12.762
Quinmerac	Dec-22	34.000	0.051
Terbutylazin	Dec-24	2.500	8.693
Terbutryn	-	0.000	0.022
Bifenox free acid	-	0.000	0.000
DCPMU	-	0.000	0.001
DCPU	-	0.000	0.000
Diuron	-	0.790	0.011
Mecoprop	Dec-20	0.900	0.217
Propyzamid	Dec-19	34.000	0.021

*Table TA 4 – List of 15 herbicide metabolites analyzed by the BfG.*

“End of License” could not be stated as the metabolites are the intermediate end product of their parent substance. The Dimethenamid-OA was the only metabolite not found in the samples.

Metabolite	End of License	RAC Value Germany (in µg/L)	Max Concentration in [in µg/L]
Dimethenamid-OA	-	0.000	0.000
Flufenacet-OA pos	-	0.000	0.084
Metazachlor-ESA	-	0.000	0.666
Metazachlor-OA	-	0.000	0.342
Metolachlor-ESA pos	-	0.000	0.613
Metolachlor-OA pos	-	0.000	0.519
Terbutylazin-2-Hydroxy	-	0.000	0.219
Terbutylazin-desethyl	-	0.000	0.473
Dimethachlor-ESA	-	0.000	0.047
Dimethachlor-OA	-	0.000	0.013
Dimethenamid-ESA	-	0.000	0.054
Flufenacet-ESA	-	0.000	0.072
Flufenacet-OA	-	0.000	0.059
Metolachlor-ESA	-	0.000	0.654
Metolachlor-OA	-	0.000	0.699

*Table TA 5 – List of 5 insecticides analyzed by the BfG.*

The only substance not found in the samples was Acetamiprid, whose license expired in February 2019.

Substance	End of License	RAC Value Germany (in µg/L)	Max Concentration in [in µg/L]
Acetamiprid	Feb-19	0.180	0.000
Clothianidin	-	0.007	0.011
Imidacloprid	Dec-22	0.009	0.005
Thiacloprid	Dec-20	0.004	0.705
Thiamethoxam	Apr-19	0.043	0.092

Table TA 6 – List of *Daphnia magna* LC 50 values and TU for every chemical found during sampling in Langder Flutgraben

Substance	LC50 <i>Daphnia magna</i>	TU_LF 30.03.	TU_LF 15.05.	TU_LF 24.05.	TU_LF 31.05.	TU_LF 12.06.
<b>Acetamiprid</b>	0	-	-	-	-	-
<b>Aclonifen</b>	0	-	-	-	-	-
<b>Carbendazim</b>	0.15		3.18762E-05	4.44571E-05	3.47444E-05	1.43333E-05
<b>Chloridazon</b>	132	-	7.93939E-08	7.90909E-08	-	-
<b>Chlortoluron</b>	0	-	-	-	-	-
<b>Chlomazon</b>	0	-	-	-	-	-
<b>Clothianidin</b>	40	-	-	-	-	-
<b>Desamino-Metamitron</b>	0	-	-	-	-	-
<b>Difenoconazol</b>	0.77	-	2.86364E-06	3.40445E-06	1.66234E-06	-
<b>Diflufenican</b>	0.24	5.26615E-05	8.06667E-05	0.000105708	0.000123944	4.6776E-05
<b>Dimethachlor</b>	24	-	-	-	-	-
<b>Dimethenamid</b>	16	1.35625E-07	1.10578E-06	2.43991E-06	6.19375E-07	1.83125E-07
<b>Dimethenamid-OA</b>	0	-	-	-	-	-
<b>Dimethomorph</b>	10.6	-	-	-	1.07594E-06	-
<b>Epoxiconazol</b>	8.69	-	-	-	-	-
<b>Fenpropimorph</b>	2.24	-	1.10638E-05	3.42028E-06	3.30729E-06	-
<b>Fluazifop</b>	0.62	-	1.12258E-05	1.27661E-05	-	-
<b>Flufenacet</b>	30.9	8.52751E-08	5.40858E-07	2.13139E-06	6.51672E-07	1.23786E-07
<b>Flufenacet-OA pos</b>	0	-	-	-	-	-
<b>Flurtamon</b>	13	1.20067E-06	2.3575E-06	4.19495E-06	3.87705E-06	4.71442E-07
<b>Imidacloprid</b>	85	5.90588E-08	-	-	-	-
<b>Cybutryn (Irgarol)</b>	2.4	-	-	-	-	-
<b>Isoproturon</b>	0.58	-	5.15086E-06	6.52217E-06	3.43391E-06	1.91379E-06
<b>Metamitron</b>	5.7	-	-	-	-	-
<b>Metazachlor</b>	33	4.22727E-08	1.84811E-07	2.97143E-07	6.86869E-08	
<b>Metazachlor-ESA</b>	0	-	-	-	-	-
<b>Metazachlor-OA</b>	0	-	-	-	-	-
<b>(S)-Metolachlor</b>	0.707	3.20721E-06	0.005056186	0.011769234	0.005514932	0.00027226
<b>Metolachlor-ESA pos</b>	0	-	-	-	-	-
<b>Metolachlor-OA pos</b>	0	-	-	-	-	-
<b>Napropamid</b>	14.3	-	-	-	-	-
<b>Prochloraz</b>	4.3	-	-	-	-	-
<b>Propiconazol</b>	10.2	2.89869E-07	9.20748E-06	4.9084E-06	5.10229E-06	7.11887E-07
<b>Prosulfocarb</b>	0.51	-	0.006959696	0.014598518	0.007261752	0.000325338
<b>Prothioconazol-desthio</b>	0	-	-	-	-	-
<b>Quinmerac</b>	100	4.8225E-08	3.6675E-08	3.03333E-08	2.682E-08	
<b>Tebuconazol</b>	2.79	1.08929E-06	4.9539E-05	2.75878E-05	3.05145E-05	3.87149E-06
<b>Terbutylazin</b>	21.2	1.16509E-07	6.72017E-05	0.000239467	0.000164165	1.20971E-05
<b>Terbutylazin-2-Hydroxy</b>	0	-	-	-	-	-

Substance	LC50 Daphnia magna	TU_LF 30.03.	TU_LF 15.05.	TU_LF 24.05.	TU_LF 31.05.	TU_LF 12.06.
Terbutryn	2.66	-	9.9087E-07	2.5956E-06	4.01253E-06	1.28045E-06
Thiacloprid	85.1	-	2.04054E-07	7.92345E-08	5.85194E-08	-
Thiamethoxam	100	-	5.58E-08	-	-	-
Triadimenol	51	-	4.46667E-07	-	-	-
Bifenox free acid	0	-		-	-	-
DCPMU	5.7	-	1.23684E-07	1.52632E-07	-	-
DCPU	0	-	-	-	-	-
Dimethachlor-ESA	0	-	-	-	-	-
Dimethachlor-OA	0	-	-	-	-	-
Dimethenamid- ESA	0	-	-	-	-	-
Diuron	5.7	-	5.07456E-07	6.54825E-07	-	5.5614E-07
Flufenacet-ESA	0	-	-	-	-	-
Flufenacet-OA	0	-	-	-	-	-
Mecoprop	200	-	7.19643E-08	7.405E-08	-	2.6325E-08
Metolachlor-ESA	0	-	-	-	-	-
Metolachlor-OA	0	-	-	-	-	-
Propyzamid	5.6	2.38839E-07	2.86161E-07	2.25765E-07	1.93304E-07	3.03571E-07
logSum		-4.23	-1.91	-1.57	-1.88	-3.17

Table TA 7 – List of Daphnia magna LC 50 values and TU for every chemical found during sampling in Waschbach grouped by substance and date of event.

Substance	LC50 Daphnia magna	TU_WB 30.03.	TU_WB 15.05.	TU_WB 24.05.	TU_WB 31.05.
Acetamiprid	0	-	-	-	-
Aclonifen	0	-	-	-	-
Carbendazim	0.15	1.00222E-05	5.17333E-05	2.97083E-05	4.42167E-05
Chloridazon	132	-	1.12008E-07	1.83561E-07	1.35045E-07
Chlortoluron	0	-	-	-	-
Chlomazon	0	-	-	-	-
Clothianidin	40	0.000000168	2.0825E-07	-	-
Desamino- Metamitron	0	-	-	-	-
Difenoconazol	0.77	1.75325E-06	2.06753E-06	2.06061E-06	1.52381E-06
Diflufenican	0.24	0.0000091	1.31786E-05	1.20313E-05	8.60938E-06
Dimethachlor	24	3.55417E-07	1.8756E-07	3.7526E-07	5.1349E-07
Dimethenamid	16	1.26825E-06	1.14446E-06	2.93203E-06	1.67672E-06
Dimethenamid- OA	0	-	-	-	-
Dimethomorph	10.6	-	2.21384E-07	2.07233E-07	2.26415E-07
Epoxiconazol	8.69	2.1256E-07	7.08142E-07	8.95857E-07	1.66643E-06
Fenpropimorph	2.24	-	-	-	-
Fluazifop	0.62	-	1.35215E-05	1.48387E-05	-
Flufenacet	30.9	2.44466E-07	1.26741E-06	9.65129E-07	6.19417E-07
Flufenacet-OA pos	0	-	-	-	-
Flurtamon	13	1.13462E-07	3.62308E-07	1.37538E-07	1.44231E-07

Substance	LC50 Daphnia magna	TU_WB 30.03.	TU_WB 15.05.	TU_WB 24.05.	TU_WB 31.05.
<b>Imidacloprid</b>	85	-	6.22353E-08		
<b>Cybutryn (Irgarol)</b>	2.4	-	5.875E-07	5.58333E-07	4.16667E-07
<b>Isoproturon</b>	0.58	0.000003	7.68719E-06	4.72414E-06	3.81897E-06
<b>Metamitron</b>	5.7		4.88518E-05	2.51982E-05	1.00947E-05
<b>Metazachlor</b>	33	2.6928E-07	2.29351E-07	3.04318E-07	3.26629E-07
<b>Metazachlor-ESA</b>	0	-	-	-	-
<b>Metazachlor-OA</b>	0	-	-	-	-
<b>(S)-Metolachlor</b>	0.707	3.56436E-06	4.26662E-05	3.98851E-05	7.07797E-05
<b>Metolachlor-ESA pos</b>	0	-	-	-	-
<b>Metolachlor-OA pos</b>	0	-	-	-	-
<b>Napropamide</b>	14.3	-	-	-	-
<b>Prochloraz</b>	4.3	-	-	-	-
<b>Propiconazole</b>	10.2	2.76716E-07	5.44678E-07	8.47672E-07	7.11152E-07
<b>Prosulfocarb</b>	0.51	-	0.000310882	5.88725E-05	7.97108E-05
<b>Prothioconazole-desthio</b>	0	-	-	-	-
<b>Quinmerac</b>	100	6.22875E-08	1.71143E-07	2.018E-07	3.84663E-07
<b>Tebuconazole</b>	2.79	2.42351E-06	1.42512E-05	1.36967E-05	9.32875E-06
<b>Terbutylazin</b>	21.2	9.58726E-08	8.19575E-07	2.78107E-06	3.09458E-06
<b>Terbutylazin-2-Hydroxy</b>	0	-	-	-	-
<b>Terbutylazin-desethyl</b>	0	-	-	-	-
<b>Terbutryn</b>	2.66	1.05714E-06	2.78258E-06	2.76817E-06	1.47697E-06
<b>Thiacloprid</b>	85.1	-	1.474E-07	1.98543E-06	1.1886E-07
<b>Thiamethoxam</b>	100	-	2.7696E-07	8.515E-08	8.05143E-08
<b>Triadimenol</b>	51	8.7549E-07	1.1551E-06	-	5.1652E-07
<b>Bifenox free acid</b>	0	-	-	-	-
<b>DCPMU</b>	5.7	-	1.70175E-07	9.47368E-08	1.2924E-07
<b>DCPU</b>	0	-	-	-	-
<b>Dimethachlor-ESA</b>	0	-	-	-	-
<b>Dimethachlor-OA</b>	0	-	-	-	-
<b>Dimethenamid-ESA</b>	0	-	-	-	-
<b>Diuron</b>	5.7	-	1.15088E-06	3.04825E-07	1.00058E-06
<b>Flufenacet-ESA</b>	0	-	-	-	-
<b>Flufenacet-OA</b>	0	-	-	-	-
<b>Mecoprop</b>	200	-	3.17514E-07	3.11975E-07	3.26588E-07
<b>Metolachlor-ESA</b>	0	-	-	-	-
<b>Metolachlor-OA</b>	0	-	-	-	-
<b>Propyzamide</b>	5.6	1.5308E-06	4.125E-07	4.2433E-07	1.85714E-07
<b>logSum</b>	-	-4.44	-3.29	-3.67	-3.6

Table TA 8 – List of *Daphnia magna* LC 50 values and TU for every chemical found during sampling in Weidgraben

Substance	LC50 <i>Daphnia magna</i>	TU_WG 15.5	TU_WG 12.06.
Acetamiprid	0	-	-
Aclonifen	0	-	-
Carbendazim	0.15	-	-
Chloridazon	132	-	-
Chlortoluron	0	-	-
Chlomazon	0	-	-
Clothianidin	40	-	-
Desamino-Metamitron	0	-	-
Difenoconazol	0.77	-	-
Diflufenican	0.24	-	5.9375E-06
Dimethachlor	24	-	-
Dimethenamid	16	1.675E-07	4.84766E-07
Dimethenamid-OA	0	-	-
Dimethomorph	10.6	-	2.30189E-07
Epoxiconazol	8.69	1.82106E-07	4.06933E-07
Fenpropimorph	2.24	-	-
Fluazifop	0.62	-	-
Flufenacet	30.9	8.78641E-08	6.59264E-07
Flufenacet-OA pos	0	-	-
Flurtamone	13	-	-
Imidacloprid	85	-	-
Cybutryne (Irgarol)	2.4	-	-
Isoproturon	0.58	-	-
Metamitron	5.7	0.000149054	4.45833E-06
Metazachlor	33	-	-
Metazachlor-ESA	0	-	-
Metazachlor-OA	0	-	-
(S)-Metolachlor	0.707	0.000161379	3.40877E-06
Metolachlor-ESA pos	0	-	-
Metolachlor-OA pos	0	-	-
Napropamide	14.3	-	-
Prochloraz	4.3	-	-
Propiconazol	10.2	-	3.0049E-07
Prosulfocarb	0.51	0.000198297	-
Prothioconazol-desthio	0	-	-
Quinmerac	100	-	-
Tebuconazole	2.79	1.72733E-06	2.65342E-06
Terbutylazin	21.2	2.03833E-06	2.46044E-06
Terbutylazin-2-Hydroxy	0	-	-
Terbutylazin-desethyl	0	-	-
Terbutryn	2.66	-	-
Thiacloprid	85.1	3.98523E-08	2.47944E-08
Thiamethoxam	100	5.60838E-07	-
Triadimenol	51	-	-
Bifenoxy free acid	0	-	-
DCPMU	5.7	-	-
DCPU	0	-	-
Dimethachlor-ESA	0	-	-

<b>Substance</b>	<b>LC50 Daphnia magna</b>	<b>TU_WG 15.5</b>	<b>TU_WG 12.06.</b>
<b>Dimethachlor-OA</b>	0	-	-
<b>Dimethenamid-ESA</b>	0	-	-
<b>Diuron</b>	5.7	-	-
<b>Flufenacet-ESA</b>	0	-	-
<b>Flufenacet-OA</b>	0	-	-
<b>Mecoprop</b>	200	-	-
<b>Metolachlor-ESA</b>	0	-	-
<b>Metolachlor-OA</b>	0	-	-
<b>Propyzamid</b>	5.6	-	-
<b>logSum</b>	-	-3.29	-4.68

*“Do what you can, with what you have, where you are.”*

*Theodore Roosevelt*

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