THE “KLEINGEWÄSSER-MONITORING” (KgM) –

A MONITORING OF GERMAN SMALL STREAMS AND ITS IMPLICATIONS FOR THE ENVIRONMENTAL RISK ASSESSMENT OF PESTICIDES

by

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2. Liess, M.; Liebmann, L.; Vormeier, P.; Weisner, O.; Altenburger, R.; Borchardt, D.; Brack, W.; Chatzinotas, A.; Escher, B.; Foit, K.; Gunold, R.; Henz, S.; Hitzfeld, K.L.; Schmitt-Jansen, M.; Kamjunke, N.; Kaske, O.; Knillmann, S.; Krauss, M.; Küster, E.; Link, M.; Lück, M.; Möder, M.; Müller, A.; Paschke, A.; Schäfer, R.B.; Schneeweiss, A.; Schreiner, V.C.; Schulze, T.; Schüürmann, G.; von Tümpling, W.; Weitere, M.; Wogram, J.; Reemtsma, T. (2021): Pesticides Are the Dominant Stressors for Vulnerable Insects in Lowland Streams. In: *Water Research.*

3. Weisner, O.; Frische, T.; Liebmann, L.; Reemtsma, T.; Roß-Nickoll, M.; Schäfer, R.B.; Schäffer, A.; Scholz-Starke, B.; Vormeier, P.; Knillmann, S.; Liess, M. (2021): Risk from Pesticide Mixtures – the Gap between Risk Assessment and Reality. In: *Science of the Total Environment.*

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5. Weisner, O.; Arle, J.; Liebmann, L.; Link, M.; Schäfer, R.B.; Schneeweiss, A.; Schreiner, V.C.; Vormeier, P.; Liess, M. (2021): Three Reasons Why the Water Framework Directive (WFD) Fails to Identify Pesticide Risks. In: *Water Research*.

Annotation

This cumulative dissertation is based on five scientific publications written by multiple authors. For this reason, the first-person plural is used throughout this thesis.

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Abbreviations

AA-EQS	Annual Average – Environmental Quality Standard
AC_{field}	Field-based Acceptable Concentration
AhR	Aryl Hydrocarbon Receptor
ASPT	Average Score Per Taxon
BEQ	Bioanalytical Equivalent Concentration
BMWP	Biological Monitoring Working Party
EBT	Effect-Based Trigger value
EFSA	European Food Safety Authority
EQS	Environmental Quality Standard
EPT	Ephemeropterans, Plecopterans and Trichopterans
ER	Estrogen Receptor
ERA	Environmental Risk Assessment
ERO	Ecological Recovery Option
ESC	Ecological Status Class
ETO	Ecological Threshold Option
FOD	Frequency Of Detection
KgM	Kleingewässer-Monitoring
LC-HRMS	Liquid Chromatography - High Resolution Mass Spectrometry
LC-HRMS/MS	Liquid Chromatography - High Resolution tandem Mass Spectrometry
LOD	Limit Of Determination
LOQ	Limit Of Quantification
MAC-EQS	Maximum Acceptable Concentration – Environmental Quality Standard
MCR	Maximum Cumulative Ratio
NAP	National Action Plan for sustainable use of plant protection products
PEC	Predicted Environmental Concentration
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
PPP	Plant Protection Product
PS	Priority Substance
RAC	Regulatory Acceptable Concentration
RBSP	River Basin-Specific Pollutant
REP	Relative Effect Potency
RQ	Risk Quotient
SI	Saprobic Index
SPE	Solid-Phase Extraction
$SPEAR_{pesticides}$	SPEcies At Risk Index
TU	Toxic Unit
UBA	Umweltbundesamt
WFD	Water Framework Directive
WWTP	Wastewater Treatment Plant

Abstract

Today's agriculture heavily relies on pesticides to manage diverse pests and maximise crop yields. Despite elaborate regulation of pesticide use based on a complex environmental risk assessment (ERA) scheme, the widespread use of these biologically active compounds has been shown to be a threat to the environment. For surface waters, pesticide exposure has been observed to exceed safe concentration levels and negatively impact stream ecology leading to the question whether current ERA schemes ensure a sustainable use of pesticides. To answer this, the large-scale "Kleingewässer-Monitoring" (KgM) assessed the occurrence of pesticides and related effects in 124 streams throughout Germany, Central Europe, in 2018 and 2019.

Based on five scientific publications originating from the KgM, this thesis evaluated pesticide exposure in streams, ecological effects and the regulatory implications. More than 1,000 water samples were analysed for over 100 pesticide analytes to characterise occurrence patterns (publication 1). Measured concentrations and effects were used to validate the exposure and effect concentrations predicted in the ERA (publication 2). By jointly analysing real-world pesticide application data and measured pesticide mixtures in streams, the disregard of environmental pesticide mixtures in the ERA was evaluated (publication 3). The toxic potential of mixtures in stream water was additionally investigated using suspect screening for 395 chemicals and a battery of in-vitro bioassays (publication 4). Finally, the results from the KgM stream monitoring were used to assess the capability to identify pesticide risks in governmental monitoring programmes (publication 5).

The results of this thesis reveal the widespread occurrence of pesticides in non-target stream ecosystems. The water samples contained a variety of pesticides occurring in complex mixtures predominantly in short-term peaks after rainfall events (publications 1 & 4). Respective pesticide concentration maxima were linked to declines in vulnerable invertebrate species and exceeded regulatory acceptable concentrations in about 80% of agricultural streams, while these thresholds were still estimated partly insufficient to protect the invertebrate community (publication 2). The co-occurrence of pesticides in streams led to a risk underestimated in the single substance-oriented ERA by a factor of about 3.2 in realistic worst-case scenarios, which is further exacerbated by a high frequency at which non-target organism are exposed to pesticides (publication 3). Stream water samples taken after rainfall caused distinct effects in bioassays which were

only explainable to a minor extent by the many analytes, indicating the relevance of unknown chemical or biological mixture components (publication 4). Finally, the regulatory monitoring of surface waters under the Water Framework Directive (WFD) was found to significantly underestimate pesticide risks, as about three quarters of critical pesticides and more than half of streams at risk were overlooked (publication 5).

Essentially, this thesis involves a new level of validation of the ERA of pesticides in aquatic ecosystems by assessing pesticide occurrence and environmental impacts at a scale so far unique. The overall results demonstrate that the current agricultural use of pesticides leads to significant impacts on stream ecology that go beyond the level tolerated under the ERA. This thesis identified the underestimation of pesticide exposure, the potential insufficiency of regulatory thresholds and the general inertia of the authorisation process as the main causes why the ERA fails to meet its objectives. To achieve a sustainable use of pesticides, the thesis proposes substantial refinements of the ERA. Adequate monitoring programmes such as the KgM, which go beyond current government monitoring efforts, will continue to be needed to keep pesticide regulators constantly informed of the validity of their prospective ERA, which will always be subject to uncertainty.

1 Introduction

1.1 Pesticide Use and Its Ecological Consequences

"If pesticide use is to blame, even partially, then this raises questions both about pesticide use and the regulatory procedures that are used to protect the environment" Topping et al. (2020)

Pesticides are an integral part of conventional agriculture today and their intensive use has become the standard in all parts of the world (Tang et al., 2021). Agriculture occupies a large fraction of about 38% of the earth's terrestrial surface – more than any other land use (settlement, forest etc.) (Foley et al., 2011). In Germany, even 47% of land is agriculturally used (UBA, 2020b). Except for permanent grasslands (13%) and organic farming (5%), the remaining 29% refer to conventional arable and permanent crop farming (UBA, 2020a). Almost a third of land surface is thus commonly treated with a broad spectrum of pesticides in order to maximize yields. Therefore, pesticide treatments pursue various objectives ranging from weed control using herbicides, avoidance of plant diseases using fungicides or elimination of pest infestation using insecticides, acaricides, rodenticides, molluscicides etc. Depending on weather conditions and cultivated crops, farmers employ a series of different pesticide applications, often prophylactically, to ensure beneficial growing conditions (Sybertz et al., 2020). In Germany alone, 285 different pesticides were applied in 2018 resulting in 2.8 kilograms active substance per hectare (280 mg/m^2) (UBA, 2021). Similarly intense use statistics apply to past decades with German sales quantities remaining fairly constant ranging from 24,000 to 31,000 tons per year (Figure 1).

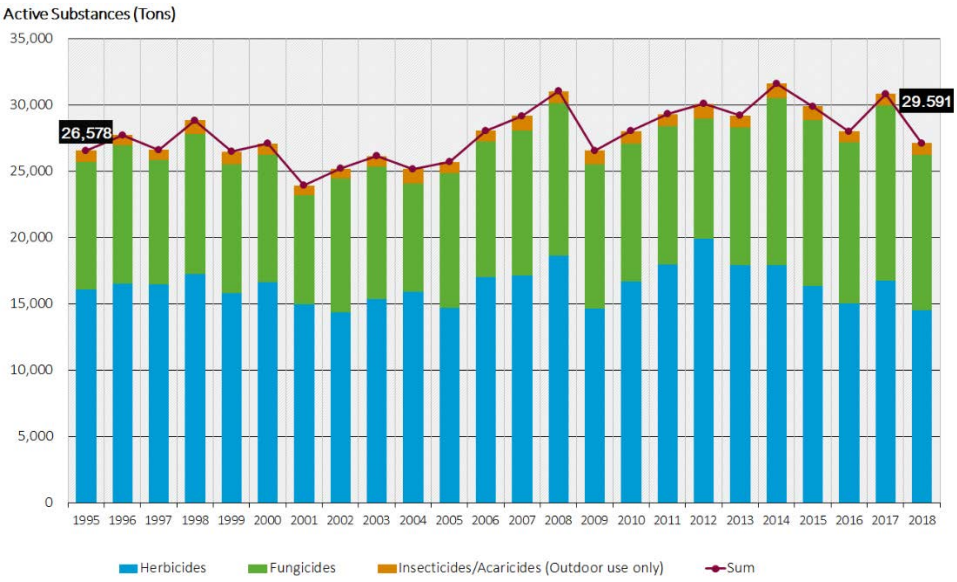

Figure 1: Stagnant domestic sales quantities of individual active substance groups over the last years. Modified from UBA (2021).

However, novel pesticide active ingredients that entered the market and dominated use patterns more recently tend to be more toxic than pesticides applied before the turn of the millennium (Schulz et al., 2021). While the amount of pesticides applied may stagnate, the ecological risk may thus have increased over the past decades.

Once a pesticide is applied, only a small fraction of estimated 0.1% reaches its intended target site, while the vast majority is subject to environmental fate processes (Pimentel, 1995). These are determined by substance-specific physico-chemical properties and local environmental conditions. Environmental pesticide fate is highly complex – temperature, sunshine intensity and molecular structure, among others, affect a substance's persistence. Sorption properties, water solubility, volatilization, field slope and many other factors further influence a substance's mobility (Nowell et al., 1999). Transportation processes such as drift, leaching or runoff distribute pesticides also into non-target ecosystems (Jong et al., 2008; Schulz et al., 1998). There, pesticide residues occur in complex mixtures (often referred to as "pesticide cocktails") so that single soil or water

samples may comprise up to 20 or more different mixture components (Schreiner et al., 2016; Silva et al., 2019; Vallotton and Price, 2016).

Consequently, ecologically relevant pesticide concentrations were measured in numerous studies in terrestrial and aquatic ecosystems in Europe (Beketov et al., 2013; Larras et al., 2017; Liess et al., 2021; Liess and Ohe, 2005; Liess and Schulz, 1999; Schäfer et al., 2011; Silva et al., 2019) , but also worldwide like in Africa (Ganatra et al., 2021), Australia (Burgert et al., 2011; Wood et al., 2019) as well as North and South America (Chiu et al., 2016; Hunt et al., 2017; Miller et al., 2020). Also in various biota tissues, pesticides were detected in harmful concentration levels (Llorca et al., 2017; Shahid et al., 2018; Xu et al., 2016). This ubiquitous occurrence of biologically active substances causes a variety of undesired direct and indirect effects by harming non-target organisms and disrupting essential food web structures (Brühl and Zaller, 2019). These effects may scale up and lead to alterations on community level and impairment of entire ecosystem functions. Accordingly, insecticide pressure, for instance, was associated with decreased pollination by lethally or only sublethally affecting bees or alterations in stream invertebrate assemblages as sensitive species declined (Liess and Schulz, 1999; Menon and Mohanraj, 2018). Fungicides can drive aquatic fungi community composition impacting leaf litter degradation representing a major energy resource for aquatic ecosystems (Zubrod et al., 2019). Herbicides can decrease herbivore biomass and hence indirectly cause bird declines in agricultural landscapes due to food scarcity (Hahn et al., 2014).

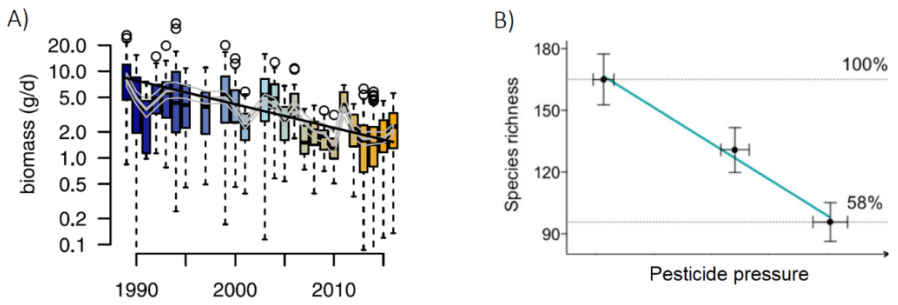

Figure 2 A) Temporal trend depicting decline in flying insect biomass of 76% from 1989 until 2016 in Germany (Hallmann et al., 2017); B) Species richness reduction by an average of 42% in pesticide polluted German and French streams. Modified from Beketov et al. (2013).

For these reasons, pesticides are linked to one of the major environmental concerns nowadays – the decline in biodiversity and more specifically in insects. Multiple studies have affirmed substantial losses in terrestrial and aquatic insect populations in the last years (Geiger et al., 2010; Sánchez-Bayoa and Wyckhuys, 2019; Seibold et al., 2019). Most prominently, Hallmann et al. (2018) observed flying insect biomass to dwindle by 76% in German nature protection areas (Figure 2A) (Hallmann et al., 2017). All authors more or less come to the same conclusion, that negative impacts of agricultural intensification are responsible for these ecological effects in agricultural as well as remote, less intensively cultivated landscapes. Besides habitat loss and fertilizer excess, pesticide use is specified as a principle driver of this ecological crisis. To which extent pesticides contribute to the diverse ecological effects observed is still unknown, though. The ubiquitous co-occurrence of confounding stressors makes it scientifically and technically challenging to specifically quantify the contribution of a single stressor and to disentangle the role of pesticides from all other stressors associated with biodiversity decline. Multiple studies, however, were able to directly link pesticide pressure to the observed ecological effects (Figure 2B) (Beketov et al., 2013; Liess and Ohe, 2005; Schäfer et al., 2011). This raises the question how pesticide use is regulated and why these procedures apparently fail to protect the environment. This thesis particularly focuses on the risk by pesticides for aquatic ecosystems. An introduction to the aquatic risk assessment within

the EU is presented below after an explanation pointing out the relevance of freshwaters in the context of biodiversity crisis.

1.2 The Eco(toxico)logical Relevance of Streams

Freshwater ecosystems in general provide essential ecosystem services to both the society and the environment. These go beyond provisioning services such as clean drinking water, fishery, irrigation and also involves transportation routes, climate and flood regulation and recreation (Malaj et al., 2014; Vörösmarty et al., 2010). From a more ecological point of view, freshwaters play a crucial role in nutrient cycling by breaking down organic matter and reinvesting it into primary production. Finally, they also represent hotspots for biodiversity hosting at least 126,000 different animal species (Balian et al., 2007). This makes up for approximately 9.5% of the globally recognized animal species despite the small fraction of 0.01% that freshwaters contribute to the earth's surface.

Especially headwater or small streams are characterized by a wide range of microclimatic, hydrological, morphological and biological conditions, which is why they offer numerous diverse small-scale habitats for plant, microbial and animal life (Meyer et al., 2007). Accordingly, a single 1 metre wide stream was found to accommodate more than 1,000 invertebrate taxa (Allan, 1995). These small streams thus play an important ecological role for the whole river network also enabling recolonization of impaired downstream reaches (Knillmann et al., 2018). Another reason for the particular attention that small streams deserve is that they comprise the major fraction of running waters (Nadeau and Rains, 2007). Each higher order stream drains multiple smaller upstream reaches. Accordingly, streams showing a width of less than 3 metres are estimated to make up for 90% of flow length of the entire German river system (Brinke et al., 2017).

Textbox 1: Pesticide Entry Pathways into Surface Waters	
Point Source Pollution	Diffuse, Nonpoint Source Pollution
• **Spills:** Accidental inputs of pesticides as for instance observed by Reiber et al. (2021). • **Wastewater treatment plants (WWTPs):** Entry of WWTP effluents often containing elevated pesticide concentration levels as pesticide compounds are only removable to a limited extent by common WWTP treatment stages (Le et al., 2017). • **Farms:** Effluents of farms where pesticide application material (containers, tanks, spray apparatus) is rinsed after usage and used water is not collected in a specific tank or sprayed on the field. • **Drainage:** Input via artificial pipes installed to drain excessive soil water of fields collecting runoff and leachate.	• **Surface runoff:** Rainfall-induced surface water runoff washing out pesticide residues from the field into adjacent surface waters, often while being sorbed to organic material carried away by the runoff (Wauchope, 1978). Most common cultures like cereals, rape and beets are sprayed downwardly causing a contamination of upper soil layers susceptible to wash-out by runoff. • **Spray drift:** Off-drift of pesticide droplets during spray application onto adjacent water surfaces. This pathway is of particular relevance for vertical cultures such as orchards, vine or hop, where PPPs are sprayed horizontally (Ganzelmeier et al., 1995). • **Leaching:** Sub-surface leaching of pesticides from point of application into surface waters. The relevance of leaching as an entry pathway largely depends on the local soil type and the chemical's sorption behavior and solubility (Fenelon and Moore, 1998).

At the same time, streams are often located in direct proximity to arable land and are thus likely to face high loads of pesticide pollution (see **Textbox 1** and Figure 3) (Liess et al., 1999). In contrast to bigger water bodies, these streams lack dilution capacity yielding higher pollutant concentrations. That is why elevated pesticide concentrations and effects were shown to occur especially in streams draining smaller catchments (Liess and Schulz, 1999; Szöcs et al., 2017). This delicate combination of sensitivity and ecological relevance makes streams an ecosystem particularly worth protecting. This is why the aquatic risk assessment of pesticides in the EU has been subject to corrections and refinements over the past decades resulting in complex practices and multi-layered approaches in scope of pesticide regulation.

Figure 3: Small stream in the agricultural landscape facing diffuse pesticide pollution (red arrows) via surface runoff, drift or leaching after pesticide application (sketch on the left). Photo by André Künzelmann.

1.3 Environmental Risk Assessment of Pesticides in Surface Waters

More and more voices were being raised recently criticizing a gap between the intended protection level related to pesticide use and environmental reality (Boyd, 2018; Brühl and Zaller, 2019; Frische et al., 2018; Liess et al., 2019; Schäfer et al., 2019; Stehle and Schulz, 2015b; Topping et al., 2020). This directly addresses the environmental risk assessment (ERA) of pesticides, which aims to realize protection goals by regulating pesticide use to ensure that the benefit tops a tolerable harm. In the EU, pesticide ERA is legally defined by the European Plant Protection Products Regulation (EC) No 1107/2009 (European Union, 2009). In a first step, it requires the approval of the pesticide active substances at the EU level by the European Food Safety Authority (EFSA). In a second step, each EU member state is responsible for the authorisation of plant protection products (PPPs) within their territory. PPPs are the products sold to and applied by farmers containing one or more pesticide active ingredients mixed with additives to improve applicability and performance. The EFSA published the Aquatic Guidance Document describing the detailed ERA procedure for pesticides and PPPs in surface waters implemented by all EU member states (EFSA, 2013). Comparable to other areas of risk assessment (e.g. industrial chemicals under REACH), the risk of pesticides in surface waters is estimated by comparing an environmental concentration of a substance with its concentration level that is expected to exclude "unacceptable effects on the environment" (European Parliament and Council of the European Union, 2009).

As pesticide risk needs to be assessed prior to its approval ("prospective risk assessment") both (i) the environmental concentration and (ii) the ecologically acceptable concentration under field conditions have to be predicted. These predictions follow a tiered approach accounting for the availability and real-world transferability of the underlying data and include the remaining uncertainty (see Textbox 2). Regarding the first, exposure models are fed with expected pesticide application amounts and yield a substance-specific predicted environmental concentrations (PEC) in

surface waters. For this purpose, model algorithms account for different application techniques, environmental/climatic conditions, pesticide properties, risk mitigation measures and a generalized scenario of a theoretical stream adjacent to a treated field. While the FOCUS model (FOCUS, 2012) is used to predict pesticide active ingredient surface water concentrations in the EU, Germany relies on the similar EXPOSIT and EVA models during PPP authorisation on national level (BVL, 2021). The application of a specific PPP may only be authorized under the condition of implementing certain risk mitigation measures that lead to a reduced PEC. Such measures include untreated riparian buffer strips, runoff reducing soil tillage, vegetated ditches and constructed wetlands in which pesticides are retained and their degradation is promoted (Reichenberger et al., 2007).

Deriving the regulatory acceptable concentration (RAC), on the other hand, relies on test systems studying the effects of pesticides or PPP on non-target organisms in more or less complex laboratory experiments. Simple experimental designs comprise acute (i.e. 48 - 96h) single surrogate species tests under simplified, laboratory conditions equalling the minimum of toxicity data required for registration. More complex test systems aim to approximate field conditions by observing chronic effects or including multiple species in more realistic experimental setups (e.g. micro- or mesocosms). In order to derive a RAC, the effect concentration observed in such an experiment is divided by an assessment factor (AF) expected to balance out the test system-related uncertainty: While complex mesocosm effect concentrations are extrapolated to field scale applying AFs of 1-5 (test system approximate field conditions – no or little extrapolation required), simple laboratory study results are extrapolated by an AF of up to 100.

Textbox 2: Tiered Approach in the ERA of Pesticides and PPPs

The ERA of pesticides and PPP relies on many data describing the environmental fate and ecotoxicity towards different organism groups, the collection of which is costly, time consuming and ethically critical (animal testing). Hence, data availability is limited, which is why the ERA considers different tiers at which the exposure (PEC) and the effect assessment (RAC) are performed. According to this concept, a simple and conservative assessment requiring few data (tier 1) is the starting point (see Figure 4). Even though described as "conservative", the first tier "may not be protective in 100 % of the cases" (EFSA, 2013). Only if a risk cannot be excluded (PEC>RAC) within this conservative approach, more complex higher-tier assessments (tier2-4) are to be performed. These higher-tier assessments incorporate more data, more realistic experimental designs and/or more elaborate computer models. These in turn replace conservative assumptions within the higher-tier assessments ultimately leading to a decrease of the PEC/RAC ratio and a potentially tolerable risk (PEC<RAC).

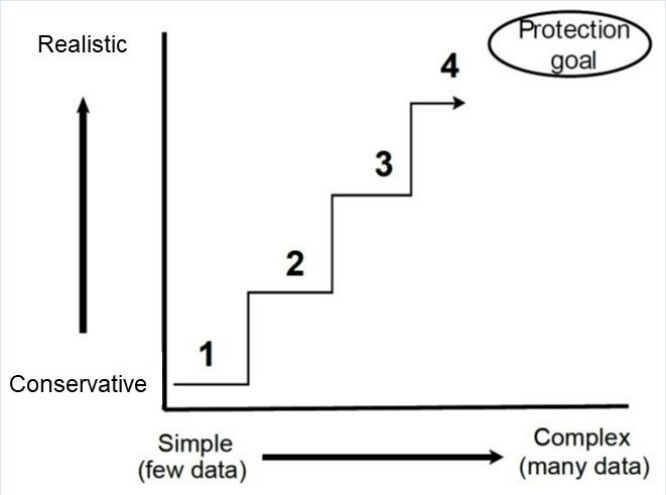

Figure 4: Schematic depiction of the ERA tiered approach (EFSA, 2013)

In order to be approved by posing an acceptable ecological risk, a PEC must not exceed the respective RAC. Both the prediction of the PEC and of the RAC and therefore of the ecological risk itself, however, rely on a number of assumptions and simplifications. These are practically necessary to

predict the complex and partly unknown fate and effects of a pesticide for the many potential environmental settings and receptors (= non-target organisms). Such assumptions involve, for example, the consideration of a model stream of a specified water volume in which specified fractions of pesticide residues are transported for the PEC modelling or the selective toxicity testing of species which are expected to be among the most sensitive organisms in the field for the RAC derivation. All these assumptions and simplifications are driven by the claim to be highly conservative by assuming realistic worst-case conditions and to account for related uncertainties. However, previous studies question this conservativeness by pointing out several flaws and blind spots concerning the current ERA, its assumptions and its simplifications potentially causing the gap between the intended protection level and environmental reality. These involve

- the restriction of toxicity test systems to single or few species disregarding indirect effects between organism groups or interactions of different trophic levels (Brühl and Zaller, 2019). Intra- and interspecific competition among test organisms may increase pesticide effects (Kattwinkel and Liess, 2014) and indirect top-down and/or bottom-up effects within the food web may lead to alterations in ecosystem functioning (McMahon et al., 2012).
- the neglect of vulnerable and ecologically relevant non-target organism groups. An example are aquatic fungi that play a crucial role in stream ecosystem functioning (Zubrod et al., 2015). These were shown to be affected by fungicides under realistic environmental concentration levels but are not adequately covered by any standard toxicity test.
- the disregard of parallel and repeated pesticide exposure by only assessing single applications of a single PPP (with the exception of mesocosm experiments treated with repeated exposure pulses) (Frische et al., 2018; Topping et al., 2020). In the environment, the intense pesticide use practices have led to complex pesticide mixtures so that non-target organisms are likely

to be exposed to multiple pesticides at a time. Additionally, organisms characterised by generation times of a few weeks or longer or wide-ranging terrestrial animals are likely to face multiple pesticide exposure peaks during their lifetime.

- inaccurate exposure predictions underestimating actual environmental concentrations (Knäbel et al., 2012).
- the neglect of co-occurring environmental stressors (e.g. temperature, salinity or food scarcity) potentially exacerbating the ecological pesticide effects (Heugens et al., 2001; Liess et al., 2016). In presence of such environmental stress organisms revealed an increased sensitivity to pesticide toxicity.

In order to assess the validity of the assumptions, the adequacy of simplifications and the sufficiency of AFs to cover uncertainties, field investigations represent the ultimate tool. In line with numerous scientific studies (Liess et al., 1999; Liess and Schulz, 1999; Müller and Hitzfeld, 2020; Schäfer et al., 2019; Stehle and Schulz, 2015b), the EFSA's Aquatic Guidance accordingly emphasizes "a need to validate/calibrate the RA scheme to the field situation. […] Field investigations need to exemplarily verify exposure and effect predictions." How these field investigations need to be designed is explained in more detail in the following chapter.

1.4 Monitoring of Pesticide Exposure and Effects in Streams

1.4.1 Chemical Monitoring

Independent of the entry pathway, pesticide input into streams occurs episodically and the in-stream concentration pattern of pesticides follow a mostly low background level with short-term, transient peaks (Liess et al., 1999). Therefore, linking any observed effects on lotic communities to pesticide exposure requires suitable and specifically adjusted pesticide sampling strategies (Liess and Schulz, 1999). Event-driven sampling (EDS) aims to account for the high spatio-temporal variance of pesticide concentrations by sampling stream water during peak exposure events. Whether and when a particular pesticide is present in a stream depends, among other things, on the catchment characteristics, the time and type of application, the weather conditions and the substance properties, which makes adequate monitoring of pesticide contamination a major challenge (Lorenz et al., 2017).

A successful EDS approach to capture pesticide peak concentrations in agricultural streams relies on sampling during rainfall events when surface runoff occurs. Common sampling approaches are automated and triggered by an increase of water level or discharge (see Figure 5). Sampling time points and periods are designed to optimally capture peak concentrations, which differ among substances, rain events and monitoring sites. This method thus only allows approximating the actual concentration peaks while always measuring less as the momentary peak is missed or diluted within the sample.

Figure 5: Automatic, water-level triggered EDS sampler installed in a typical agricultural stream to measure surface runoff-induced pesticide peak concentrations in the Kleingewässer-Monitoring (KgM).

While multiple scientific studies deployed EDS approaches, governmental monitoring of pesticides in the EU member states is enacted by the Water Framework Directive (WFD) and is based on regular grab sampling (European Union, 2000). Here, samples are taken on a regular, mostly monthly basis meaning a significantly reduced temporal and financial effort. The comparably high material and personnel requirements restrain the practicability of EDS for any monitoring authority.

In accordance with the WFD, measured environmental pesticide concentrations are then compared to Environmental Quality Standards (EQS). These are thresholds for substances that were identified to be of concern for European surface waters and partly comprise general priority substances (PS) as well as river basin-specific pollutants varying throughout EU member states. To reach the desired “good chemical status” required under the WFD, measured environmental concentrations must not exceed their EQS.

1.4.2 Biological Monitoring

Biological monitoring (or biomonitoring) uses biological responses to identify and evaluate environmental changes and stressors to assess

stream health (Karr, 1999). Depending on the type of change or stressors, respective effects can be observed at different levels of biological organisation. Suitable aquatic organism groups range from fish, invertebrates, macrophytes and algae to bacteria and fungi. More recent approaches aim to study effects on the ecosystem level by quantifying ecosystem functioning like aquatic organic matter breakdown (Berger et al., 2018; Cornejo et al., 2020). And while part of biological endpoints responds to specific stressors, others indicate overall environmental conditions as a combination of stressors. Either way, due to the prolonged persistence of biological responses, biomonitoring has the great advantage that stressors do not have to be measured at the exact time they occur.

Regarding ecological quality assessment of streams and rivers, benthic invertebrates have become the most monitored organism group for the following reasons: (i) Invertebrates reflect local stream conditions due to their comparably sessile mode of life, (ii) show aquatic life-cycle stages long enough to face short-term stressor pulses, (iii) promptly respond to a wide range of pollutants due to a relatively general sensitivity, (iv) are omnipresent in high abundancy, and (v) easy to sample and identify (Barbour et al., 1999). Accordingly, various environmental stressor effects are indicated by the invertebrate community ranging from habitat degradation, eutrophication and salinization to pesticide toxicity (García et al., 2017; Liess and Schulz, 1999; Miler and Brauns, 2020; Timpano et al., 2018). Under the WFD, the assessment of invertebrate assemblages represents one so called “biological quality element” and thus a major pillar of the ecological quality assessment of streams and rivers (European Union, 2000).

The community level in particular provides well-measurable endpoints like overall abundance, species richness and diversity which are closely linked to ecosystem functioning (Clements and Rohr, JR, 2009). However, these general community endpoints are accompanied by two drawbacks: (i) Shifts in the community structure resulting from the replacement of stressor-sensitive species by more stressor-tolerant ones remain undiscovered (Geiszinger et al., 2009). (ii) Attributing an observed

ecological effect to a specific stressor among a set of environmental parameters is challenging. The ubiquitous co-occurrence of natural and anthropogenic, so called confounding factors, such as toxic pressure, habitat structure, biotic and abiotic conditions may bias or mask single stressor-related effects (Alexander et al., 2013; Kattwinkel and Liess, 2014; Münze et al., 2017).

To avoid these difficulties, trait-based biomonitoring approaches came into play that focus on species properties rather than taxonomic composition. These traits specify the physical characteristics, ecological niche, and functional role of species or other taxonomic entities within the ecosystem (Baird et al., 2008; Usseglio-Polatera et al., 2000). Trait-based approaches take advantage of the fact that biotic and abiotic habitat conditions determine the trait profile of all organisms present at a site. Typical benthic macroinvertebrate traits are organism size, feeding type, voltinism or locomotion. While stressors may not impact overall abundance, they may shape communities showing a shifted set of such traits. This in turn may decrease a community's functional diversity ultimately limiting ecosystem functions (Voß and Schäfer, 2017).

The $SPEAR_{pesticides}$ index is a trait-based biological indicator to quantify pesticide effects on the aquatic invertebrate community (Liess and Ohe, 2005). It integrates the pesticide-specific traits (i) physiological sensitivity to pesticides, (ii) low migration and recolonization potential, (iii) long generation time and (iv) presence of aquatic life-cycle stages. $SPEAR_{pesticides}$ has shown to respond to pesticide pressure while largely disregarding confounding factors (Knillmann et al., 2018; Schäfer et al., 2012). It therefore enables an explicit link between pesticide exposure and ecological effect and represents a highly relevant tool regarding pesticide risk assessment in streams.

Besides invertebrates, the aquatic flora plays a central role in stream ecology and hence comprise focal species studied in the effect assessment in the ERA of pesticides. They also reflect the second pillar of the WFD's ecological status assessment. The aquatic flora comprises algae including

phytobenthos and diatoms that grow on hard substrate (i.e. rocks) and macrophytes. Nutrients are a dominant driver for the composition of the freshwater flora, but especially the diatom community is considered a valuable indication for a multitude of natural and environmental stressors (Kelly, 2013). Lastly, fish represent the third organism group that are the focus of the ERA of pesticides and the ecological status assessment under the WFD. These are less abundant in such small streams as the occurrence of fish is positively correlated with the stream size (Magalhaes et al., 2002). They represent a biological quality element of minor relevance in these ecosystems and are disregarded here.

1.5 The „Kleingewässer-Monitoring“ Project (KgM)

1.5.1 Background

In 2009, the European Parliament and European Council have imposed each EU member state to develop National Action Plans in accordance with the Plant Protection Framework Directive 2009/128/EC (European Parliament and European Council, 2009). Within these Action Plans, respective governments were to commit to self-set goals and measures to reduce pesticide risks aiming at a sustainable use of pesticides. In 2013, the German Action Plan for Sustainable Plant Protection was published. Among the 12 objectives aiming to reduce pesticide risks in aquatic ecosystems, one prescribed a monitoring of small agricultural surface waters by 2018 to evaluate agricultural pesticide nonpoint source pollution (BMEL, 2013). A representative selection of surface waters characterized by hydrological catchment areas smaller than 10 km^2 should be targeted, an ecosystem type that has been widely ignored so far in governmental monitoring under the WFD. By 2023, 99% of EDS samples collected within this monitoring project should reveal concentrations below the RACs, another goal defines.

Two sub-projects were carried out, which formed the basis for the practical implementation of the monitoring. In the first sub-project, monitoring data on the pesticide pollution of small agricultural water bodies were collected from federal states, harmonized and jointly analyzed (Brinke et al., 2017). Respective results once more emphasized the vulnerability of small

streams and the demand for a specifically adjusted pesticide monitoring. The second sub-project was dedicated to planning the concrete implementation of the monitoring by e.g. defining monitoring site requirements and a list of pesticides to be analyzed (Wick et al., 2019).

1.5.2 Implementation

After the imposition of the monitoring to federal states failed, the Helmholtz Centre for Environmental Research (UFZ) in cooperation with the German Environment Agency (UBA) and the University of Koblenz-Landau took over the implementation of the monitoring. While similar studies have been carried out on smaller scale before, the KgM is unique with respect to its extent and effort so far: 124 monitoring sites extending over 12 federal states (see Figure 6), three-month campaigns during the main pesticide application period from April to July in 2018 and 2019, consistent recording of driving environmental stressors (nutrients, heavy metals, hydromorphology, temperature, pH, dissolved oxygen, flow regime, electric conductivity, upstream land use, stream type), grab-, event-driven and passive sampling analysing for a broad spectrum of pesticides via target analysis and suspect screening of water samples for more than 500 pollutants complemented by comprehensive effect monitoring including invertebrate and diatom community sampling, biotests and in-vitro bioassays.

The KgM project should tackle the following questions:

- Is a national monitoring programme that can record all relevant ecological drivers and potential effects feasable?
- Do short-term peak concentrations sampled via EDS significantly exceed pesticide concentration levels recorded in previous governmental monitoring programmes?
- Can measured pesticide exposure be related to effects on the aquatic communities?
- Based on these findings, what conclusions can be drawn for the ERA of pesticides?

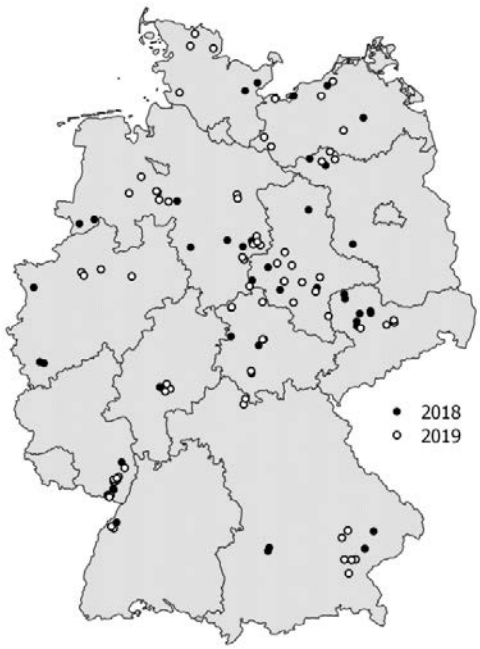

Figure 6: 124 Kleingewässer-Monitoring (KgM) sampling sites distributed over 12 German federal states.

As the entire project concentrated on agricultural pesticide nonpoint source pollution, the selection of sites aimed at excluding any potential upstream point sources (e.g. wastewater treatment plants). In addition, the fraction of urban land cover within the catchment should not exceed 5%, while agricultural land cover should mount up to 40% or more. By including pristine reference sites, a full gradient of agricultural land use intensity was covered allowing to link pollution stress to ecological effects. Three different risk indicators were chosen in advance to quantify pesticide risk. (i) Exceedances of RAC thresholds as a measure of how often and by what factor the ERA's PNECs are exceeded. (ii) Exceedances of EQS thresholds reflecting how often and by what factor WFD monitoring PNECs are exceeded. Besides these chemical monitoring approaches, (iii) $SPEAR_{pesticides}$ was applied to indicate pesticide induced effects on the invertebrate community. The KgM represents a fundamental part of this thesis as all publications involved originate from or partially include the data and results of this project.

1.6 Thesis Objectives

Various scientific studies investigated pesticide pollution as well as related effects in streams and underpinned the vulnerability of these ecosystems during the last 25 years (Gustavsson et al., 2017; Knillmann et al., 2018; Liess and Schulz, 1999; Schäfer et al., 2012; Stehle and Schulz, 2015a; Szöcs et al., 2017). Building on this scientific foundation, the KgM takes this area of research to a new level due to its scope, thus providing an unprecedented opportunity to representatively study the patterns of pesticide risks in small streams and derive general conclusions for the ERA of pesticides. The large selection of monitoring sites in combination with a consistent recording of further environmental and anthropogenic factors allows to representatively determine the status of these ecosystems and to disentangle pesticide stress from the confounding factors. Correspondingly, the overarching aim of this thesis is the investigation of pesticide exposure in small agricultural streams including the assessment of related ecological effects (see concept in Figure 7). On the basis of these findings, conclusions are drawn for the ERA and the monitoring of pesticides in surface waters. This overarching aim was subdivided among the below listed publications, where each publication tackles partial aspects of the research objective that are jointly discussed afterwards (see Discussion).

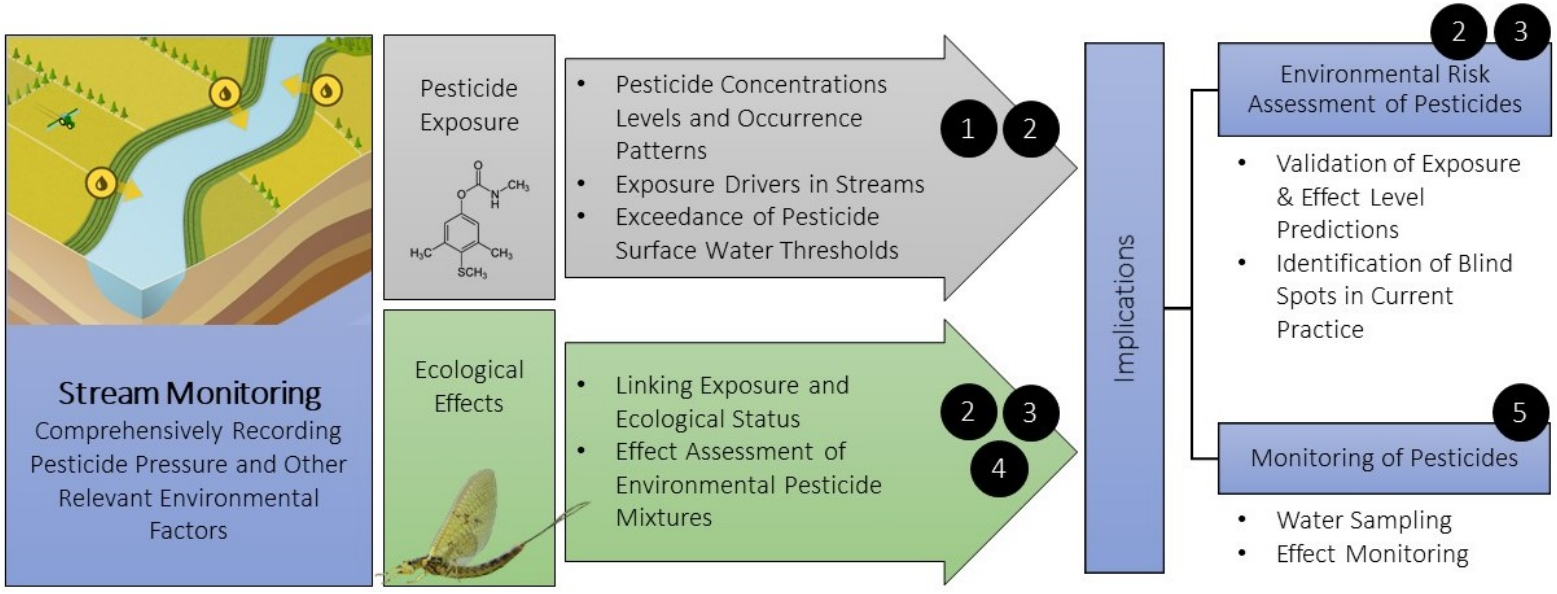

Figure 7: Graphical outline of the concept underlying this thesis. The single research objectives contributing to the overarching aim and their interrelations are displayed. The numbers 1-5 indicate the respective publications addressing a particular research objective (see list of publications below). The publications are provided in chapters 2-6. The main results and implications are jointly discussed further below in chapter 7. Ephemeroptera image modified from Böhringer (2013).

1 Halbach, K.; Möder, M.; Schrader, S.; Liebmann, L.; Schäfer, R. B.; Schneeweiss, A.; Schreiner, V.C.; Vormeier, P.; Weisner, O.; Liess, M.; Reemtsma, T. (2021): Small Streams – Large Concentrations? Pesticide Monitoring in Small Agricultural Streams in Germany during Dry Weather and Rainfall. In: *Water Research.*

This publication focuses on the occurrence patterns of pesticide active ingredients and metabolites in small streams in detail. It analyses the impact of weather conditions, upstream land use and the substances' physico-chemical properties on the measured concentrations. In addition, it uses monitoring data besides the KgM data to compare pesticide exposure in small and large streams.

2 Liess, M.; Liebmann, L.; Vormeier, P.; Weisner, O.; Altenburger, R.; Borchardt, D.; Brack, W.; Chatzinotas, A.; Escher, B.; Foit, K.; Gunold, R.; Henz, S.; Hitzfeld, K.L.; Schmitt-Jansen, M.; Kamjunke, N.; Kaske, O.; Knillmann, S.; Krauss, M.; Küster, E.; Link, M.; Lück, M.; Möder, M.; Müller, A.; Paschke, A.; Schäfer, R.B.; Schneeweiss, A.; Schreiner, V.C.; Schulze, T.; Schüürmann, G.; von Tümpling, W.; Weitere, M.; Wogram, J.; Reemtsma, T. (2021): Pesticides are the dominant stressors for vulnerable insects in lowland streams. In: *Water Research.*

Using statistical models, this publication estimates the relative influence of environmental factors on the streams' invertebrate community composition. Moreover, it compares measured pesticide concentrations with PECs and RACs in order to validate exposure model predictions and to assess the ecological risk. By linking the estimated pesticide pressure in a stream to its ecological status indicated by the invertebrate community, field-based thresholds for invertebrate-toxic pesticides are derived.

3 Weisner, O.; Frische, T.; Liebmann, L.; Reemtsma, T.; Roß-Nickoll, M.; Schäfer, R.B.; Schäffer, A.; Scholz-Starke, B.; Vormeier, P.; Knillmann, S.; Liess, M. (2021): Risk from Pesticide Mixtures – the Gap between Risk Assessment and Reality. In: *Science of the Total Environment.*

Pesticides are commonly applied multiple times per season on a single field, where a single application often comprises multiple PPPs at a time, which also often contain multiple pesticide active ingredients. That is why terrestrial and aquatic non-target organisms are exposed to manifold pesticide mixtures. The single PPP-oriented ERA, however, widely ignores these pesticide mixtures and thus underestimates the actual ecological risk. By jointly analysing a comprehensive dataset on pesticide applications schemes and the measured concentrations during the KgM, this publication aims at quantifying the risk of multiple pesticides co-occurring in the environment for aquatic invertebrates and algae overseen by the single PPP-oriented ERA. The analysis comprises the characterisation of pesticide mixtures in PPP, PPP applications often involving multiple PPP, and stream water samples. The manuscript ultimately addresses the frequency at which organisms are exposed to pesticide pulses in agricultural fields and streams.

4 Neale, P.A.; Braun, G.; Brack, W.; Carmona, E.; Gunold, R.; König, M.; Krauss, M.; Liebmann, L.; Liess, M.; Link, M.; Schäfer, R.B.; Schlichting, R.; Schreiner, V.C.; Schulze, T.; Vormeier, P.; Weisner, O.; Escher, B.I. (2020): Assessing the Mixture Effects in In Vitro Bioassays of Chemicals Occurring in Small Agricultural Streams during Rain Events. In: *Environmental Science & Technology.*

Instead of studying the in-stream invertebrate community or estimating ecological effects on the basis of measured concentrations in combination with toxicity data, this manuscript provides an analysis of the toxic potency of EDS stream water

samples using in-vitro bioassays. The five different bioassays (cytotoxicity, activation of the estrogen, aryl hydrocarbon and peroxisome proliferator-activated receptors and oxidative stress response) allow for assessing a wide range of chemicals and their effects as a mixture also covering pollutants not included in the chemical target or suspect screening analysis.

5 Weisner, O.; Arle, J.; Liebmann, L.; Link, M.; Schäfer, R.B.; Schneeweiss, A.; Schreiner, V.C.; Vormeier, P.; Liess, M. (2021): Three Reasons Why the Water Framework Directive (WFD) Fails to Identify Pesticide Risks. In: *Water Research*.

The KgM monitoring strategy was designed to assess pesticide risks in surface waters realistically and exceeds the WFD monitoring efforts (e.g. complementation of grab sampling by EDS). This publication uses the KgM findings to evaluate the WFD's monitoring and assessment strategy with a focus on pesticides. It compares the pesticide risks identified according to the KgM and WFD-compliant monitoring and assessment and discusses implications on the monitoring of pesticides in surface waters in general.

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2 Small Streams – Large Concentrations? Pesticide Monitoring in Small Agricultural Streams in Germany during Dry Weather and Rainfall

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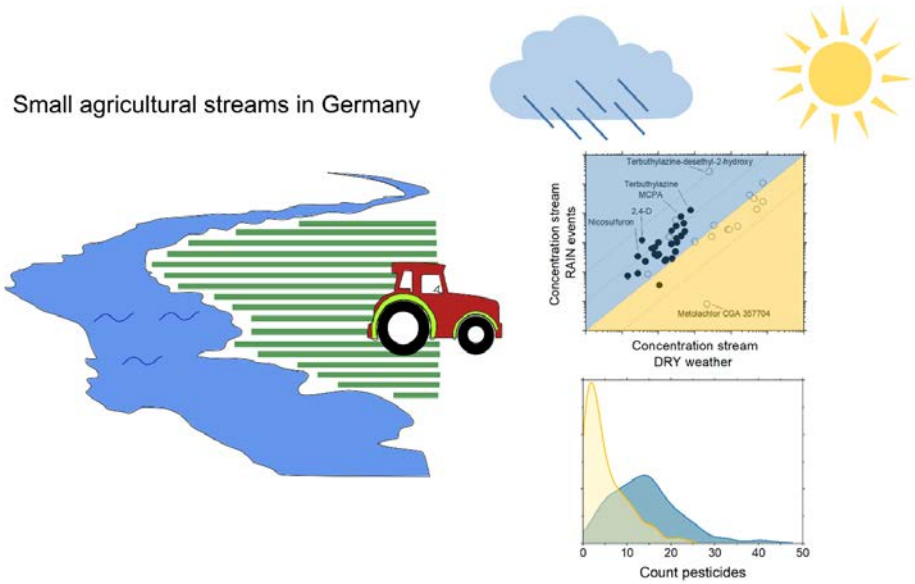
Abstract

Few studies have examined the exposure of small streams (< 30 km^2 catchment size) to agriculturally used pesticides, compared to large rivers. A total of 105 sites in 103 small agricultural streams were investigated for 76 pesticides (insecticides, herbicides, fungicides) and 32 pesticide metabolites in spring and summer over two years (2018 and 2019) during dry weather and rainfall using event-driven sampling. The median total concentration of the 76 pesticides was 0.18 µg/L, with 9 pesticides per sample on average (n = 815). This is significantly higher than monitoring data for larger streams, reflecting the close proximity to agricultural fields and the limited dilution by non-agricultural waters. The frequency of detection of all pesticides correlated with sales quantity and half-lives in water. Terbuthylazine, MCPA, boscalid, and tebuconazole showed the highest median concentrations. The median of the total concentration of the 32 metabolites exceeded the pesticide concentration by more than an order of magnitude. During dry weather, the median total concentration of the 76 pesticides was 0.07 µg/L, with 5 pesticides per sample on average. Rainfall events increased the median total pesticide concentration by a factor of 10 (to 0.7 µg/L), and the average number of pesticides per sample to 14 (with up to 41 in single samples). This increase was particularly strong for 2,4-D, MCPA, terbuthylazine, and nicosulfuron (75 percentile). Metabolite concentrations were generally less responsive to rainfall, except for those of terbuthylazine, flufenacet, metamitron, and prothioconazole. The frequent and widespread exceedance of the regulatory acceptable concentrations (RAC) of the 76 pesticides during both, dry weather and rainfall, suggests that current plant protection product authorization and risk mitigation methods are not sufficient to protect small streams.



2.1 Introduction

Most surface waters are subject to the input of anthropogenic chemicals. These may stem from discharges of treated municipal wastewater, combined sewer overflows, cooling waters, industrial wastewaters, and diffuse sources such as road runoff or agricultural fields (Wittmer et al., 2010). Pesticides applied to agricultural fields for the protection of crops can enter water bodies by surface runoff, subsurface drainage systems, groundwater inflow, and spray drift (Bundschuh et al., 2014; Leu et al., 2010; Liess et al., 1999). Important parameters that influence the extent of pesticide input into surface waters are weather, soil type, pesticide properties, and application method (Gramlich et al., 2018). Pesticides are biologically active compounds, and it is known for long that their input into surface waters can affect aquatic biota from single species to community level and the whole river ecosystem (Beketov et al., 2013; Liess and Ohe, 2005; Stehle and Schulz, 2015). The input of pesticides into surface waters is particularly high during the main application period in spring and summer and has been shown to increase during rain events (Leu et al., 2004; Szöcs et al., 2017).

In the European Union (EU), the first regulation of pesticide concentrations in aquatic compartments dates back to 1980 (The Council of the European Communities, 1979) and was directed to groundwater as an important source of drinking water, with a limit of 0.1 µg/L for any pesticide. With the Water Framework Directive (WFD) of 2000 pesticide concentrations in surface waters were regulated (European Parliament and Council of the European Union, 2020). To date, maximum allowable concentration Environmental Quality Standards (MAC-EQS) are derived for only 14 of the 463 pesticides that are currently approved in the European Union (European Commission, 2020).

According to the WFD, official monitoring programs are in place in the EU to surveil surface water quality. The ten major river basin districts in Germany have to be monitored representatively and sampled at defined intervals independent of weather conditions. Small basins with less than 100 km^2 are sampled less frequently than larger basins (Wick et al., 2019), and small catchments < 10 km^2 are not specifically considered by the WFD. However, small streams are important habitats, and, they comprise the majority of running waters. In Germany, for example, almost 2/3 of the total length of running waters is represented by small streams with catchment sizes < 10 km^2 (approx. 258 000 of 400 000 km) (Bundesamt für Naturschutz, 2004). Small agricultural streams are characterized by immediate proximity to agricultural fields and a low dilution capacity of field runoff by other waters compared to larger rivers further downstream (Szöcs et al., 2017). Such stream sections are denoted as "edge-of-field" surface waters in the EU-EFSA risk assessment of plant protection products for aquatic organisms (European Food Safety Authority, 2013). Small streams have been shown to be specifically exposed to high pesticide concentrations. Spycher et al. reported that risks in five small streams in Switzerland were underestimated by current monitoring strategies with low temporal resolution (Spycher et al., 2018). In Germany, a so-called National Action Plan on Sustainable Use of Plant Protection Products (NAP) was implemented that demands monitoring of the pesticide burden of small streams to better account for their risks (Federal Ministry of Food and Agriculture Germany, 2013).

The protection of the environment is a primary aim of pesticide regulation. For the approval of plant protection products in the EU, exposure models are used to derive predicted environmental concentrations (PEC) in surface waters for a given application. Furthermore, regulatory acceptable concentrations (RACs) of pesticides are derived for surface waters based on available effect data (European Food Safety Authority, 2013) to exclude "unacceptable effects on the environment" (European Parliament, Council of the European Union, 2009). RACs can vary between EU member states and are subject to change if new effect data become available. Eventually, only those applications of plant protection products are approved, for which the PEC remains below the RAC. Consequently, exceedances of RACs should not occur. Pesticide monitoring data can be used to check for compliance with the respective RACs and, thus, serve as reality check for the approaches established in the approval of plant protection products.

Such a reality check needs to consider also ecologically relevant and potentially critical situations and, thus, to include small agricultural streams and rainfall events. To become representative, such monitoring would also have to include a large diversity of settings in terms of catchment morphology, land use, and crops grown in the catchment and to cover diverse weather conditions.

In this study, pesticides were monitored at 105 sites in spring and summer of two years (2018, 2019) by a combination of sampling at regular intervals and event-driven sampling to account for rain events (Liess et al., 2021). This sampling combines high temporal resolution with high spatial coverage in Germany, resulting in > 800 samples processed in the same way to generate highly comparable concentration data for 76 pesticides and 32 metabolites, plus 4 indicator compounds to account for inputs from non-agricultural sources. This large set of monitoring data is interpreted involving information on the catchments (land use, crops growth) and the physico-chemical properties and use characteristics of the pesticides under study.

This work aims at answering the following questions: What are the concentration levels of pesticides and pesticide metabolites in small agricultural streams compared to larger ones during the period of pesticide application? How do rainfall events affect pesticide concentrations in these streams? Which pesticides reacted most sensitively to rainfall events? Can we explain the concentrations of pesticides based on their physico-chemical properties or their use characteristics? Can the concentrations be predicted from the fraction of agricultural land use in a catchment? How do pesticide metabolites compare to their parent compounds? Do pesticide concentrations during dry weather and rainfalls comply with the concentration levels derived during the approval of plant protection products? By answering these questions, this study aims at supporting risk assessment as well as risk management of pesticides, with a focus on small streams.

2.2 Materials and Methods

2.2.1 Sampling

In total, 886 samples were taken in small agricultural streams in spring and summer (April to July) of 2018 and 2019, covering the application period of intensive pesticide use (Wick et al., 2019). Sampling was carried out at 105 sampling sites in 103 streams, selected based on catchment size, high percentage of agricultural land use and expected low urban influence (Wick et al., 2019), distributed over twelve federal states in Germany (Figure S1). The mean catchment size was 17.6 km^2. The sampling strategy comprised regular grab water sampling (every third week), following the approach employed in monitoring according to WFD, and event-driven sampling to cover rain events. Of the regularly taken 551 samples, only 480 samples are included in this study. These were categorized as taken during dry weather (referred to as “DRY” hereafter), because less than 10 mm rainfall was reported on the day of sampling and no rainfall event was noted on this day or the day before. The event-driven sampling was conducted with automated samplers (MAXX TP5, Rangendingen, Germany) triggered by a rise of water level in the respective stream that corresponded to precipitation > 10 mm/day in the respective catchment (“RAIN”, 335

samples). Further details on sampling and site characteristics were provided in a previous publication (Liess et al., 2021).

2.2.2 Sample Preparation

The water samples were filtered with a disposable syringe filter (a combination of glass fiber filter and 0.45 µm regenerated cellulose acetate (Altmann Analytik, Munich, Germany)). One mL of the filtered sample was spiked with five isotope-labeled internal standards of the pesticides with very low RAC values of 0.00077 – 0.01 µg/L (spiking concentrations are provided in Table S2). Three further internal standards were added to check for instrumental performance.

The reference substances at purities of 98 % and higher were delivered from HPC Standards (Borsdorf, Germany) and Chemos (Altdorf, Germany) dissolved in acetonitrile at concentrations of 100 µg mL^{-1}. The dilutions to build the calibration curves were prepared in Milli-Q-water.

2.2.3 LC-MS/MS Analyses

In total, 76 pesticides (40 herbicides, 24 fungicides, and 12 insecticides), 32 pesticide metabolites, and 4 indicator compounds were analyzed in the water samples. The target analytes changed slightly from 2018 to 2019. Amino-bifenox acid, bifenox acid, and sulcotrione were excluded from the monitoring in 2019, whereas chlorantraniliprole (insecticide), hexamethoxymethylmelamine (HMMM, a marker for road runoff), and the metabolite R471811 of chlorothalonil (fungicide metabolite) were included.

The analyses were carried out with an LC-MS/MS system involving an Agilent 1290 infinity liquid chromatography system coupled to a QTrap6500+ tandem mass spectrometer equipped with an electrospray ionization (ESI) interface (Sciex) by direct injection of the aqueous samples and multiple-reaction-monitoring. Quantification was performed by external calibration in ultrapure water, except for the five analytes for which labelled internal standards were added (see above). Details on the analytical method, linear calibration range, and validation data are provided in the supplement information (Table S1 to S3).

2.2.4 Data Analysis

For data processing, the MultiQuant™ software version 3.0 (Sciex, Darmstadt, Germany) was used. While most of the pesticides were quantified with external calibration curves (concentration levels between 0.005 µg L^{-1} to 0.75 µg L^{-1}), those marked by low RACs (clothianidin, imidacloprid, thiacloprid, methiocarb, and fipronil) were quantified using the isotope-labeled internal standards listed in Table S2. The linear calibration range between the limit of detection (LOD) and 0.75 µg L^{-1} allowed the quantification of the DRY samples. If pollutant concentrations exceeded the linear calibration range, e.g., in RAIN samples, the quantification was carried out after repeated analysis injecting a smaller sample volume of 10 µL instead of 80 µL. All concentration data will be available under https://doi.org/10.1594/PANGAEA.931673 from the 30.09.2022 onwards.

Further data analysis was carried out with OriginPro 9.7.0.185 (OriginLab). The frequency of detection (FOD) was calculated based on values larger than the limit of quantification (LOQ, FOD_{LOQ}) as well as the limit of detection (LOD, FOD_{LOD}). For calculations of concentration ranges, values below the LOQ were also considered (specified where applicable). Quantiles were interpolated based on the method "empirical distribution with averaging" in Origin. K-means (k=5) clustering was conducted to group the pesticides and their metabolites based on the physico-chemical properties charge and logD_{ow} (pH=7.4, chemicalize (ChemAxon)), see results in Table S9 and Figure S8.

To test the explanatory power of different variables on the FOD_{LOQ} values, we conducted a correlation analysis (Spearman's rank correlation analysis) with the sales quantities per pesticide (Federal Office of Consumer Protection and Food Safety (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, 2020)), half-life in water and soil (retrieved from the Pesticide Properties DataBase (University of Hertfordshire, 2020)), and the polarity (logD_{ow}) values (chemicalize (ChemAxon)). A correlation was considered significant at $p < 0.05$. To further explore the relationship between pesticide concentrations and their physico-chemical properties

and use characteristics, a multiple linear regression was performed followed by an ANOVA in OriginPro 9.7.0.185 (OriginLab).

Land use within the hydrological catchments of the studied streams was derived using the CORINE land cover data (Copernicus). All land use subtypes of the classes agriculture, forest, urban, and grassland were aggregated at class level, and the respective area share was calculated. Land use and cultivated crops were mapped in-situ within a buffer zone reaching 3 km upstream and 500 m to each streamside. The four main land use classes were not further differentiated and no pesticide application data for the grassland and forests were available. Thus, only the agriculture and urban land use are further discussed in this manuscript. Cultivated crops are displayed as the percentage of the agricultural area (Figure S9).
The land use data were tested for correlation (Spearman's correlation) with the measured concentration per site. For sites with different land use in 2018 and 2019, the years were considered separately, resulting in a total number of 119 observations. The median and mean values of the measured concentrations per compound at one site were calculated from all samples taken (DRY and RAIN, n = 815), and the correlation was tested for both values (Table S10). The five pesticides with the highest correlation coefficient are shown in Table S10a with the urban land use plus the marker substances and in Table S10b with the six most representative (area-wise) crops (wheat, corn, rape, barley, viticulture, sugar beet). Information on typically applied pesticides per crop for 2018 and 2019 was provided by the Julius-Kühn Institute (Julius-Kühn Institute).

2.3 Results and Discussion

2.3.1 Frequency of Detection and Concentration Ranges of the Monitored Pesticides

A total of 815 samples from 105 sampling sites in 103 small agricultural streams taken in spring and summer of 2018 and 2019 are considered in this study (480 samples denoted as "DRY"; 335 samples denoted as "RAIN") (Figure S1). They were analyzed for 76 pesticides (40 herbicides, 24 fungicides, and 12 insecticides) and 32 pesticide metabolites. Pesticide

selection was based on previous monitoring data, use, and ecotoxicity (Wick et al., 2019).

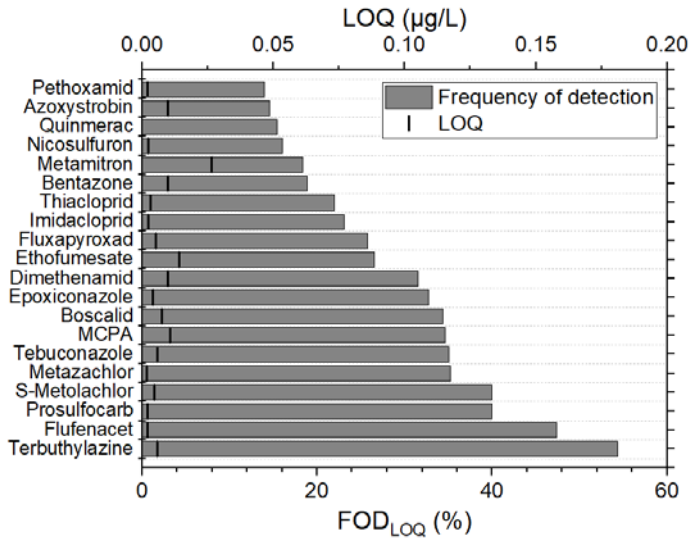


Figure 8: Frequency of detection (FOD_{LOQ}) of pesticides in all samples (n = 815) at agricultural sites for the 20 parent substances with the highest FOD_{LOQ} (grey bars). Straight bold lines represent the LOQ value (average value of LOQ 2018 and 2019). For full all data, please refer to Table S4.

In this set of > 50,000 concentration data, terbuthylazine, flufenacet, prosulfocarb, and S-metolachlor showed the highest frequency of detection (FOD_{LOQ}) of > 40% (Figure 8, complete list in Table S4). Considering all values above the LOD, these compounds were detected in more than 64% of all samples (Table S4). FODs of all pesticides correlated with their sales quantity for Germany (r_s = 0.51, $p < 0.001$) and, less pronounced, with their half-lives in water (r_s = 0.28, $p < 0.05$) (Figure S2) but not with their half-lives in soil, their water solubility or polarity expressed as logD_{ow}. The latter finding may point to the fact that pesticides can be exported from the agricultural field in dissolved form as well as particle-bound during runoff events. Their application amounts and persistence, then, remain as the decisive factors for the occurrence in agricultural streams. A multiple linear regression (Figure S3) also confirmed the significance of the sales quantity and the half-lives in water, explaining 32% of the variance. This agrees to a previous study for one defined

catchment that also found the applied amount as the major determinant of occurrence in adjacent streams, although for a lower number of pesticides (Kreuger and Törnqvist, 1998). The FOD_{LOQ} in this study with 815 samples were comparable or higher than those reported in an earlier study in Germany, based on samples taken between 2005 and 2015 as part of the regular monitoring according to the WFD (Szöcs et al., 2017). Higher FODs are presumably partially due to lower LOQs in this study compared to regular monitoring. Much higher FODs were found for picoxystrobin (factor 45), prosulfocarb, and bromoxynil (factor 16 and 13). Lower FODs were recorded for dimethachlor and isoproturon (factor 11 and 5 lower FODs than Szöcs et al., respectively, Figure S4). The latter may be explained by the fact that the approval of isoproturon ended in 2016 in the EU and that the sales quantity of dimethachlor declined by a factor of 6 between this earlier study and the years 2018 and 2019 (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, 2020).

The pesticides detected most frequently were also often those determined with the highest median concentration (Figure 9). Terbuthylazine was detected in concentrations ranging up to 0.56 µg/L (95 percentile, median = 0.0056 µg/L, Figure 9). Other predominant pesticides in this study were MCPA (95 percentile = 0.38 µg/L, median = 0.0035 µg/L), boscalid, tebuconazole, S-metolachlor, dimethenamid, flufenacet, and fluxapyroxad (95 percentile = 0.041 µg/L, median = 0.0013 µg/L). Also, for the concentrations determined in the streams, a significant correlation with sales quantity ($r_s = 0.46$, $p < 0.001$) and half-lives in water ($r_s = 0.26$, $p < 0.001$) was found (Figure S6).

For 45 pesticides, concentrations measured here could be compared with governmental monitoring data for the years 2018 and 2019 of two Federal States in Germany; these data have a higher share of larger streams (Figure 10). Pesticide concentrations of this study were significantly higher (Kolmogorov-Smirnov test, $p < 0.05$, p-values in Table S5) for 28 (federal state A) and 25 (federal state B) of the 45 pesticides. The higher concentration may be attributed to i) the small stream sizes of this study, ii) the high number of RAIN samples or iii) the collection of samples only

during the application period of most pesticides. However, within the 105 sites of this study, a decrease of pesticide concentrations with increasing catchment size was not visible (r_s = 0.082, p = 0.40 for the mean and r_s = 0.051, p = 0.61 for the median). This may be due to the limited span of catchment sizes (from 9 km^2 to 19 km^2 for the 25 – 75 percentile). Higher concentrations in smaller streams have been reported previously, for example in a study on 42 Danish streams in three size classes with > 1000 samples analyzed. This increase was particularly pronounced for the peak concentrations (95 percentiles) (Lorenz et al., 2017).

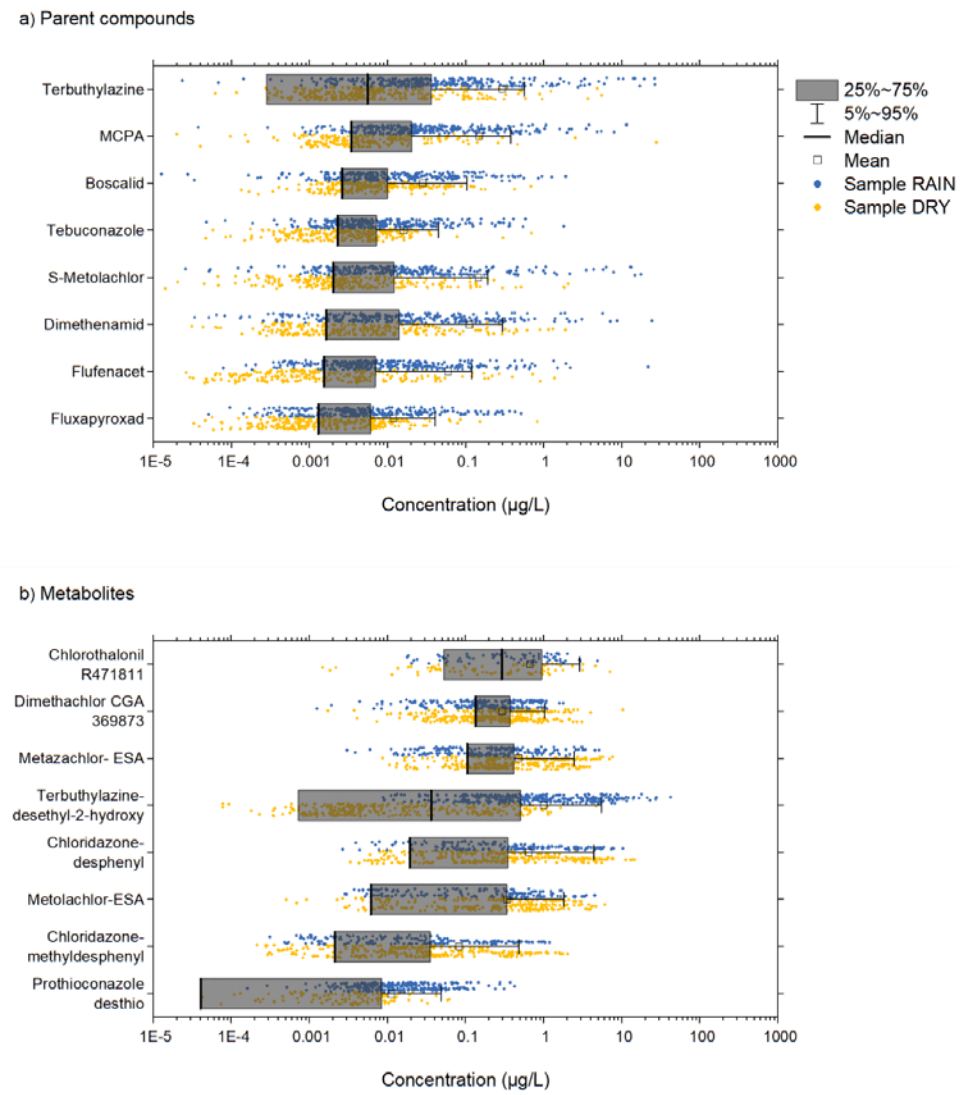


Figure 9: Boxplots and underlying data of the eight compounds with the highest median values in the whole sample set (n = 815) for a) the parent substances and b) the metabolites. Colors indicate the weather conditions at sampling: yellow (DRY) and blue (RAIN). Due to the logarithmic scale, only data > 0 are displayed. Values between LOD and LOQ were considered.

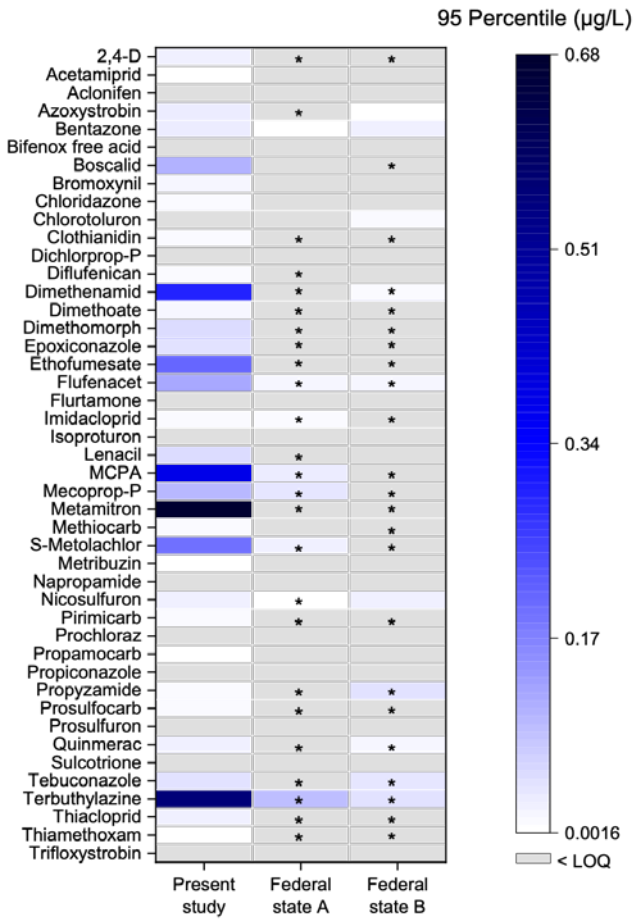


Figure 10: Comparison of the 95 percentile concentrations for selected pesticides of the present study (n = 815) and samples taken in two Federal states in Germany in 2018 and 2019 as part of the monitoring for the WFD (range of n = 98-1,680 for Federal State A and n = 241-516 for Federal State B). Displayed are the pesticides that were measured in the present study and monitored in both Federal States. Limits of quantification (LOQ) did not differ by more than a factor of 10 between this study and the two states. Data only above the LOQ were available from one Federal state, thus for percentile calculation values below the LOQ in all three datasets were set to zero. * indicates a significantly different distribution of the measured concentration data from the present study ($p < 0.05$, Kolmogorov-Smirnov test, see for exact p-values Table S5).

Taken together, the FOD data (Figure 8) and the concentration data (Figure 10) for the 76 pesticides at the 105 sites confirm the previous notion that pesticide concentrations in small agricultural streams of catchment sizes of 10 km^2 and below are not well reflected in the official monitoring program

(Leu et al., 2004; Szöcs et al., 2017). It should be noted in this context that the small streams make up almost 2/3 of the running water in Germany (Bundesamt für Naturschutz, 2004).

The formation of metabolites from parent pesticides is inevitable for non-persistent pesticides (Fenner et al., 2013). These metabolites are often more polar as well as mobile and can also be more persistent (Gassmann et al., 2013). Monitoring of pesticide metabolites supports the understanding of pesticide fate in agricultural systems. The median of the total concentration of the 32 metabolites included in this study was 2.4 µg/L and, thus, exceeded the total pesticide concentration by a factor of 13. Given the comparatively low number of metabolites, the concentration of all metabolites must be even higher. The most frequently detected metabolites in this study originate from chlorothalonil (FOD_{LOQ} 94%), terbuthylazine, chloridazon, and dimethachlor (FOD_{LOQ} > 40%; Figure S5). The chlorothalonil metabolite R471811 was only recently reported as the predominant pesticide metabolite in groundwater and surface water samples from Switzerland (Kiefer et al., 2020). Two other studies reported chloridazon-desphenyl in between 43% and 77% of surface water samples in Germany and with mean concentrations comparable to this study (Buttiglieri et al., 2009; Szöcs et al., 2017). Dimethachlor CGA 369873 was previously described as an emerging metabolite in German ground and surface waters in 2013, but at a much lower concentration (median 0.02 µg/L for groundwater and surface water vs. 0.14 µg/L in the present study) (Reemtsma et al., 2013).

The mean concentrations of some metabolites were two to three orders of magnitude higher than their parent compounds (factor 89 – 530) for metazachlor-ESA, chloridazon-desphenyl, and dimethachlor CGA 369873 (Table S6). In single samples, the concentration of metazachlor-ESA, metolachlor-ESA, terbuthylazine-desethyl-2-hydroxy, and chloridazon-desphenyl even exceeded their parent compounds by a factor > 1000 (Table S6). Chloridazon-desphenyl was previously detected in higher concentrations than chloridazon in the Hesse region, Germany, in 2007 (Buttiglieri et al., 2009).

The so-called 'relevant metabolites' are of the highest regulatory concern, as these still cause pesticidal, toxic, or ecotoxicological effects (European Commission, 2003). Among the monitored metabolites, the two metazachlor metabolites BH 479-11 and BH 479-9 (6% and 0.1% FOD_{LOQ}) and terbuthylazine-desethyl-2-hydroxy (40% FOD_{LOQ}) are classified as 'relevant metabolites' (Banning et al., 2019; LAWA, 2019). Furthermore, the European Food Safety Authority (EFSA) recently recommended considering all metabolites of chlorothalonil as 'relevant metabolites' (Kiefer et al., 2020). Chlorothalonil R471811 (median = 0.29 µg/L) was also classified as relevant in Switzerland (Bundesamt für Landwirtschaft, 2020). Especially, the high FODs of the relevant metabolites of chlorothalonil and terbuthylazine raise concern.

It should be noted, however, that due to their mobility and persistence, also "non-relevant" metabolites in surface water can affect water quality and the downstream use of surface water, e.g., as a resource for drinking water via bank filtration.

2.3.2 Effects of Rainfall on the Frequency of Detection and Concentrations

Previous studies, more limited in pesticide number or spatial extent than this study, have shown that pesticide concentrations in streams can be strongly elevated during rainfall compared to those found during dry weather due to inputs by surface runoff, macropore flow, or subsurface drainage (Chow et al., 2020; Liess et al., 1999). The large number of sites, samples, and pesticides of this study allows evaluating the effects of rain events on pesticide export in more detail.

The total concentration of pesticides in surface waters drastically increased during rainfall events (Figure 11a): the median increased by one order of magnitude, from 0.072 µg/L to 0.70 µg/L from DRY to RAIN, and the 95 percentile from 1.7 to 24 µg/L. Correspondingly, also the number of pesticides per sample drastically increased: the maximum of the frequency distribution shifted from two pesticides per sample in the DRY sample set to 14 pesticides per sample in the RAIN sample set (Figure 11c), and the

mean number of quantified pesticides (concentration > LOQ) increased from 5.2 pesticides per sample to 14 in RAIN samples. For the detected pesticides (concentration > LOD) the number increased from 16 in the DRY to 31 in the RAIN sample set. In single RAIN samples, more than 40 different pesticides occurred above their LOQ.

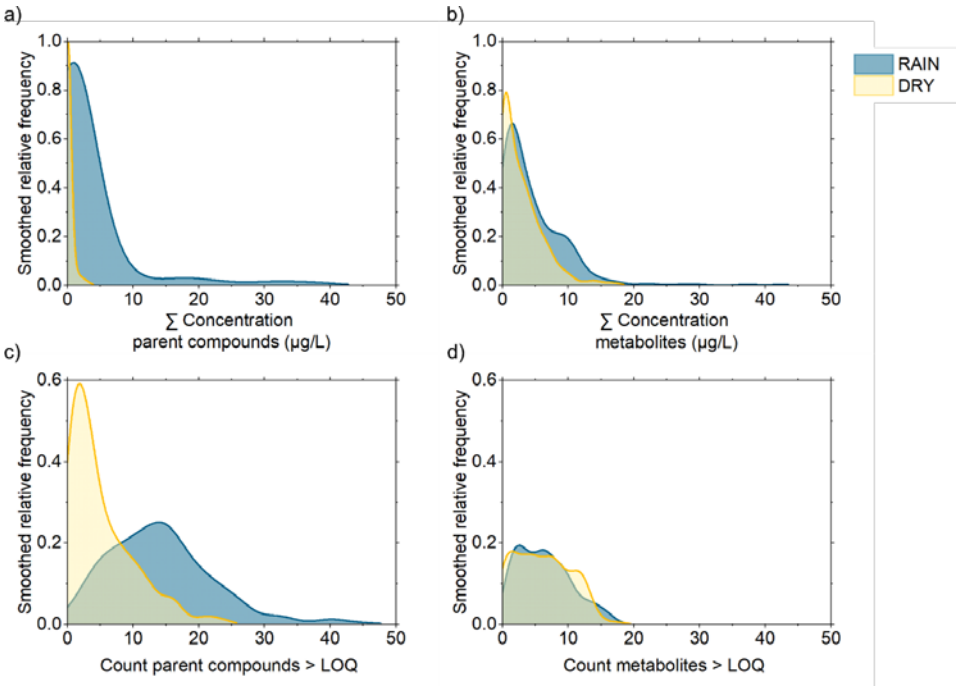


Figure 11: Smoothed (kernel) relative frequency for DRY (yellow, n = 480) and RAIN (blue, n = 335) samples of a) summed concentration of the 76 parent compounds per sample (5 samples with a summed concentration up to 96 µg/L are out of scale), b) summed concentration of the 32 metabolites per sample, and c) the total number of parent compounds and d) of metabolites per sample detected above the limit of quantification (LOQ).

Correspondingly, the mean FOD_{LOQ} for the 76 parent pesticides tripled from 6.9% in the DRY samples to 18.6% in the RAIN samples. Thus, rainfall events lead to a drastic increase in both pesticide concentration and pesticide number in the small agricultural streams. This matches with a study on 10 Danish streams, in which an increase of the total pesticide concentration from 0.19 µg/L at base flow to 1.8 µg/L at storm flow was recorded (Rasmussen et al., 2015).

For a quantitative description of pesticide dynamics, it would be useful to calculate rainfall-dependent pesticide fluxes (Wittmer et al., 2010). However, the continuous recording of water fluxes in the streams, as necessary for that purpose, was not feasible at the 105 sites under study. As this study was directed to studying the effects of pesticide export on stream water quality, concentrations are more important than fluxes.

The more frequent detection of higher concentrations of pesticides during rainfall events is already seen in Figure 9 (RAIN, blue data points). However, this trend is not equally strong for all pesticides, as visible by comparing the 75 and 90 percentiles of the RAIN and DRY samples for the 76 pesticides (Figure 12 and S5). As data for 105 sampling sites are included in this comparison, these values were expected to be largely independent of site characteristics and mainly relate to physico-chemical properties or use characteristics of the pesticides.

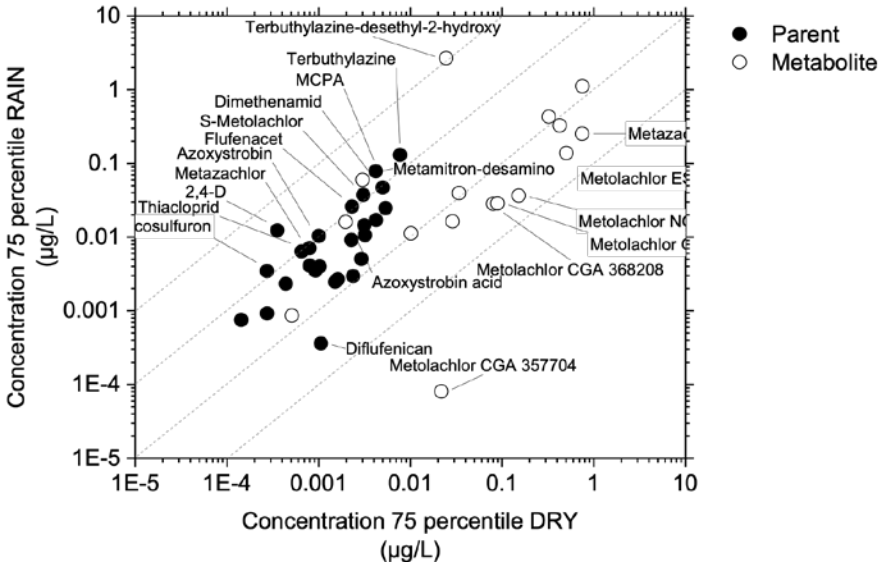


Figure 12: Comparison of the 75 percentile concentrations of pesticides and metabolites in RAIN samples (n = 335) versus the DRY samples (n = 480).

The pesticides 2,4-D, terbuthylazine, flufenacet, metamitron, trifloxystrobin, MCPA, ethofumesate, pirimicarb, nicosulfuron,

methiocarb, fluroxypyr, S-metolachlor, mecoprop-P, dimethenamid, isoproturon, thiacloprid, and azoxystrobin exhibited strongly elevated concentrations during rainfall (> one order of magnitude) for both, 75 and 90 percentiles compared to dry weather (Figure 12, S7 and Table S7). These are mainly substances of medium polarity (average logD_{ow} 2.2) and either neutral or with a single negative charge (Tables S8 and S9, Figure S8). The same is true, however, for the compounds that do not show a clear increase during rain events (Figure 12), like bentazone, clothianidin, and diflufenican (Table S7).

Contrary to the parent pesticides, the summed concentrations of metabolites and the number of detected metabolites per sample were hardly affected by rainfalls (Figure 11b and d). The formation of metabolites by (bio-) transformation takes time, so that their occurrence in surface waters is less directly linked to the application period of their parent substances. Correspondingly, the concentrations of the metabolites have been shown to be less influenced by rainfall events and to be largely exported via subsurface as previously shown for the metabolites of dimethenamid, atrazine, and metolachlor in a headwater catchment (Gassmann et al., 2013).

Nevertheless, marked differences between RAIN and DRY samples were also visible for individual metabolites (Figure 12, S5, and Table S7). For example, the metabolites terbuthylazine-desethyl-2-hydroxy, flufenacet thiadone, metamitron-desamino, and prothioconazole-desthio exhibited one to two orders of magnitude elevated concentrations during rainfall.

Conversely, the metabolites metolachlor CGA 357704 and metazachlor BH 479-12 were much less concentrated (one order of magnitude) in surface waters during rain events (Figure 12). These two metabolites of metolachlor and metazachlor are no primary metabolites but are formed only in later stages of the degradation processes (Reemtsma et al., 2013). It is reasonable to assume that their formation requires longer periods of time, which are not available when surface runoff occurs shortly after pesticide application, but when pesticides and their metabolites infiltrate

into the soil. These metabolites may, therefore, reach surface water by groundwater exfiltration (Kern et al., 2011). During rainfall, the increased surface runoff dilutes the fraction of groundwater in the surface water and, therefore, the concentration of these metabolites may decrease. The very high polarity of the two metabolites (logD_{ow} -4 to -5 and a negative charge of -2) supports the notion that they may be transported via groundwater, as sorption to organic as well as inorganic soil constituents should be negligible for such compounds.

The effects of rainfall events on the concentrations of pesticides and their metabolites in small agricultural streams are illustrated for selected compounds at selected sites in Figure 13. The concentrations of flufenacet, metamitron, MCPA, 2,4D, terbuthylazine, and metazachlor strongly increased during rainfall after application. The magnitude of this increase is clearly larger than for their metabolites (except terbuthylazine-desethyl-2-hydroxy). The terbuthylazine metabolite largely exceeded the concentration of its parent compound (1-3 orders of magnitude; Figure 13d). However, also the metabolites flufenacet thiadone and metamitron-desamino showed increased concentrations during rainfall events (Figure 13a, b). In contrast, the concentrations of the metabolites of S-metolachlor were not increased (Figure 13f). These concentration profiles highlight the strong fluctuation in concentrations evoked by rainfall events in small agricultural streams.

Site-specific RAIN/DRY ratios for the sum of all pesticides may be used to elaborate on catchment characteristics that support pesticide export into surface water, such as slope, the distance between fields and the water body, or the presence of subsurface drainage systems. In addition, the consideration of site-specific concentration ratios of certain metabolite/parent pairs may also provide information on the preferred transport pathway at that site. Such an extended data analysis may help identify characteristics of catchments that are critical for pesticide export and, in this way, point to options on how to reduce this export into surface waters.

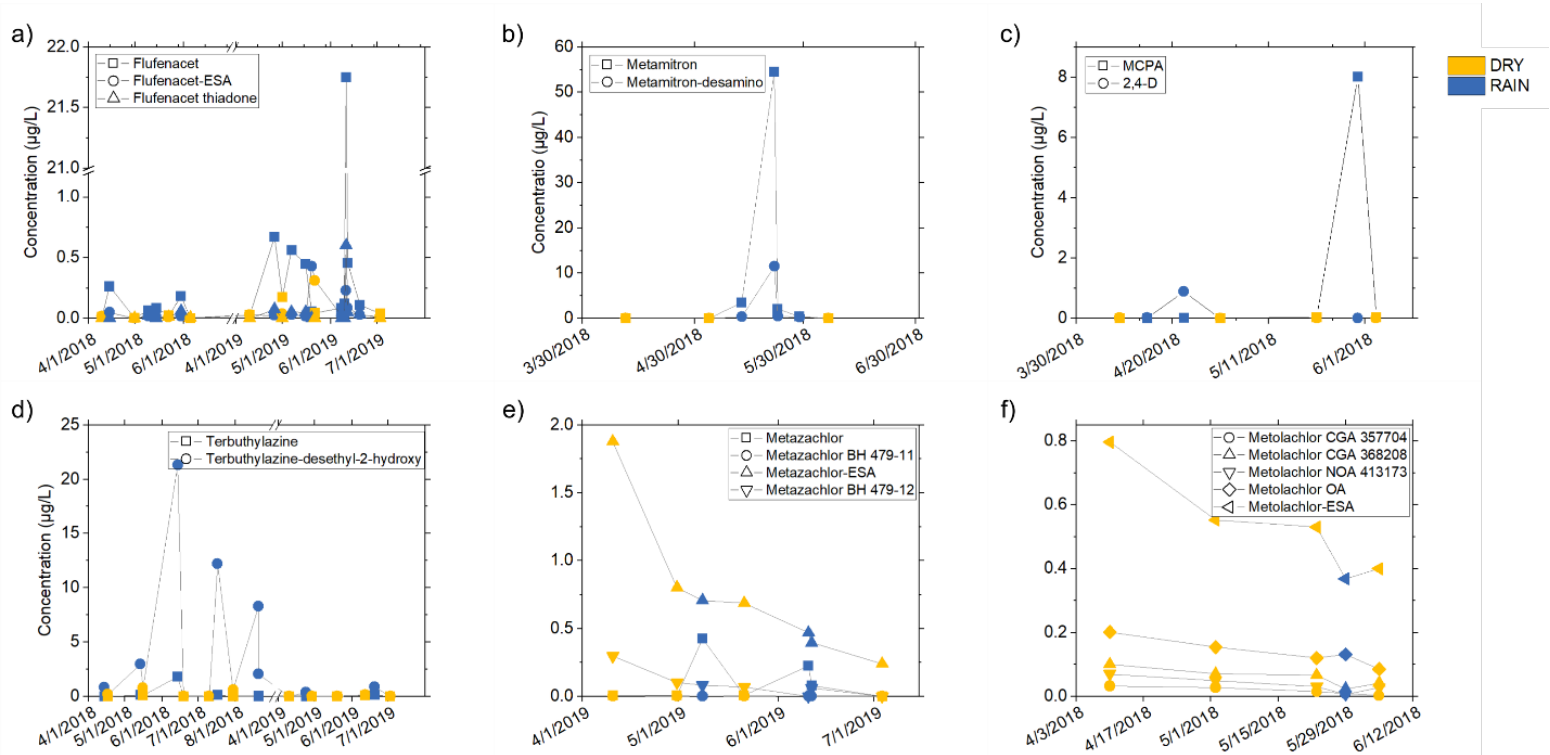


Figure 13: Concentration-time profiles for six groups of pesticides/metabolites at selected sites: a) flufenacet and its metabolites ESA and thiadone, b) metamitron and metamitron-desamino, c) MCPA and 2,4-D, d) terbuthylazine and terbuthylazine-desethyl-2-hydroxy, e) metazachlor and three metabolites BH 479-11, ESA, and BH 479-12 and f) the five metabolites of S-metolachlor CGA 357704, 368208, 413173, OA and ESA. Colors indicate the weather classification of the water samples as “DRY” (yellow) and “RAIN” (blue).

2.3.3 Influence of Land Use on Pesticide Concentrations

The land use in the 105 catchments under study was categorized into four main groups: agriculture, forest, urban, grassland (Figure S9), and the agricultural area, then further divided into crop groups. The six most representative crops (largest grown area) of agricultural land use were wheat, corn, rape, barley, vineyard, and sugar beet (Figure S9).

Many pesticides showed a significant Spearman's correlation ($p < 0.05$, Table S10) with the main crop types grown in the respective catchment: e.g., the concentrations of the herbicides ethofumesate, quinmerac, and the fungicide epoxiconazole moderately correlated with the percentage of wheat in the respective catchment (r_s = 0.54 – 0.46, 102 sites), the herbicide S-metolachlor, terbuthylazine, and nicosulfuron with corn (r_s = 0.37 – 0.28, 91 sites). The herbicides propyzamide, diflufenican, and the fungicide dimoxystrobin correlated moderately positive with areas where rape (r_s = 0.47 – 0.38; 90 sites) and the herbicides diflufenican and flurtamone where barley (r_s = 0.47 – 0.29, 85 sites) was grown. For the vineyard areas, a correlation with the fungicides metrafenone, boscalid, and dimethomorph was found (r_s = 0.79 – 0.51; 20 sites), the herbicides metamitron, ethofumesate, lenacil, quinmerac, and chloridazon correlated with the area where sugar beet was grown (r_s = 0.60 – 0.42, 47 sites). Many of these pesticides were also listed for the respective crop by the so-called "PAPA-survey" in Germany, which collects pesticide application data from selected agricultural farms for 2018 and 2019 (Julius-Kühn Institute). These results outline the strong link between the agricultural activity in a catchment and the occurrence and concentration of pesticides in the respective stream. Hence, agricultural practice is the key to reduce pesticide concentrations in small streams. Also, the practice in the past years may affect present pesticide loads in streams (Rasmussen et al., 2015).

A few pesticides could be linked to urban activities. To account for input from sources other than agriculture, a few indicator compounds were also monitored, such as the pharmaceutical diclofenac and the corrosion

inhibitor benzotriazole for municipal wastewater discharges and hexamethoxymethylmelamine (HMMM) for road runoff (Alhelou et al., 2019; Reemtsma et al., 2010; Seitz and Winzenbacher, 2017); (Table S10). HMMM (r_s = 0.56 – 0.61) and the phenoxyacid herbicides mecoprop P (r_s = 0.42 – 0.49) and, although weaker, 2,4-D (r_s = 0.27 – 0.35) showed a significant correlation with the percentage of the urban area but not with the agricultural area. These are pesticides that are especially used as a weed killer in urban areas and are also described as an indicator for urban runoff waters (Jekel et al., 2015; Raina et al., 2011). Benzotriazole and diclofenac also correlated with urban land use (r_s = 0.24 – 0.38) but weaker than HMMM.

2.3.4 Frequency of RAC Exceedances

The RAC value represents the environmental concentration below which no unacceptable effects on the environment are expected in regulation. The approval of plant protections aims at avoiding RAC exceedances by requiring the farmers to implement certain risk management measures such as keeping distances to water bodies. However, a companion paper analyzing the monitoring data of this study shows that RAC-exceedances are frequently occurring in small agricultural streams especially during rainfall and outlined the consequences for aquatic invertebrate communities (Liess et al., 2021).

A comparison of RAC exceedances separately for the DRY and RAIN samples is performed here to assess the relevance of rain events (Table 1). For the set of 480 samples taken during dry weather conditions, RACs were exceeded 143 times in 23% of the samples and at 50% of the sites. The situation became significantly worse during rainfall events (335 samples): then, a total of 448 RAC-exceedances were recorded (on average 1.3 times per sample) in 60% of the samples (Figure S10) and at 73% of the 105 sites. In other words, only 27% of the sampling sites at small agricultural streams were left without a RAC-exceedance during rainfall during the sampling period (April-July) in two years.

These data show that RAC exceedances in small streams occur widely and frequently. Although rain events are especially critical, RAC exceedances also occur frequently during dry weather: in that phase, only 50% of the sites covered in this study did not show a RAC exceedance. Overall, this suggests that streams of small size are especially susceptible to RAC-exceedances, amplified by rain events.

The almost systematic exceedance of RACs questions the approval process of plant protection products that aims at preventing such exceedances. Three factors may explain the discrepancy between the regulatory aim and agricultural reality: a) some assumptions underlying the models for predicting environmental concentrations are too optimistic so that PEC modeling systematically underestimated the real environmental concentrations (Bach et al., 2017; Knäbel et al., 2012), b) the risk management measures that should be taken in agricultural practice are either not taken or have less benefit than expected, with the consequence that concentrations in agricultural streams exceed the PECs on a broad scale, and c) the application of a plant protection product that was approved several years ago for a certain culture fails to comply with more recently derived (lower) RACs of the respective pesticide (Liess et al., 2021).

The RAC-exceedances encountered in this study also outline the importance of an adequate post-approval monitoring. This would inform to which extent the real-world situation agrees to the predictions made in the approval or plant protection products.

The insecticides thiacloprid, clothianidin, and fipronil were the three compounds with the most frequent RAC exceedances during dry weather as well as during rainfall events (Table 1). The RACs for thiacloprid, clothianidin, and fipronil are in the low ng/L or even pg/L range (0.007 – 0.00077 µg/L) as these insecticides are also highly toxic to aquatic insects. It appears generally challenging to comply with such low RACs. The EU has reacted with a ban on clothianidin and thiacloprid field applications (approval expired in 2019 and 2020). Therefore, a decline in the concentrations of these two compounds in surface waters may be

observed in the future. The approval of the insecticide fipronil as seed treatment expired in 2017 already. Its frequent RAC exceedances observed in 2018 and 2019 may be due to the stock of fipronil in the agricultural soils remaining from its previous application (previously recommended amount of 10 kg/ha in potato). The importance of legacy pesticides for current streamwater quality has been outlined earlier (Rasmussen et al., 2015). Alternatively, the ongoing use of fipronil as a biocide and as a veterinary product may explain these findings; this option is corroborated by the correlation of fipronil concentrations with the percentage of urban land use in this study (r_S = 0.39; Table S10). The use of fipronil as veterinary flea products was recently suggested to cause elevated concentrations in rivers in England (Perkins et al., 2021).

Furthermore, the carbamate pesticide methiocarb and the neonicotinoids imidacloprid and thiamethoxam (approval expired in the EU in 2019 and 2020), the herbicides lenacil, terbuthylazine, metolachlor, and nicosulfuron, also exceeded their RAC-value in up to 5% of the samples taken during rain events (Table 1). Frequent exceedance of the RACs by neonicotinoids has been recognized earlier (Casado et al., 2019; Szöcs et al., 2017)

Beyond the 76 pesticides selected for this study, another 387 pesticides are approved in the EU (European Commission, 2020); furthermore, not all crop cultures could be covered representatively by the 815 samples of this study. Therefore, further pesticides than those listed in Table 1 and Tables S11 may lead to RAC exceedances.

It may seem obvious to reduce the pesticide burden of agricultural streams by reducing the application of those pesticides with frequent RAC exceedances (Table 1) and recommending using pesticides with a similar application domain but a lower number of exceedances. However, such a strategy may eventually lead to an increasing frequency of RAC exceedance for the substitute with overall little if any positive effect on the pesticide burden of agricultural streams (Boyd, 2018). Consequently, more holistic

approaches have been proposed to reduce the environmental burden of pesticide application (Topping et al., 2020).

2.4 Conclusions

- The total median pesticide concentration at 105 sites in small agricultural streams (median catchment size 13 km^2) in Germany in spring and summer 2018 and 2019 was 0.18 µg/L. This concentration was considerably higher than recorded during governmental monitoring, according to the WFD.
- The local agricultural use was linked to the pesticide concentration in the streams.
- Current official monitoring strategies in Germany underestimate the input of pesticides into small streams.
- Across all sites, the FOD_{LOQ} was highest for terbuthylazine, flufenacet, prosulfocarb, S-metolachlor, and metazachlor; for the 76 pesticides of the study, FODs correlated with their sales quantity and aqueous half-lives.
- Rainfall induced a strong increase of pesticide concentration by a factor of 10 in the small streams compared to dry weather to a median total concentration of 0.7 µg/L. Also, the average number of quantified pesticides increased to 14 per sample. Concentration increase with rainfall was strongest for 2,4-D (factor 35), MCPA, and terbuthylazine (factor 17).
- Pesticide metabolites occurred in much higher concentrations at dry weather than their parent compound (total median 2.0 vs. 0.07 µg/L) but were, in general, less affected by rain events.
- RAC exceedances in small agricultural streams are frequent and widespread. They are very high during rainfall events but do also occur frequently during dry weather at 50% of the sites. This outlines that the present approval of plant protection products fails to ensure compliance of pesticide concentrations in small agricultural streams.

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3 Pesticides Are the Dominant Stressors for Vulnerable Insects in Lowland Streams

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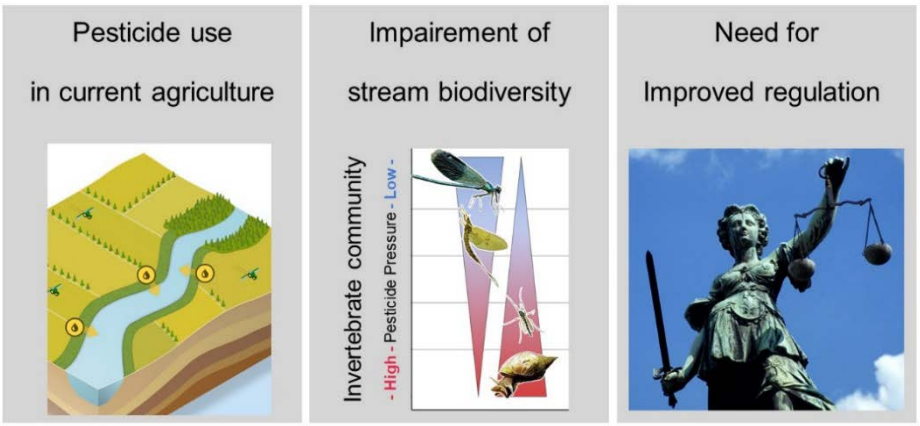
Abstract

Despite elaborate regulation of agricultural pesticides, their occurrence in non-target areas has been linked to adverse ecological effects on insects in several field investigations. Their quantitative role in contributing to the biodiversity crisis is, however, still not known. In a large-scale study across 101 sites of small lowland streams in Central Europe we revealed that 83% of agricultural streams did not meet the pesticide-related ecological targets. For the first time we identified that agricultural nonpoint-source pesticide pollution was the major driver in reducing vulnerable insects in aquatic invertebrate communities, exceeding the relevance of other anthropogenic stressors such as poor hydro-morphological structure. We revealed that the current authorisation of pesticides, which aims to prevent adverse effects, underestimates the actual ecological risk as (i) measured pesticide concentrations exceeded current regulatory threshold levels in 81% of the agricultural streams investigated, (ii) for several pesticides the inertia of the authorisation process impedes the incorporation of new scientific knowledge and (iii) existing thresholds of invertebrate toxicity drivers are not protective by a factor of 5.3 to 40. To provide more reliable thresholds, the authorization process needs to include monitoring-derived information on pesticide effects at the ecosystem level. Here, we derive thresholds that ensure a protection of the invertebrate stream community.



3.1 Introduction

The ongoing biodiversity crisis is caused by a variety of anthropogenic stressors including pesticides (Agency, 2015). However, great uncertainty remains about the respective contribution of various stressors to ecosystem degradation. This debate also relates to agricultural pesticides as some investigations have identified strong impacts of nonpoint-source pesticide pollution on streams in Australia (Beketov et al., 2013), Europe (Beketov et al., 2013; Liess and Von Der Ohe, 2005), North America (Chiu et al., 2016) and South America (Hunt et al., 2017) while others only identified comparatively low impacts of pesticides (Noges et al., 2016). Accordingly, the question remains how severe the effects of pesticides are compared to other stressors and, more specifically, at which concentrations ecosystem effects occur and which species and functional parameters are affected. Only with this knowledge it is possible to prioritize and manage stressors effectively.

The regulatory authorisation of agricultural pesticides is supposed to prevent unacceptable effects in the environment. For example in Australia, the EU and the US, an extensive test-system based assessment scheme to protect communities in non-target aquatic ecosystems has been established (Australian Environment Agency, 2009; EFSA, 2013; US Goverment, 2004). This regulatory framework is based on the concept of scaling the effect of individual pesticides in single-species test systems or model ecosystems to the effect in the ecosystem. On this basis, pesticide

concentrations are determined at which damage to aquatic communities can be excluded. However, the natural and anthropogenic stressors present in the ecosystem are not systematically included. Nor has there been any validation of the prediction of ecosystem effects to date.

In this investigation we therefore performed a monitoring in a large geographical area that allows us to quantify all relevant anthropogenic stressors with high temporal resolution. Additionally, we identified the stream invertebrate community as a measure of ecological quality. On this basis, we aimed (i) to model the relative contribution of environmental variables determining the occurrence of aquatic invertebrates and to attribute measured pesticide pressure to ecological status, (ii) to evaluate the protectivity of the aquatic pesticide risk assessment and (iii) to derive evidence-based thresholds for the effects of pesticides considering the presence of additional stressors relevant to the ecosystem.

3.2 Materials and Methods

3.2.1 Site Selection

A total of 101 stream sections distributed over Germany were sampled in April and July for 2018 and 2019 (see map in SI Figure 1), 11 sites were monitored both years. The initial selection comprised 124 stream sections, however, we omitted those stream sections that were affected by drought (lack of flow, drying out) or where the automatic rain Event-Driven Samplers (EDS) did not function (EDS: SI chapter 3). The catchment areas of the monitoring sites were characterized by a gradient of agricultural land use (agricultural land cover in hydrological catchment 0- 100%) and less than 5% of urban areas to focus on agricultural diffuse source pollution. 86 streams were located in agricultural environments (agricultural land cover in hydrological catchment > 20%, referred to as “agricultural” streams) whereas 15 streams were located in areas with less agricultural influence (agricultural land cover in catchment < 20%, see SI chapter 1 for land use analyses). Catchment sizes were generally below 30 km² to represent small lowland streams where those with a catchment greater than 10 km^2 (n = 60) correspond to the reporting requirements of the WFD (Commision,

2000); stream sections with a catchment size of less than 10 km^2 (n = 41) corresponding to the "edge-of-field" surface waters of the EU-EFSA risk assessment of plant protection products for aquatic organisms (EFSA, 2013). Detailed site characteristics are listed in Tab. SI 1.

3.2.2 Water Sampling and Chemical Analyses

Streams were sampled from April to July in 2018 and 2019 during the main application period of pesticides in spring and early summer for most crops (Szöcs et al., 2017). During this time period grab samples (n = 520) were taken regularly in a three-week cycle. This sampling method followed the monthly sampling in governmental monitoring practices under the WFD regardless of weather conditions. EDS samples (n = 320) were taken with automated (MAXX TP5, Rangendingen, Germany) and bottle samplers (Liess and Von Der Ohe, 2005) in order to capture runoff-induced exposure peaks associated with heavy rainfall (Liess et al., 1999), (see Figure SI 5). Small streams with agricultural catchment area are subject to short-term water level rise (Liess and Von Der Ohe, 2005) with the occurrence of storm events exceeding approximately 10 mm/d (Schulz et al., 1998). EDS sampling was triggered by a rise of water level of more than 5 cm so that waves did not trigger the sampling and every runoff event could be captured. (further details see SI chapter 3). The total of 840 samples of both field campaigns 2018 (n = 411) and 2019 (n = 429) were analyzed for pesticides, trace elements and nutrients.

For pesticide analysis, water samples were filtered and analyzed via direct injection into LC-MS/MS without enrichment by multiple-reaction-monitoring (Reemtsma et al., 2013) (details see SI chapter 4). The target analysis tested for 75 pesticides and 33 pesticide metabolites. Pyrethroid insecticides and the herbicide Glyphosate were not included due to analytical limitations. The compound selection was established by prioritization according to active substance-related sale quantities, the consideration of current environmental quality standards (EQS) and the regulatory acceptable concentrations (RAC) (Brinke et al., 2017), (see Tab. SI 2).

To test for further urban toxicants, the samples of 2018 were additionally subjected to LC-HRMS/MS screening analytics (details see SI chapter 6). This screening analyses tested for 257 substances, which were grouped into 16 compound classes including pharmaceuticals, industrial chemicals, rubber additives, stimulants, corrosion inhibitors, plastic additives, sweeteners, biocides, UV filters, bitterns, repellents, per- and polyfluorinated compounds, food ingredients, surfactants, dyes and flame retardants (see Tab. SI 4).

The concentrations of trace elements (arsenic, cadmium, copper, zinc, lead, mercury) were analyzed in water samples using Agilent's ICP-MS 8000 Triple Quad. At the site the samples were pre-filtered (20 ml, 0.45 µm) for arsenic, cadmium, copper, zinc, lead, while mercury samples were bottled unfiltered in a stabilizing solution of nitric acid and potassium dichromate.

3.2.3 Scaling Concentrations for Toxicity

Concentrations of pesticides and trace elements were converted to invertebrate toxicity by calculating Toxic Units (TUs), where measured substance concentrations are normalized to their respective LC_{50} in acute standard laboratory test systems (Sprague, 1969). These LC_{50} values were derived from *Daphnia magna* or *Chironomus sp.* whose acute sensitivity, when considering a wide range of organic toxicants, is approximately equal or slightly less than the acute sensitivity of many insects (Morrissey et al., 2015; von der Ohe and Liess, 2004). For the TU calculation, the LC_{50} of the most sensitive species was considered and retrieved from the Pesticide Property Data Base (PPDB) and in few cases the US EPA ECOTOXicology knowledgebase, if the PPDB lacked respective data (see Tab. SI 3), (Lewis et al., 2016). In case no experimental data was available (0% of target analytes, 57% of non-target analyte LC_{50} values, mostly urban contaminants also including rubber additives as street-runoff indicators), Quantitative Structure Activity Relationship (QSAR)-derived effect concentrations were used to estimate TUs (Busch et al., 2016).

Pesticide peak exposure (TU_{max}) in streams toxic to invertebrates was determined by the maximum single substance insecticidal toxicity measured (Liess and Von Der Ohe, 2005) (TU_{max}, see Tab. SI 1). Extending

this calculation method, we identified that exceptionally toxic samples, that are highly unusual in the exposure profile of the respective stream, did not reflect the ecological situation ($SPEAR_{pesticides}$) and were therefore not considered in the TU_{max} calculation. These exceptional exposure peaks, encountered in 20% of streams (n = 20), were defined by a TU_{max} exceeding the mean TU_{max} of the five subsequent samples (ranked by TU_{max}) by a factor of more than 100. An inclusion of exceptionally high single pulses led to a weaker correlation between the toxic pressure and the ecological effect on vulnerable species ($SPEAR_{pesticides}$) (R^2 = 0.34 versus R^2= 0.43 with and without high pulses considered). The authors are not aware of studies that have identified the reduced significance of an exceptionally high toxicant pulse compared to many, significantly lower pulses. In contrast, the great ecotoxicological significance of several successive toxicity pulses was recognized; the "culmination“ of low-dose pesticide effects (Liess et al., 2013). Analogously, the typical peak pesticide mixture toxicity (TU_{sum}) was determined by summing all individual substance TUs detected in a sample. To assess regulatory thresholds, pesticide concentrations were also scaled by the RACs instead of the LC_{50} values (see SI chapter 11). The toxicity of urban toxicants was determined in the same way as for pesticides (see Tab. SI 4). The toxicity of trace elements was calculated using literature LC_{50} values (Liess et al., 2017; Tsui and Wang, 2005), see Tab. SI 3). Here, the local maximum of summed TUs (TU_{sum}) including all trace elements per sample is considered in the multiple linear regression.

3.2.4 Further Abiotic Parameters

Ortho-phosphate, nitrate, nitrite and ammonium concentrations were determined in all grab and EDS samples using either colorimetric tests by "Visicolor" (MColortest, Merck KGaA; Darmstadt, Germany) or a UV spectrophotometer (PF-12 and visocolor ECO tests, Machery-Nagel, Düren, Germany) in 2018 and a UV spectrophotometer (DR 1900, Hach Lange GmbH; Düsseldorf, Germany) in 2019. Furthermore, total phosphorus (TP) and total nitrogen (TN) contents of all water samples were analyzed (ICP-MS 8800 Triple Quad from Agilent). Oxygen, temperature, water level was continuously measured throughout the sampling period from April to June

in a 3-minute interval using multi-parameter probes (LogTrans7-compact measuring system SENSOdive CTDO2, UIT; Dresden, Germany and O2-Log3055-INT and CTD3100-10 Logger, Driesen+Kern, Bad Bramstedt, Germany). PH was measured with every grab samples using pH-meter (Greisinger G 1500, Regenstauf, Germany and Xylem Analytics WTW Multi 3620 IDS Set G, Weilheim, Germany). The continuous discharge was derived from a stage-discharge relation calculated based on manually measured reference values for flow velocities and water depth for a subset of 31 streams. Hydromorphology was recorded in-situ according to the official procedure by the German Länderarbeitsgemeinschaft Wasser (LAWA) quantifying all hydromorphological criterions required under the WFD. These include among others meandering of the watercourse, variation in stream depth and width as well as riparian conditions (Commision, 2000). Additionally, bed habitat structure described the presence of potential holding substrate for invertebrates (Gieswein et al., 2017). This parameter represents the combined fraction of coarse particulate organic matter, plants, debris and stones > 2 mm in the stream bed. See SI chapter 2 for site-specific data and variable aggregation.

3.2.5 Invertebrate Sampling

Benthic macroinvertebrates were sampled at the beginning of June towards the end of the main pesticide application period for most crops and therefore suitable for ecological effect identification (Liess and Von Der Ohe, 2005) (SI Invertebrate list). Standardized multi-habitat sampling(Meier et al., 2006) as prescribed under the WFD ensured comparable observations. A 50 m long section of each stream was divided into its substrate types on a percentage basis. A total of 20 subsamples (100%) were subdivided into frequencies of the occurring substrate types (smallest unit 5%). Each unit (5%) was sampled by kick sampling ten times using a net with a surface of 0.0625 m^2 and a mesh size 0.5 mm. Sampled organisms were separated the from coarse organic debris using a column sieve set, preserved in 90% ethanol, and later determined in the laboratory generally down to the lowest taxonomic level possible under the binocular.

The invertebrate determination level, abundance and occurrence at sampling sites is provided in the SI chapter 8.

3.2.6 Biological Metrics of Invertebrates

We applied a wide range of biological indicator systems to assess the ecological effects of the stressors measured. Some of the invertebrate based indicators selected were developed to unspecifically respond to stressors. These are taxa number, number of insect taxa, insect and EPT% biomass - estimated using average taxa body volumes approximated by simple geometries (cylinder, ellipsoid, rotational ellipsoid or cone depending on taxon body shape) and a density of 1.06 g/mL (SMIT et al., 1993), Shannon taxa diversity (Shannon and Weaver, 1949), proportion of ephemeroptera, plecoptera and trichopteran (Lenat, 1988), Ecological Status Class (ESC) as multimetric index applied under the WFD considering individual indicators for morphological structure, organic pollution and acidification (Commision, 2000), the biological monitoring working party (BMWP) index and the Average Score Per Taxon (ASPT) indicating general water quality(Armitage et al., 1983), the Fauna Index (Lorenz et al., 2004) and the 3 functional diversity components richness, divergence and evenness (Mason et al., 2005) considering the traits body size, feeding type, locomotion and aquatic stages (Schmidt-Kloiber and Hering, 2015; Usseglio-Polatera et al., 2000). As indicators responding to specific stressors we included the $SPEAR_{pesticides}$ (Liess and Von Der Ohe, 2005) index that relates to the toxic pressure of pesticides on invertebrates and can be calculated with an online tool (https://systemecology.de/indicate/) and the Saprobic index related to the organic pollution that is linked to oxygen deficiency (Kolkwitz and Marsson, 1909; Rolauffs et al., 2013).

We defined the desired ecological status related to pesticides as for other invertebrate metrics under the WFD; with 4 boundaries separating the 5 even quality classes equal EQR (Ecological Quality Ratio) values of 0.8, 0.6, 0.4, and 0.2 (EU Commission, 2008) and classified the resulting ecological status into the usual 5 quality classes ranging from “high” to “bad” related to $SPEAR_{pesticides}$ (for details of approach and classes see SI chapter 9).

3.2.7 Statistical Analyses

All statistical analyses were performed with the statistical software R (version 3.6.1, (R-Core Team, 2019)). Multiple linear regression was performed with all predictors for each of the above listed biological metrics of invertebrates. These include: pesticide pressure, dissolved oxygen, hydromorphology, bed habitat structure, pH, ortho-phosphate, nitrate, nitrite, ammonium, total phosphorus, total nitrogen, flow velocity, temperature, rubber additive concentration, discharge, urban toxicity, metal toxicity, stream width and stream depth (see Tab. SI 1). All predictors were checked for homoscedasticity and normality, some of which were log-transformed if necessary. Different aggregations for individual predictors were investigated to explain all biological indicators by single linear regressions. Those yielding highest coefficients of determinations compared to other aggregations were chosen (details see SI chapter 2). If parameters were only available for a subset of streams (rubber additive concentration, discharge and urban contaminants toxicity) regression analyses was reduced to the respective stream section subset.

Intercorrelation of environmental parameters was tested using the variance inflation factor (VIF). Parameters with VIF-scores greater than two were omitted. The selection of the total model was carried out by an automated forward model selection analysis and the Akaike Information Criterion (stepAIC, R-package "MASS")(Venables and Ripley, 2002). The total model is composed of significant parameters only and the explained variance is given by the adjusted R^2. The contribution of each significant parameter to the total explained variance was evaluated with the metric approach "lmg", which uses R^2 for the evaluation (Hierarchical Partitioning (Chevan and Sutherland, 1991), R-package "relaimpo" (Grömping, 2006)).The visualisation of the data and linear regression models were performed in R using ggplot2 (Wickham, 2016).

3.3 Results and Discussion

3.3.1 Assessment of Anthropogenic Stressors

3.3.1.1 Determining Relevant Anthropogenic Stressors

The 101 streams selected are a representative cross-section of small lowland streams in Central Europe (see SI chapter 1). They cover a wide gradient of agricultural pollution, include 11 small wastewater treatment plants (WWTPs) with less than 3000 population equivalents and a number of diffuse domestic discharges identified by wastewater markers. We used multiple linear regression to identify those anthropogenic stressors that determine invertebrate community composition (see SI chapter 3 for stressor distribution, chapter 8 for invertebrates sampled). Stressors with the highest explanatory power were (i) pesticide toxic pressure during exposure peaks, (ii) oxygen deficiency and (iii) poor hydromorphology (Figure 14). Stressors showing no or only minor associations with invertebrate-related endpoints include urban toxicants such as pharmaceuticals, heavy metals, and street run-off. Agricultural pesticides, related to the substance of the peak exposure events with the highest exposure to effect concentration ratio, the TU_{max} (maximum TU), were on average 91 times more toxic than urban contaminants (related to the sum of all toxicants (TU_{sum}) 76 times more toxic). We also found that TUs measured at 11 stream sections with WWTPs were similar to those without WWTPs (SI chapter 7) comparable to a study related to WWTP in Switzerland (Munz et al., 2017). Agricultural nonpoint-source pesticide pollution was thus identified as a major driver of invertebrate community composition in the ecosystems under investigation (see chapter 3.3.3.2. on the ecological processes of the low-concentration effects of pesticides).

Non-additive interactions between stressors were investigated limited to relevant stressor combinations so as not to reduce statistical power. These were interactions between those stressors already known to act synergistically: toxicants and water temperature (Arambourou and Stoks, 2015; Verheyen and Stoks, 2020) and oxygen deficiency (Ferreira et al., 2008; Gupta et al., 1983; Van der Geest et al., 2002). We also added the

remaining stressor that proved to be relevant for many of the ecological endpoints; the deficiency of morphological structure. Interactions between these three stressor combinations were all additive; none resulted in measurable antagonistic and synergistic ecological effects. Other investigations yielded comparable results for the minor relevance of interactions (Birk et al., 2020; Gieswein et al., 2017) explaining them with community adaptation processes which reduce non-additive stressor interactions (Romero et al., 2019).

3.3.1.2 Assessment of Ecological Endpoints

Ecological endpoints best responding to the measured anthropogenic stressors were: (i) the $SPEAR_{pesticides}$ index, identifying the degradation of invertebrate communities by pesticide toxicity (Liess and Von Der Ohe, 2005), (ii) the proportion of vulnerable insects %EPT (Ephemeroptera, Plecoptera, Trichoptera), identifying the general degradation of the community (Lenat, 1988) and (iii) the saprobic index, identifying the oxygen deficiency (Kolkwitz and Marsson, 1909) (Figure 14). Other common indicators of community disturbance were only marginally associated with any of the anthropogenic stressors quantified, namely the BMWP and ASPT (Armitage et al., 1983). Also the Ecological Status Class (ESC) for the biological quality element invertebrates under the EU water framework directive (WFD) (Völker et al., 2016) seems unable to reflect anthropogenic stressor effects in small lowland streams. An extended list of endpoints and their association to stressors is displayed in Figure 14.

Our results show that indicators of function were only marginally associated with any of the anthropogenic stressors quantified. These include invertebrate biomass, taxa number and also diversity indices as functional richness, evenness and divergence (Mason et al., 2005). Similar results were revealed for other small lowland streams (Voß and Schäfer, 2017). The weak association of anthropogenic stressors and several indicators of function is likely due to compensatory processes (Frost et al., 1995). Obviously such “integrating endpoints” that describe a system in its entirety (i.e. total abundance or biomass) are subject to compensatory

processes and therefore respond less to stressors compared to "differentiating endpoints" (Liess and Foit, 2010). The loss of sensitive species may be compensated through tolerant species (Dornelas et al., 2019). Accordingly, "differentiating endpoints" that include structural community measures and can reflect declines of the fraction of vulnerable taxa – increased by competitive processes between taxa (Liess et al., 2013) – show strong associations with stressors. These measures describe biological systems by grouping its elements (individuals and populations) according to contrasting traits (Liess and Foit, 2010). Examples are the endpoints $SPEAR_{pesticides}$, %EPT, and the Saprobic index that differentiate community composition according to the vulnerability of taxa towards pesticides, general stressors or oxygen depletion. It follows that measures describing the community without reference to competitive processes, the "integrating endpoints" such as total invertebrate biomass, taxa number and the Shannon index are not capable of indicating anthropogenic stress. It is precisely the exclusive use of integrating endpoints that carries the risk of overlooking actual stressor effects and signs of ecological degradation. One example is a recent comprehensive meta-study that reported an increase in freshwater insect abundances over the last decades, based only on integrating endpoints (Klink et al., 2020). Accordingly, total biodiversity without reference to contrasting traits such as size, longevity or sensitivity may not be a sensitive indicator of global change.

3.3.1.3 Characterization of the Agricultural Pesticide Pollution

In terms of pesticide toxic pressure, regular grab samples, mainly taken during base-flow conditions, revealed a background contamination with an average of 17 detected pesticides and 10 pesticide metabolites per sample, whereas event-driven sampling (EDS) revealed an increased average of 31 pesticides and 11 metabolites per sample. Pesticide concentrations (95% percentiles) sampled by EDS events exceeded grab sample derived background concentrations by a factor of 54 on averaging, with a median of 6.3. A detailed overview of the detected pesticides and their concentrations is reported in the SI chapter 4.

Figure 14: Relative importance of stressors for biological endpoints - multiple linear regression to determine the explained variance, R^2 (numbers below dots). Significance levels p < 0.05*; < 0.01**; < 0.001***. Red dots indicate a deterioration of the biological endpoints with increasing stress, blue dots an improvement.

Pesticides contributing dominantly to the toxic pressure of peak events on invertebrates included the neonicotinoids thiacloprid (mean share of TU_{sum} = 46.6%), imidacloprid (9.5%) and clothianidin (3.6%) as well as the biocide fipronil (9.9%) and the carbamates methiocarb (5.1%) and pirimicarb (4.8%). These 6 pesticides drove the invertebrate toxicity in 91.3% of the peak exposure events when considering the pesticide with the highest exposure to effect concentration ratio, the TU_{max}. On average, TU_{max} accounted for 69% of the invertebrate mixture toxicity assuming concentration addition (TU_{sum}). Accordingly, we show that the pesticide causing the highest toxic pressure out of the complex mixture of numerous pesticides is a good proxy of the total toxic pressure from a peak event. This was also confirmed by the linear regression depicted in Figure 16A which showed no improved association between the toxic pressure and $SPEAR_{pesticides}$ when using TU_{sum} instead of TU_{max} (both R^2 = 0.43). This finding matches previous studies, which compared the relevance of the dominant compound to the mixture for the environmental impact of pesticides in agricultural streams (Knillmann et al., 2018; Liess and Von Der Ohe, 2005; Schäfer et al., 2007). Here it is necessary to recognize that the dominant compound in each event can be a different one. Several such pesticide peak exposure pulses with at least a tenth of the TU_{max} occurred on average 3.7 times per site and sampling period.

3.3.2 Current Risk Assessment Underestimates Exposure and Effects of Pesticides

3.3.2.1 Exceedances of Regulatory Acceptable Concentrations (RACs)

The authorisation of a pesticide requires that its regular application results in an environmental exposure below the safe level for non-target organisms within the ecosystem (EFSA, 2013). Exposure models are applied to derive predicted environmental concentrations (PEC). The level of exposure considered to be safe is determined in a tiered approach identifying regulatory acceptable concentrations (RAC) for each pesticide. Our monitoring-based findings show that these regulatory requirements (PEC < RAC) are often not met in reality:

The measured environmental concentration (MEC) was higher than the predicted environmental concentrations (MEC > PEC, Figure 15B). For 11 out of 16 pesticides that frequently exceeded RACs (selection see Tab. SI 2) we observed PECs being exceeded in more than 1% of EDS samples (Figure 15B).

The RACs in place during the monitoring were exceeded in the majority of streams (Figure 15A). Even pesticides no longer approved at the time of the investigation (2018, 2019) were present in concentrations above their RAC (SI Tab. 2). At least one exceedance of a RAC was detected in the 81% of sites in catchments with agricultural land use exceeding 20% (Figure 15A). More than 5 RAC exceedances within one sampling period were identified in 41% of agricultural streams. EDS with a total n = 296 from agricultural streams revealed RAC exceedances in 59%, grab samples with a total n = 440 in 26% of samples. This is similar to the results obtained by the most comprehensive meta-study to date, which found that 45% of the 1566 cases of measured insecticide concentrations in EU surface waters exceeded their respective RACs (Stehle and Schulz, 2015). On the substance level, 37 pesticides and 2 metabolites exceeded their RAC (Figure 15B, for the 20 pesticides with most exceedances, Tab. SI 2 for all substances). Moreover, in this current investigation we identified 41% of the 17 streams with less than 20% of agricultural land use where RACs were still exceeded. 4 out of 7 streams without any agriculture or known point sources within their catchment showed RAC exceedances of 3 pesticides (Imidacloprid, Clothianidin, Fipronil; see Figure SI 4A). Although the authorisation of spray applications for 3 neonicotinoids had already expired in 2019, similar high exceedances as in 2018 were measured (clothianidin, imidacloprid, thiamethoxam).

3.3.2.2 Reasons for Non-Compliance with Regulatory Thresholds

For the 20 pesticides that most frequently exceeded the RACs, the following potential reasons for non-compliance with the regulatory thresholds were identified (Figure 15B and SI chapter 4).

- For 11 of these pesticides PECs were exceeded, possibly either due to unauthorised application rates, faulty exposure modelling, failure to consider multiple applications in the river basin, or overestimation of the predicted effectiveness of risk reduction measures (thiacloprid, terbuthylazin, nicosulfuron, lenacil, diflufenican, thiamethoxam, S-metolachlor, foramsulfuron, dimethenamid-P, pirimicarb, mesotrione).
- Due to regulatory updated effect information after pesticide approval the RAC has been lowered for 8 pesticides after approval of available products. However, this updated effect information does not have an impact on the already authorised products on the marked. This leads to the situation, that products are available for use even if the expected PEC is above the updated RAC and an authorisation would not have been granted (EU Commission, 2011). However, due to the inertia of the risk assessment practice where re-evaluation is generally intended only every 10 to 15 years, this incorporation of new knowledge had not been performed for several products containing the pesticides thiacloprid, clothianidin, methiocarb, imidacloprid, thiamethoxam, acetamiprid, dimoxystrobin and bromoxynil.
- The measured environmental concentrations of 2 pesticides exceed their RAC without having a PEC assigned as authorisation assumed that there is no discharge into streams. For methiocarb, no PEC run-off was modelled due to the exclusive use as seed treatment. Although this assumption has proven wrong years ago, the new assessment practice in place did not have an impact on authorized products already on the market. fipronil on the other hand is only approved for biocidal and veterinary use and therefore has no PEC for agricultural use assigned.

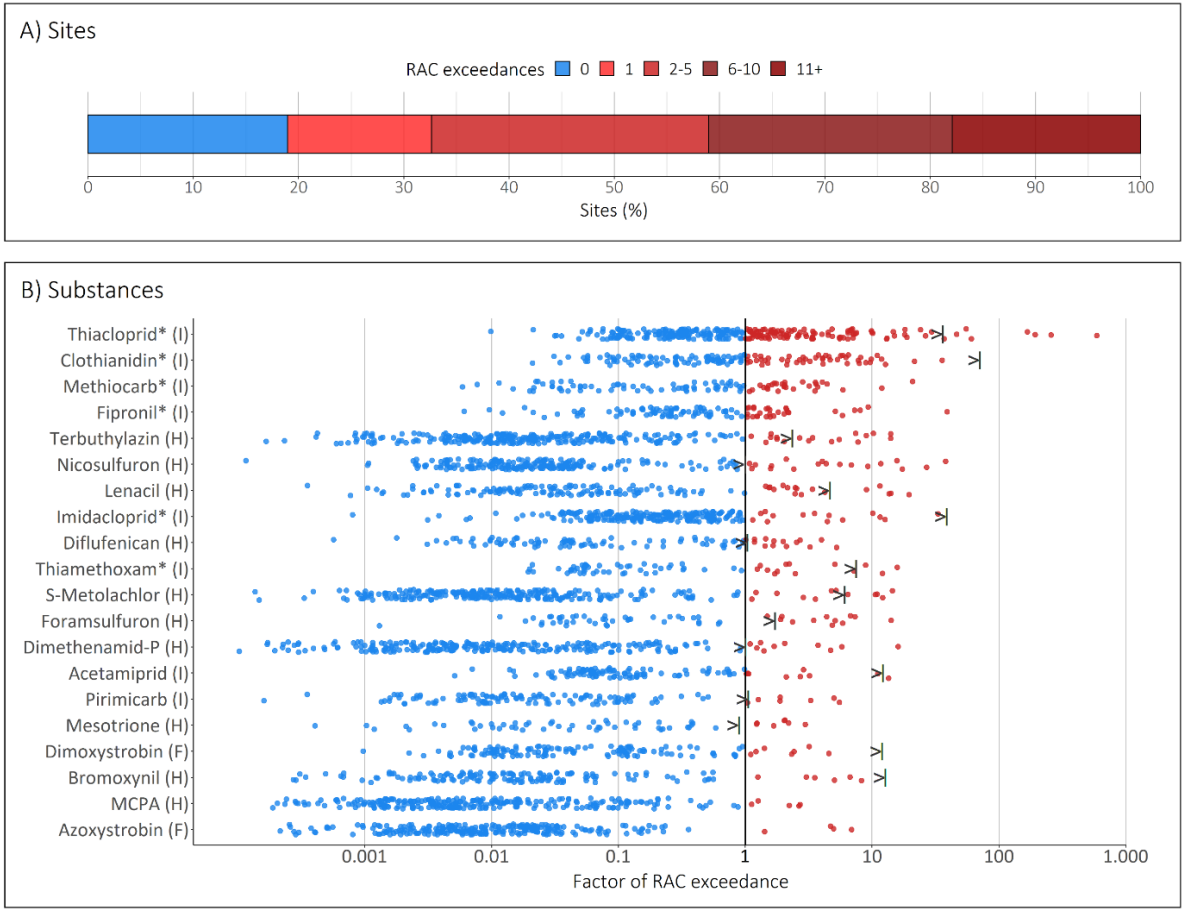


Figure 15: Measured exceedances of regulatory acceptable concentrations (RAC), Event-driven samples (EDS) from streams with >20% agricultural land use within the catchment. A) RAC exceedances per site and year (n = 95). No exceedances in 19% of sites, 1 in 14%, 2-5 in 23%, and more than 11 in 18%. B) Substance-related RAC exceedances in EDS samples (n = 296) of RACs for those 20 pesticides with most exceedances. Regulatory approval of marked (*) substances expired by December 2020. The ratio of predicted environmental concentrations (PEC) to the respective RAC including risk mitigation measures is shown by black ">|" symbols. For MCPA and Azoxystrobin no single PEC value could be identified.

3.3.2.3 Contradiction to the Pesticide Regulation and the Water Framework Directive (WFD)

The environmental situation as revealed in the current investigation related to agricultural streams shows an impairment of vulnerable populations, represented by a reduction of the $SPEAR_{pesticides}$ index. This situation does not comply with the Regulation (EU) 546/2011 that states "Member States shall ensure that use of plant protection products does not have any long-term repercussions for the abundance and diversity of non-target species." (EU Commission, 2011). This also contradicts the requirements of the EU regulation 1107/2009 that pesticides must not exert "unacceptable effects on the environment" considering "particularly contamination of surface waters," with regards to "non-target species" and "impact on biodiversity and the ecosystem" (EU Parliament, 2009). As required by the European parliament, no authorization to pesticides shall be granted "unless it is clearly established through an appropriate risk assessment that under field conditions no unacceptable impact on the viability of exposed species … occurs" (EU Commission, 2011). Whereas unacceptability is defined within the specific protection goal for the "ecological threshold option" as "negligible population-level effects" on the "most sensitive populations". "The term negligible is used since it is difficult to demonstrate that no effect is occurring" (EFSA, 2013). Furthermore, the responsible authorities themselves are questioning the extent to which these environmental protection requirements are being implemented in practice. For example, the European Court of Auditors noted "limited progress in measuring and reducing risks" of plant protection products (European Court of Auditors, 2020). Furthermore, the German Federal Environment Agency (UBA) criticizes "the current intensity of chemical plant protection in Germany as ecologically unsustainable and thus threatening the achievement of key targets of environmental protection and nature conservation policies" (Frische et al., 2018).

The Water Framework Directive (WFD) also requires a good chemical status of water bodies by not exceeding Environmental Quality Standards (EQS). The respective exceedances of these thresholds point a similar picture, see SI chapter 10 and SI Table 2.

3.3.3 Deriving Protective Thresholds for Pesticides

3.3.3.1 Deriving the Acceptable Concentration (AC_{field})

The extensive dataset generated here allows to identify field-based safe concentrations at which no unacceptable adverse ecological effects on invertebrate communities are expected, the field validated Acceptable Concentration (AC_{field}). For the first time, this enables a validation of regulatory effect thresholds. The AC_{field} is based on 3 components: (i) the indicator system $SPEAR_{pesticides}$, (ii) an identification of the desired ecological status related to pesticides, (iii) the quantification of the uncertainty of the exposure-effect relationship.

(i) As a specific biological indicator, we applied the $SPEAR_{pesticides}$ index that uses pesticide-specific traits (pesticide sensitivity, generation time, migration ability, presence during the time of contamination) characterising the aquatic invertebrate community to establish a link between test-system based toxicity (LC_{50}; *D. magna, C. riparius*) and ecological impact (Liess and Von Der Ohe, 2005). The index responds primarily to toxic pressure and is largely independent of other environmental factors as shown earlier (Knillmann et al., 2018; Liess et al., 2008) and also here (Figure 14). The approach has been successfully applied in various geographical regions including Europe (Knillmann et al., 2018; Schäfer et al., 2012), Australia (Burgert et al., 2011) and South America (Hunt et al., 2017) enabling a widespread adoption of the presented approach.

(ii) To define the ecological status related to pesticides we derived an EQR (Ecological Quality Ratio) following the respective EU-WFD procedure (EU Commission, 2008) and as detailed within the methods section and the SI chapter 9. The respective quality classes are indicated in Figure 16A, where

the boundary between a “good” and “moderate” status was set to a $SPEAR_{pesticides}$ value of 0.6 resulting in 83% of agricultural streams that did not reach the pesticide related ecological targets.

(iii) The uncertainty of the exposure-effect relationship is quantified by the variance of the relationship (Figure 16A). Causes for this variance are likely to include site-specific environmental factors and their interaction with pesticides as well as inaccurate exposure and effect assessment. The linear regression between toxic pressure (TU_{max}) and community response ($SPEAR_{pesticides}$) intersects the transition between the “good” and “moderate” quality class at a log TU_{max} of -3.27, identifying the threshold where 50% of sites below the regression line fail to meet a “good” ecological quality for invertebrates (Figure 16A). To establish a reliable ecosystem-based exposure-effect relationship we assume that all the variance observed is not related to the effects of pesticides but to other factors. This approach will considerably underestimate the true impact of pesticides. Accordingly, the $SPEAR_{pesticides}$ benchmark for an acceptable ecological status is reduced by the variance observed and should therefore be considered a conservative indicator of pesticide exposure (1.645σ corresponding to a one-sided confidence level of 95%, see Figure 16A, line *a*). Thus, a log TU_{max} of -3.27 marks the toxic pressure at which only 5% of sites will show an unacceptable $SPEAR_{pesticides}$ with a 95% confidence level (Figure 16A & B, line $b_{5\%}$). With this framework we consider the pesticide effects and as well as the related variability existing in the field and transform an adaptive cause-effect relationship of toxic pressure ($SPEAR_{pesticides}$) into a benchmark-related ecological cause-effect relationship (95% of streams protected), termed the AC_{field}. Accordingly, the threshold value for a pesticide that adversely affects invertebrates equals the substance-specific acute LC_{50} divided by an extrapolation factor of about 2000 (AC_{field} see Tab. SI 2). This measure describes the typical short-term exposure of primarily invertebrate-toxic pesticides at which no adverse effect on the invertebrate community is expected in 95% of the streams. The relationship displayed in Figure 16B additionally allows to

identify the toxic pressure of a pesticide that relates to any percentage of streams affected.

The approach presented here presupposes that the extrapolation factor from the laboratory-based LC_{50} to the field-effect is similar for all pesticides. Only then is it possible to include all peak loads to derive a common extrapolation factor, regardless of the dominant pesticide in a given mixture. The exceptionally good association between toxic pressure (TU) and invertebrate response ($SPEAR_{pesticides}$) for an ecological context shows that this assumption can obviously be made. Furthermore, pesticides that do not cause the highest toxicity are also contributing to the overall ecological impact. As for other environmental factors, for the ecological assessment they are considered as a constant effect-determining factor that is included in the extrapolation factor. The good correlation identified in Figure 16A indicates that these assumptions are valid for the majority of the pesticides investigated. Nevertheless, significant deviations from this rule may occur in individual cases, so that the AC_{field} values are merely an indication of the ecological potency of a toxicant. With this restriction in mind a prospective assessment of the ecosystem impact of new pesticides is possible. Accordingly, this approach integrates prior knowledge into the derivation of ecologically effective concentrations in a similar way as other studies have based the probability of occurrence of taxa on habitat suitability (Vermeiren et al., 2020) and toxicant concentration (Liess and Von Der Ohe, 2005). The AC_{field} allows an effect assessment for a pesticide on the basis of the other pesticides typically present in agricultural streams. Therefore, the AC_{field} can only be compared with the RAC when considering that RAC values were derived without taking into account the presence of other pesticides.

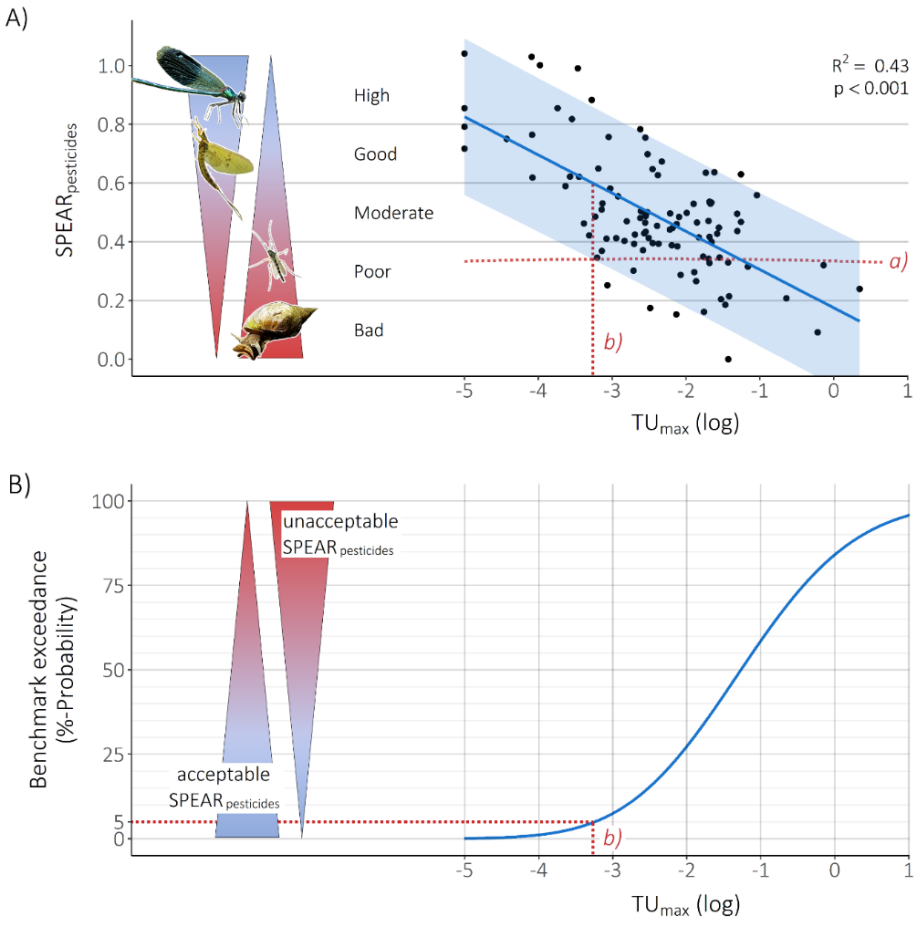


Figure 16: Field-based adaptive (A) and benchmark-related (B) cause-effect relationship for pesticides. A) Adaptive cause-effect relationship of toxic pressure (TU_{max}) and ecological effect ($SPEAR_{pesticides}$) observed in the 101 streams. The blue band corresponds to the 90% prediction interval. Line $a_{95\%}$ depicts the $SPEAR_{pesticides}$ benchmark to identify unacceptable pesticide effects with a confidence of 95% ("good"-"moderate" benchmark reduced by 1.645σ of the linear regression). Line $b_{5\%}$ represents the log TU_{max} threshold of -3.27, where 5% of streams show an unacceptable ecological status according to $SPEAR_{pesticides}$ with a confidence of 95%. B) Benchmark-related ecological cause-effect relationship: Resulting probability of exceeding the $SPEAR_{pesticides}$ benchmark as a function of TU_{max}.

The AC_{field} that is available for 22 primarily invertebrate-toxic pesticides identifies an extrapolation factor related to acute LC_{50} values of about 2,000 protecting 95% of streams; a factor exceeding the acute regulatory Tier 1 "assessment factor" (100) by 20. To protect 99% of streams the respective extrapolation factor would amount to 18,000, a log TU_{max} of -

4.25 (Figure 16B). However, the exposure to RAC ratio was found to explain $SPEAR_{pesticides}$ equally well as the exposure to LC_{50} ratio (R^2=0.44 versus R^2=0.43, see Figure SI 8A). This shows that the RAC values are related to the ecological effect as shown in the cause-effect relationship in Figure SI 8A. Nonetheless, their compliance would cause unacceptable effects in 14% of agricultural stream sections; 86% would be protected (Figure SI 8B). To protect 95% or 99% of streams, respectively, the RAC for invertebrate-toxicity driving pesticides (SI chapter 11) required an additional assessment factor of 5.3 or 40.2. It must to be taken into account that these results refer primarily to the pesticides with the greatest RAC exceedances. These include particularly 4 different neonicotinoids as well as fipronil, methiocarb and terbuthylazine (Figure SI 4).

3.3.3.2 Mechanisms for the Observed Low-Concentration Effects of Pesticides

We hypothesize the following ecological processes as the reason for the high field sensitivity of vulnerable species and the associated increased extrapolation factor identified here:

- The multitude of pesticides present in the streams may not only result in additive effects (Loewe and Muischnek, 1926) but also in a synergistic increase of pesticide toxicity due to the presence of additional toxicants that may exceed the additive effects by a factor of up to 660 as identified in laboratory investigations (Liess et al., 2020) or by an increase of single-substance toxicity by more than one order of magnitude as identified in field investigations (Rydh Stenström et al., 2021).
- Environmental stressors may act synergistically when acting in concert. Examples include the combined effects of nutrients, suspensions and temperature frequently producing synergistic effects on abundance at the population level of periphyton communities (Piggott et al., 2015) and the combined effects of nutrients, suspensions and chloride inducing invertebrate drift in streamside mesocosms (Beermann et al., 2018). Additionally,

stressors such as predator pressure, competition and suboptimal environmental conditions may increase the sensitivity of populations to pesticides by a factor of up to 100 as revealed in microcosm (Liess et al., 2016) and mesocosm studies (Liess and Beketov, 2011).

- Repeated insecticide pulses leading to multiple exposure of individuals within a generation (within a spray season for annual species), increases the impact compared to a single insecticide pulse (Wiberg-Larsen et al., 2021). Also repeated pesticide pulses leading to multiple exposure of populations between generations (between spray seasons for annual species), increases the impact compared to a single insecticide pulse and may result in a multigenerational culmination of low-concentration effects (Liess et al., 2013).

The effect-determining factors and their related processes described here are generally not considered in the aquatic risk assessment. Thus, neither for individual-based lower-tier studies nor for mesocosm-based higher-tier studies effect-determining factors are taken into account that are comparable in their expression with the field. Calibration of existing assessment factors by means of traditional higher-tier studies has been successfully carried out (Brock et al., 2016; Rico et al., 2019), but does not allow prediction of pesticide effects in the field. We therefore suggest to calibrate the assessment factors applied in pesticide regulation integrating field-based findings. For example, a relevant candidate for such an exercise is the insecticide chlorantraniliprole, a pesticide that may replace the widely used neonicotinoids and could therefore gain high relevance in the near future (Schmidt-Jeffris and Nault, 2016). For chlorantraniliprole the RAC is a factor of 50 higher than the respective AC_{field}. Accordingly, regular authorities could review the derivation of the current RAC in order to avoid future environmental problems with this pesticide.

3.4 Conclusions

- In this study of 101 small lowland stream sections, we revealed for the first time the prime relevance of agricultural pesticide pressure for the composition of invertebrate communities.
- In this study of 101 small lowland stream sections, we revealed for the first time the prime relevance of agricultural pesticide pressure for the composition of invertebrate communities.
- The diversity and number of vulnerable species was already reduced at very low pesticide concentrations, so that most of agricultural streams did not meet the pesticide-related ecological targets.
- We revealed that the current authorisation of pesticides underestimates the actual ecological risk, as measured pesticide concentrations exceeded current regulatory threshold levels in most of the agricultural streams and even existing thresholds were not protective for invertebrates.
- By including monitoring-derived information on pesticide effects within the ecosystem we identified pesticide threshold concentrations that will ensure a protection of the invertebrate stream community.
- Future research should extend this concept developed here to other groups of aquatic organisms such as amphibians, fish, plant and fungi communities, and also to terrestrial ecosystems. This identification of field validated Acceptable Concentrations for the ecosystem (AC_{field}) can then be used to review the existing thresholds of the Pesticide Risk Assessment (RAC) and the Water Framework Directive (MAC-EQS).

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4 Risk from Pesticide Mixtures – The Gap between Risk Assessment and Reality

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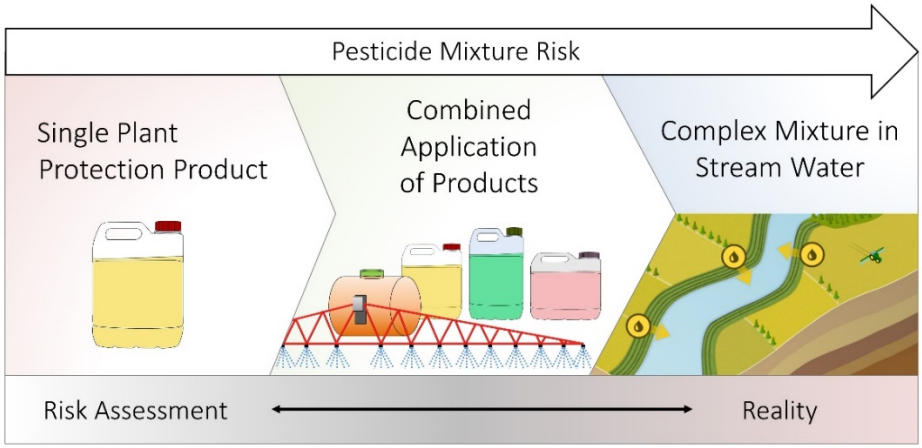
Abstract

Pesticide applications in agricultural crops often comprise a mixture of plant protection products (PPP), and single fields face multiple applications per year leading to complex pesticide mixtures in the environment. Restricted to single PPP, the current European Union PPP regulation, however, disregards the ecological risks of pesticide mixtures. To quantify this additional risk, we evaluated the contribution of single pesticide active ingredients to the additive mixture risk for aquatic risk indicators (invertebrates and algae) in 464 different PPP used, 3,446 applications sprayed and 830 water samples collected in Central Europe, Germany. We identified an average number of 1.3 different pesticides in a single PPP, 3.1 for complete applications often involving multiple PPP and 30 in stream water samples. Under realistic worst-case conditions, the estimated stream water pesticide risk based on additive effects was 3.2 times higher than predicted from single PPP. We found that in streams, however, the majority of regulatory threshold exceedances was caused by single pesticides alone (69% for algae, 81% for invertebrates). Both in PPP applications and in stream samples, pesticide exposure occurred in repeated pulses each driven by one to few alternating pesticides. The time intervals between pulses were shorter than the 8 weeks considered for ecological recovery in environmental risk assessment in 88% of spray series and 53% of streams. We conclude that pesticide risk assessment should consider an additional assessment factor to account for the additive, but also potential synergistic simultaneous pesticide mixture risk. Additionally, future research and risk assessment need to address the risk from the frequent sequential pesticide exposure observed in this study.



4.1 Introduction

A total of 466 pesticide active ingredients, referred to as pesticides in the following, are currently approved for use in plant protection of the various agricultural crops within the EU (European Commission, 2021). In Germany alone, 288 different pesticides were approved ingredients in 932 plant protection products (PPP) in 2019 (UBA). PPP application schemes, referred to as spray series, comprise multiple applications per field and year, where multiple PPP are frequently applied simultaneously, which in turn often contain a mixture of pesticides. Consequently, manifold pesticide residues occur in the different environmental compartments, resulting in complex environmental pesticide mixtures (Schreiner et al., 2016; Silva et al., 2019; Stehle and Schulz, 2015b).

Small streams with agricultural catchments face particularly diverse and ecologically relevant pesticide pollution (Knauer, 2016; Stehle and Schulz, 2015a; Szöcs et al., 2017). In a Germany-wide monitoring of more than 100 lowland streams, Liess et al. (2021a) and Halbach et al. (2021) confirmed the widespread occurrence and ecological relevance of pesticides in streams on a large scale. The adjacency to agricultural fields in combination with a limited dilution capacity makes streams particularly receptive to an agricultural input of pesticide residues. These enter the water bodies via rain-induced runoff, drainage and spray drift (Jong et al., 2008; Liess et al.,

1999). The respective contribution of each pathway to the total input depends on site-specific parameters and pesticide properties; however, runoff is most likely to cause peak concentrations in typical agricultural catchment scenarios (Liess and Schulz, 1999). Especially after rainfall, streams thus represent a reservoir for recent pesticide applications within their catchments. Multiple studies have reported an increased risk due to pesticide mixtures occuring in these aquatic environments and stressed their adverse potential (Gustavsson et al., 2017; Schreiner et al., 2016; Vallotton and Price, 2016).

The current European environmental risk assessment (ERA) of pesticides, however, considers almost exclusively single applications of single PPP on a single crop (European Union, 2009; Frische et al., 2014; Frische et al., 2018; Northern Zone, 2018; Topping et al., 2020). More precisely, this means that the ERA accounts for the mixture in a single PPP, which is a formulation of one or more pesticides and additives to improve the PPP's properties such as solubility for example. If at all, PPP applications with one or more PPPs at the same time are only considered in rare cases where application mixtures of several PPPs are specifically registered as such and listed on the label of use with a clear name and dose rate. However, the ERA of PPP currently provides no concept to address all unknown PPP application mixtures, spray series and, more importantly, unintended pesticide mixtures present in the environment. To our knowledge, no country or region in other parts of the world considers the risk due to simultaneous pesticide mixtures in the environment within the authorisation or risk mitigation of PPPs.

This is problematic following the widely acknowledged assumption that exposure to multiple pesticides as a consequence of intensive PPP use represents a major disregarded ecological risk and a contribution to the biodiversity decline (Backhaus and Faust, 2012; Brühl and Zaller, 2019; Hayes et al., 2006; Silva et al., 2002). This assumption is often supported by studies testing equitoxic mixtures, in which all components contribute equally to the toxicity of the mixture based on a consistent measurement

endpoint (Altenburger et al., 2000; Backhaus et al., 2000; Silva et al., 2002). Especially under such conditions, the combined effect of the mixture significantly exceeds respective single substance effects. Accordingly, the guidance documents defining principles for the ERA generally acknowledge the need to also consider possible effects due to other chemicals already present in the environment (EFSA, 2009, 2013). However, the aquatic guidance states that "a thorough analysis of PPP usage practices in major crops […] is not yet available" and assumes that "observed effects are, in many cases, related to the effects of one or two [pesticides]". The disregard of multiple PPP exposure in the ERA is reasoned by a lacking systematic analysis of and harmonized concept how to consider real-world PPP usage practices and environmental exposure patterns (Ctgb, 2021; Garthwaite et al., 2015).

In this study, we address this knowledge gap by comparing comprehensive monitoring data sets on (i) real-world PPP applications and (ii) measured concentrations in surface waters also considering peak exposure scenarios. This allows the gap between the pesticide mixture risk considered by PPP authorisation and the actual environmental risk to be quantified. In addition, the combined dataset provides insight how often agricultural fields and streams face exposure pulses of such mixtures. We therefore aim to (i) estimate and compare the risk considered under the single PPP-oriented ERA with the risk of pesticide mixtures present in the field, (ii) evaluate stream water pesticide mixtures in the light of regulatory threshold levels, (iii) characterise environmental pesticide mixture composition and identify pesticides driving mixture risk and (iv) quantify the sequential pesticide exposure due to serial applications on fields and recurring inputs in streams.

4.2 Material and Methods

4.2.1 General Approach

In order to compare the risk considered under the single PPP-oriented ERA with the risk of pesticide mixtures present in the field, we quantified the risk of pesticide mixtures in single PPP, PPP applications (=single spray

event of one or several PPP) and water samples taken from agricultural streams. For this, we reviewed a large dataset of real-world PPP spray series comprising applied PPP and their components for common crop types. On the basis of the amount of pesticides applied, we modelled the surface water exposure as performed within the European environmental risk assessment (ERA) for individually sprayed PPP as well as combined PPP applications and estimated the resulting risk in surface waters for invertebrates and algae/macrophytes. Under real world conditions, the pesticide mixtures in surface waters are expected to show a different toxicity than estimated by exposure modelling based on single PPP applications. Most importantly, off-site transportation, parallel PPP applications on adjacent fields and degradation of pesticides result in spatially and temporally integrated environmental mixtures. In addition to the modelled pesticide exposure, we therefore analysed measured pesticide concentrations in agricultural streams and compared these with the modelled exposure of the reported PPP applications. The spray series and stream monitoring data we jointly analyzed originate from different projects and are temporarily divergent. Although the water samples were collected in 2018-2019, we expect them to match the spray series data from 2007-2015 in terms of applied and environmental pesticide toxicity given that application intensities remained stable (Julius Kühn-Institut, 2020). Single pesticide or PPP authorisations were withdrawn and new substitutes entered the market while toxicity ratios in environmental mixtures are likely to remain unchanged. The reported PPP applications and monitored streams do not cover the same hydrological catchments but are from the same geographical region.

4.2.2 Pesticide Application Data & Exposure Modelling

The pesticide application data were obtained from the INL – "Privates Institut für Nachhaltige Landbewirtschaftung" Halle, Germany, and compiled as part of the COMBITOX project (FKZ 3715 63 407 0) (Knillmann et al., 2019). The dataset included 889 real-world spray series from the years 2007-2015 (see Supporting Information/SI Figure 1). A total of 229 different pesticides were applied on twelve different crops including

different cereals, oilseed rape, potato, sugar beet, vine, and apple (see substance and crop list in SI). The 24 farms and 175 fields are mostly located in different agricultural regions in Germany and a few in neighbouring Austria that were also included due to comparable climatic conditions and the fact that both countries fall under the Central Zone for the registration of PPP.

Each spray series in the dataset describes a sequence of plant protection and plant growth regulation measures over one growing season. In each case, this covers the time from sowing (arable crops) or from leaf development (permanent crops) to harvest. One application within a series is defined as the total of all measures applied on one specific day and field. Each application is characterised by the PPP used, the pesticide(s) in the PPP, the application rate (e.g. in kg/ha) and the date of application. The application frequencies of the spray series analysed were congruent with the strongly aggregated, but publicly available pesticide statistics of the Julius Kühn-Institut for each crop type (see SI Table 1) (Julius Kühn-Institut). Therefore, we expect that the dataset on spray series well reflects the agricultural practice in recent years. To avoid bias from seasonal variability, only data from PPP applications sprayed in the stream sampling period (April until mid-July, n = 3,446) were compared with the water samples.

We modelled the predicted environmental concentrations in surface water on the basis of the amounts of pesticide applied. Exposure modelling is used to account for the pesticides' physico-chemical properties driving their tendency to enter surface waters. For this, we used FOCUS, the official model for estimating pesticide exposure at EU level (FOCUS, 2012). We performed FOCUS Step 2 calculations (unavailable case-specific data would be required for Step 3 and 4) limited to the most relevant entry pathways, runoff and drainage, to ensure comparability with measured peak concentrations after rainfall (Huber et al., 2000; Liess and Schulz, 1999). In the model, we accounted for plant interception reducing pesticide loads in the soil, depending on the culture and its stage during application (EFSA, 2014). As assumed in FOCUS models, the residues of

each application are washed out by a defined rainfall after partially degrading in soil for 4 days. The physico-chemical properties of the pesticides applied required for the calculations were retrieved from the Pesticide Properties DataBase (PPDB, experimental data) and the US EPA EPI Suite (modelled data), where experimental data was prioritised (Lewis et al., 2016; US EPA, 2015). Model parameters are described in more detail within the SI. Depending on the application scenario (e.g. treated culture, growth stage, slope of field, seasonality), PPP may only be sprayed under "mandatory conditions of use". This may include maintaining untreated buffer strips along surface waters. As this information was not available, surface water concentrations were modelled without accounting for conditions of use. This may have resulted in higher concentrations than modelled in the actual ERA.

4.2.3 Stream Water Pesticide Sampling

The information on stream water pesticide concentrations were collected as part of the "Kleingewässermonitoring", a Germany-wide monitoring of small streams (FKZ 3717 63 403 0) (Helmholtz-Centre for Environmental Research - UFZ, 2020). The monitoring involved several stakeholders as it was supported by the German Federal Environment Agency (UBA), regional water authorities and also advised by regional agricultural authorities. See Liess et al. (2021a) and Halbach et al. (submitted 2021) for a description of sampling methods and a detailed discussion of measured pesticide concentrations and observed ecological effects. In brief, this study focused on a sub-selection of 103 agricultural streams where agriculture made up at least 20 % of land cover in the hydrological catchment (Copernicus Land Monitoring Service, 2019). A total of 830 water samples were taken from the beginning of April to mid-July in 2018 and 2019. Pesticide applications are most frequent during this period, so that peak concentrations are most likely to occur (SI Figure 2). Upstream catchments were mostly smaller than 30 km^2 (mean = 17 km^2, max = 267 km^2) and characterised by a gradient of agricultural influence (agricultural land cover ranged from 22-100%, mean = 74.5%, excluding forestry). Settlements and other urban land

covers accounted for less than 5% in the majority of stream catchments (see SI for catchment characteristics).

The sampling was carried out in two different ways to capture (i) background concentrations under dry weather conditions and (ii) rainfall-driven peak concentrations. To sample the continuous background concentrations, grab samples were taken in a regular, 3-week cycle (n = 518). To sample rainfall-driven peaks, we used automatic sampling devices triggered by a water level increase resulting in sampling during or directly after rainfall. These event-driven samples (EDS, n = 312) are of high ecological relevance, capturing transient, short-term peak concentrations of pesticides in surface waters, which have been shown to especially affect stream communities and relate to biological effects (Liess and Schulz, 1999). All stream water samples were analysed for 74 pesticides and 33 pesticide metabolites using LC-MS/MS (see substance list and analytical details in SI). The selection of analytes was based on (i) pesticide use data in relation to its toxicity, (ii) substances occurring in elevated concentrations in previous monitoring programs and (iii) compatibility with a multi-substance method for chemical analysis (Wick et al., 2019). We thus assume that we have captured the main proportion of pesticide toxicity. All data are publicly available in Liess et al. (2021b).

4.2.4 Toxicity Calculations

The Toxic Unit (*TU*) concept was applied to estimate the toxicity of a substance and of mixtures in the environment (Sprague, 1971). Predicted and measured substance concentrations c_i were normalised to their respective EC_{50} – the concentration that causes a defined effect in 50% of test organisms. Hence, the toxicity of substance *i* described as TU_i is defined as

$$TU_i = \frac{c_i}{EC_{50_i}} \qquad 1$$

The mixture component resulting in the highest environmental toxicity yields the highest *TU*-value, the TU_{max}:

$$TU_{max} = max_{i=1}^{n} \frac{c_i}{EC_{50_i}} \quad (2)$$

We also aimed to predict which pesticides drive stream water toxicity by modelling surface water concentrations of the monitored PPP applications and identifying pesticides applied causing the TU_{max}. Toxicity drivers were defined as pesticides predicted to cause a log TU_{max} > -4 in at least 1% of applications. We then validated our predicted toxicity drivers to those pesticides causing a log TU_{max} > -4 in at least 1% of event-driven stream water samples.

To evaluate and quantify the risk caused by pesticide mixtures, we applied the Concentration Addition (CA) approach (Loewe and Muischnek, 1926), that has proven predictive power and is the recommended default for the ERA mixture toxicity assessment (Altenburger et al., 2000; EFSA Scientific Committee et al., 2019; Rodney et al., 2013). Following CA, the total toxicity of the mixture TU_{mix} is calculated by adding together the *TU*s of all the individual mixture components *i*:

$$TU_{mix} = \sum_{i=1}^{n} \frac{c_i}{EC_{50_i}} \quad (3)$$

Other approaches such as Independent Action (IA) require more data and have led to less conservative predictions when comparing predicted and observed laboratory experiment effects, with some exceptions where mixtures explicitly consisted of dissimilarly acting toxicants (Backhaus et al., 2000; Bliss, 1939).

*TU*s were calculated for the organism groups of aquatic invertebrates (AI) and algae/aquatic plants (AP) (for EC_{50} values see SI Table 4). Given their sensitivity to pesticides, surrogate species of these groups are ecotoxicological standard test species and therefore provide a high data availability (SI Table 5). We considered mortality for AI and growth rates or biomass for AP as effect measures considered for the EC_{50}. These

ecotoxicity data were retrieved from the PPDB database (Lewis et al., 2016). Data assigned a quality criterion equal to or less than 2 was discarded to exclude unverified data from unknown sources.

Mixture risk was also evaluated from a regulatory perspective by applying regulatory acceptable concentrations (RACs). These are defined as surface water concentrations that, if not exceeded, are assumed to ensure no unacceptable effects on the environment. RACs were retrieved from the German Federal Environment Agency (Umweltbundesamt – UBA) and reflect the state of regulation during the stream monitoring period (see SI Table 4) (European Union, 2009; UBA). By analogy with *TU*s, risk quotients (*RQ*s) relate a measured concentration to the respective RAC instead of to EC_{50} in the case of the *TU*, and indicate whether a single pesticide (RQ_{max}) or the mixture (RQ_{mix}) pose an unacceptable risk from a regulatory point of view (*RQ* > 1).

$$RQ_{max} = max_{i=1}^{n} \frac{c_i}{RAC_i} \quad (4)$$

$$RQ_{mix} = \sum_{i=1}^{n} \frac{c_i}{RAC_i} \quad (5)$$

Each RAC is based on the effect concentration observed for the most sensitive organism group for a particular pesticide and an assessment factor to account for the uncertainty when predicting field effects from experimental data. Hence, a pesticide RAC may relate to either AI, AP or fish. RQ_{mix} values were calculated separately for the organism groups AI and AP by only summing up *RQ*s of pesticides with RAC values for these groups. AI represented the most sensitive organism group of 22 pesticides analysed in this study (12 insecticides, 8 fungicides, 3 herbicides, see substance list in SI). AP represented the most sensitive organism group of 36 analysed pesticides (34 herbicides, 2 fungicides).

Finally, the maximum cumulative ratio (*MCR*) allows to identify the contribution of a single compound to the mixture by comparing the

additive toxicity of the mixture with the highest toxicity of a single component (Price and Han, 2011):

$$MCR = \frac{Toxicity\ of\ the\ mixture}{Highest\ toxicity\ of\ single\ mixture\ component} \quad 6$$

The *MCR* thus estimates the factor by which the mixture is more toxic than the highest single pesticide toxicity in terms of *TU*s. The *MCR* was calculated for the mixtures in (i) a PPP (MCR_{PPP}), (ii) an application (MCR_{app}) and (iii) water samples (MCR_{sample} and MCR_{RAC}, see equations 7-10). The *MCR* of a mixture is generally different for the endpoints AI and AP due to the deviating EC_{50} values. To generalise across the organism groups AI and AP, we calculated the arithmetic mean of the organism group-specific *MCR*s.

PPP

$$MCR_{PPP} = \frac{TU_{mix}\ of\ PPP}{TU_{max}\ of\ PPP} \quad 7$$

Application

$$MCR_{app} = \frac{TU_{mix}\ of\ application}{TU_{max}\ of\ application} \quad 8$$

Water samples

$$MCR_{sample} = \frac{TU_{mix}\ of\ sample}{TU_{max}\ of\ sample} \quad 9$$

$$MCR_{RAC} = \frac{RQ_{mix}\ of\ sample}{RQ_{max}\ of\ sample} \quad 10$$

All calculations were performed using the statistical software R (version 3.5.1), all plots were created using the "ggplot2" R package (version 3.2.0) (R Core Team, 2017; Wickham, 2009b).

4.3 Results and Discussion

4.3.1 Quantifying the Increased Risk Posed by Pesticide Mixtures

We estimated the toxicity of pesticide mixtures in single plant protection products (PPP), PPP applications and water samples. By calculating the Maximum Cumulative Ratio (*MCR*), we assessed and compared the pesticide mixture risk in these mixture categories. Regardless of the mixture category, the MCR generally increased with the number of mixture components (Figure 17). Conversely, the fewer pesticides a mixture contained, the more its risk was driven by a single component (low *MCR*). Details of the investigated mixture categories are given below:

Single PPP - The PPP that were sprayed during the main application period from April to mid-July (n = 464) contained a mean of 1.3 different pesticides (min = 1, max = 4, Figure 17 - Single PPP). 30% (n = 138) of PPP consisted of at least two pesticides. PPP applied in apple cultures generally contained fewer pesticides (mean = 1.1), whereas PPP used to treat sugar beet and cereals were more likely to contain a mixture of pesticides (mean = 1.5). PPP mixtures showed a mean MCR_{PPP} of 1.1 (10^{th} percentile = 1, 90^{th} = 1.2, Figure 17).

Single application - The PPP applications (n = 3,446) of one or several PPP at a timepoint contained a mean of 3.1 pesticides (min = 1, max = 12) and 2.2 PPP (min = 1, max = 7). In 80% (n = 2751) and 73% (n = 2513) of applications, multiple pesticides or PPP were applied simultaneously. Cereals and sugar beet in particular were characterised by the highest number of pesticides per application (mean = 3.3 and 4.3, Figure 17 - Single application). Apple and oilseed rape cultures exhibited the lowest number of pesticides per application (mean = 2 and 2.2, respectively). Pesticide mixtures in applications revealed a mean MCR_{app} of 1.3 (10^{th} = 1, 90^{th} = 1.9). Apple and rape applications were on average 1.1, cereals 1.3 and sugar beet 1.8 times more toxic than the most potent mixture component.

Stream water - Pesticide mixtures detected in the streams, by comparison with the other mixture categories, were far more complex containing a mean of 17 (27 including metabolites) detected pesticides in grab samples (n = 518) and 30 (42 including metabolites) in event-driven samples (EDS) (n = 312) taken during rainfall induced exposure peaks (Figure 17 - Stream water). A maximum of 57 pesticides was detected in a single EDS. Hence, we detected almost twice as many pesticides in an average EDS compared with the common grab sample and ten times as many as sprayed in an application. Pesticide mixtures detected in EDS were on average 2.2 times more toxic than the most potent pesticide alone (MCR_{sample}, 10^{th} = 1.5, 90^{th} = 3.1, including measured metabolites). In 69% of the grab samples (n = 360) and 43% of EDS (n = 133), a single pesticide caused a higher toxicity than all other detects in combination ($MCR_{sample} < 2$). During exposure peaks, an increased MCR_{sample} of 2.7 was shown for aquatic plants/algae (AP), whereas a minor impact of the sampling method was found for aquatic invertebrates (AI) with an MCR_{sample} of 1.7. In the grab samples, the mean MCR_{sample} yielded 1.8 (10^{th} = 1.1, 90^{th} = 2.5) and was comparable for AI and AP. Especially for AP, mixtures thus become more relevant during rain-induced exposure peaks as more pesticides occur in relatively high concentrations and contribute to the overall risk.

Generally, the additional risk by mixtures in stream water was not associated with the total estimated pesticide toxicity: The logarithmic TU_{mix} exhibited no correlation with the MCR_{sample} (for AI: $R^2 = 0.01$, $p < 0.005$; for AP: $R^2 = 0.01$, $p < 0.005$). Even at low toxic pressure, where the number of detected compounds decreased, the MCR_{sample} remained relatively constant. This suggests that the *MCR* calculation was largely unaffected by analytical constraints in terms of limits of quantification. Furthermore, no influence of the hydrological catchment size on the MCR_{sample} was observed ($R^2 < 0.01$, $p = 0.05$, area log-transformed). Within the limited gradient of studied catchment sizes, we therefore observed the pesticide mixture risk in different-sized stream or river systems to be comparable.

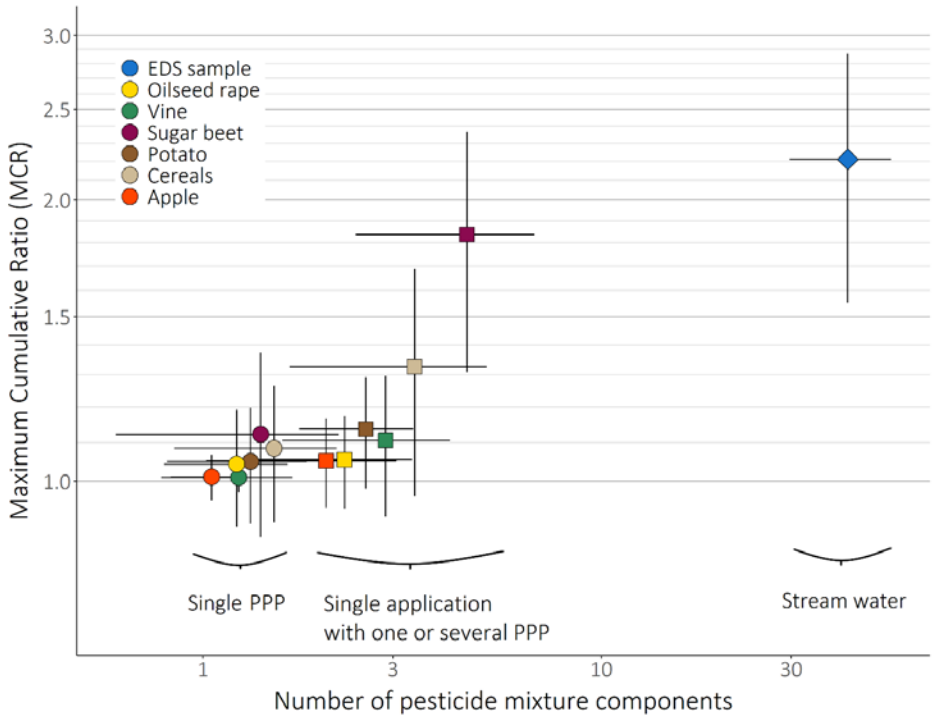


Figure 17: *MCR*s of different mixture categories against the number of pesticide mixture components: Culture-specific Plant Protection Products (PPP) applied (MCR_{PPP}, circle), applications (MCR_{app}, square) and EDS stream water samples (MCR_{sample}, diamond, including metabolites). Data points represent mean values and bars display the respective standard deviation.

Our findings match those of Vallotton and Price (2016) who derived slightly higher MCR_{sample} values from 2.4 to 2.85 for pesticide mixtures in grab samples from US American surface waters. Accordingly, Gustavsson et al. (2017) found MCR_{sample} values for AI and AP in weekly samples from Swedish small agricultural streams ranging from 2.22 to 2.86, which were constant across streams of different catchment sizes. Regional differences in PPP use and climate conditions impact the spectrum of mixture components and their environmental fate. Nevertheless, comparable pesticide contamination of surface waters has been observed in several other parts of the world, including Africa (Ganatra et al., 2021), Australia (Burgert et al., 2011), France, Finland (Schäfer et al., 2007) and South America (Hunt et al., 2017). Therefore, despite varying mixture

components, we expect the risk due to simultaneous pesticide mixtures in the environment to be comparable wherever similar agricultural practices are followed.

The *MCR* values increased from PPP to single applications and water samples indicating a stepwise increase of the pesticide mixture risk. In a first step, application practices combining multiple PPP lead to enhanced mixture risk. In a second step, pesticide residues of these sequential applications from numerous fields featuring different crops with varying PPP treatments within the catchment area enter streams resulting in more complex pesticide cocktails. As the authorisation of PPP is performed at single PPP level, the respective ERA only considers mixtures as represented by the MCR_{PPP}. In the environment, however, pesticide risk is on average twice as high when considering mixtures assuming concentration addition ($MCR_{sample} \approx 2 \times MCR_{PPP}$). We consider the 95th percentile of the event-driven sampling MCR_{sample} of 3.4 to reflect realistic worst-case pesticide mixture conditions. A factor of 3.2 would thus be required to extrapolate from single PPP risk to environmental pesticide mixture risk ($3.4 \approx 3.2 \times MCR_{PPP}$) to cover mixture risk in 95% of observed peak exposure scenarios.

This extrapolation factor relies on the assumption of additive effects from pesticide mixtures, which is recommended as default in the ERA mixture toxicity assessment (EFSA Scientific Committee et al., 2019). While the effects of most mixtures of pesticides were shown to be additive, specific pesticide combinations greatly exceeded the additive effect predictions, i.e. acted synergistically (Cedergreen, 2014). Synergistic combinations may also involve a pesticide and other pollutants like metals or antifoulants. In addition, synergisms were exacerbated when organisms were exposed to additional environmental stress, such as food limitation (Liess et al., 2016; Shahid et al., 2019). In the case of synergistic combinations, the proposed additive mixture extrapolation factor of 3.2 still underestimates the actual ecological effect.

4.3.2 Pesticide Mixtures in the Light of Regulatory Thresholds

Single PPP are generally regulated in such a way that the modelled peak concentrations remain, often only marginally, below predicted ecological threshold levels ($RQ_{max} < 1$). In the field, multiple pesticides may co-occur in concentrations close to their regulatory acceptable concentration (RAC). In combination, mixture components may then accumulate to exposure levels jointly posing an unacceptable risk to aquatic organisms ($RQ_{mix} > 1$) (Junghans et al., 2019).

We therefore assessed the likelihood of pesticides individually or jointly (sum of components primarily affecting the same organism group) causing threshold exceedances in EDS (n = 312). RAC exceedances already by single pesticides for AI and AP were detected in 53% and 18% of EDS, respectively ($RQ_{max} > 1$, see Figure 18). Adding up the risk from all mixture components affecting either AI or AP, the exceedances in EDS increased to 66% and 26% ($RQ_{mix} > 1$). On the one hand, this shows that AI, in particular, are frequently subject to RAC-exceeding pesticide concentrations. On the other hand, 81% (AI) and 69% (AP) of joint RAC exceedances were due to single pesticides, though several samples revealed MCR_{RAC} values greater than 4 or 5. The MCR_{RAC} resulted in a mean value of 1.6 (10^{th} = 1.0, 90^{th} = 2.2) for AI reflecting a 63%-contribution of a single pesticide to the RQ_{mix}. For AP, the mean MCR_{RAC} of 2.4 (10^{th} = 1.3, 90^{th} = 3.6) reflected a 42%-contribution of the dominant pesticide to the RQ_{mix} and affirmed the increased mixture risk for AP compared with AI. Rather than through the joint action of many individual mixture components, exceedances of regulatory thresholds are primarily caused by single pesticides in high concentrations. Nevertheless, the frequent exceedances of regulatory thresholds by single pesticides alone is further aggravated by the joint toxicity of mixtures in the stream water samples.

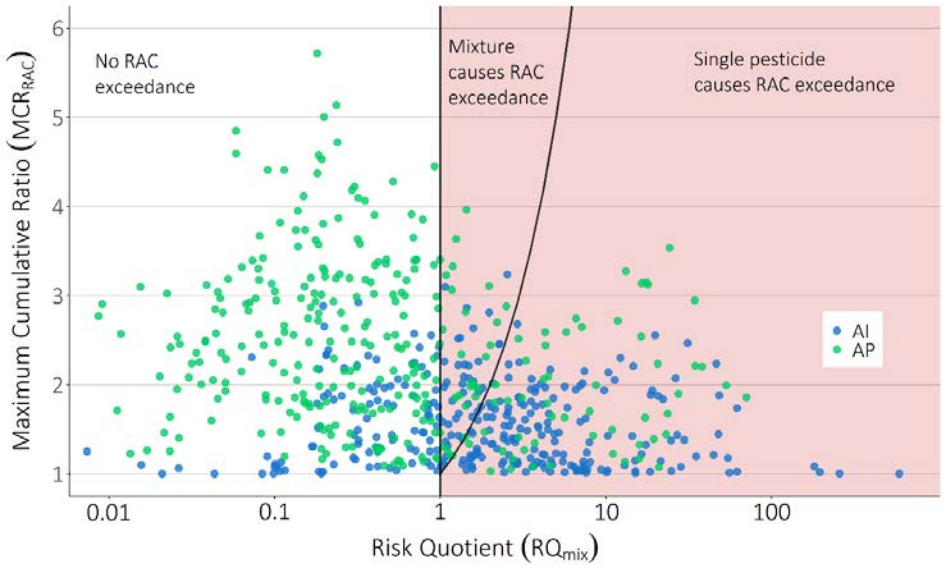


Figure 18: The additive concentration-RAC quotient (*RQmix*) indicating regulatory threshold exceedance and respective Maximum Cumulative Ratio (*MCRRAC*) derived separately for aquatic invertebrates (AI, blue dots) and aquatic plants/algae (AP, green dots) of each event-driven stream water sample (n = 312). Log *RQmix* values ≤ 0 represent samples not exceeding the RAC (34% for AI, 74% for AP). Log *RQmix* values > 0 represent samples exceeding the RAC (within red shaded area). Dots between the black lines represent samples that exceed the RAC only as a mixture (13% for AI, 8% for AP). Dots to the right of the curved, black line represent samples where single substances already exceed the respective RAC (53% for AI, 18% for AP).

To derive the *RQmix* of a sample, all *RQ*s of pesticides affecting the same organism group (AI or AP) were cumulated. This approach may underestimate the actual ecological risk as (i) indirect pesticide effects may enhance the sensitivity of another organism group and increase the overall risk faced by the aquatic ecosystem (Edge et al., 2020; Fernández et al., 2015), (ii) pesticides primarily affecting one organism group may still adversely affect other organisms (Misaki et al., 2019) and (iii) pesticides primarily affecting organisms omitted from our analysis (e.g. fish) additionally contribute to the mixture risk. This *RQmix* approach, however, relies on laboratory-based effect concentrations and can thus only estimate the actual ecological risk in the field.

To derive the RQ_{mix} of a sample, all *RQ*s of pesticides affecting the same organism group (AI or AP) were cumulated. This approach may underestimate the actual ecological risk as (i) indirect pesticide effects may enhance the sensitivity of another organism group and increase the overall risk faced by the aquatic ecosystem (Edge et al., 2020; Fernández et al., 2015), (ii) pesticides primarily affecting one organism group may still adversely affect other organisms (Misaki et al., 2019) and (iii) pesticides primarily affecting organisms omitted from our analysis (e.g. fish) additionally contribute to the mixture risk. This RQ_{mix} approach, however, relies on laboratory-based effect concentrations and can thus only estimate the actual ecological risk in the field.

4.3.3 The Variable Dominance of Single Pesticides

Both the low *MCR* values and the regulatory threshold exceedances described above indicate that the main contribution to the toxicity of a mixture could be largely attributed to a single pesticide. However, the identity of these pesticides was found to vary spatio-temporally: 55 different pesticides and 3 pesticide metabolites of the 107 analytes were dominant and ecotoxicologically relevant (log $TU_{max} > -4$) for AI or AP in at least one stream water sample. 21 different pesticides and 1 metabolite were dominant in at least 1% of the samples (see SI Table 4). Previous studies confirmed that pesticide mixture risks in aquatic ecosystems are driven by 1 to very few alternating compounds that vary among sites (Gustavsson et al., 2017; Liess and Ohe, 2005; Liess and Schulz, 1999; Markert et al., 2020; Stenström et al., 2021; Vallotton and Price, 2016). The dominance of single pesticides in the monitored PPP applications implies similar conditions in agricultural fields. This marks a departure from the many studies investigating the effect of mixtures, in which the individual components equally contribute to mixture risk (Altenburger et al., 2000; Backhaus et al., 2000; Silva et al., 2002). Assessing the risk of these equitoxic mixtures proved the combined effect of mixture components in principle, but does not reflect the observed toxic imbalance of components in the environment and thus overrates pesticide mixture relevance. Laboratory toxicity tests assessing the effects of mixtures should consider

this toxic imbalance of components for an improved simulation of environmental conditions. For pesticide monitoring programs, the variable spectrum of dominant substances observed here suggests a broad set of analytes to be measured ideally comprising all pesticides applied in a stream's catchment area.

We further assessed whether pesticides that were identified to drive stream water toxicity can be predicted based on the spray series data. Our exposure modelling led to 27 pesticides causing a log TU_{max} > -4 in at least 1% of monitored applications (see SI Table 2). However, only 5 of these matched the subset of the 21 pesticides identified as drivers in real water samples. The other 22 pesticides were not identified as drivers in the water samples (n = 9 pesticides) or were absent from the list of analytes (n = 13). Therefore, identification of pesticide toxicity drivers using our application data was limited. Reasons for this may be (i) the changing spectrum of PPP and mitigation measures applied over the years so that the time interval of several years between the monitoring of spray series and streams limits the comparability and (ii) the lack of location information for the monitored applications: We expect that georeferenced spray series data on catchment-scale are needed to account for locally specific cultures shaping mixture patterns. To enhance our predictive capacity of environmental mixtures, more precise knowledge about the timing and localisation of PPP applications is required.

4.3.4 The Frequency of Recurring Exposure Pulses

The mixtures identified in this study represent one-time snapshots of environmental conditions, but over the longer term, the investigated pesticide exposure pulses occur repeatedly. The ERA of pesticides requires that "populations of short-cyclic water organisms" and "species with contrasting life cycle traits (i.e. longer generation time) are able to completely recover in the time available between the exposure events" (Environmental Recovery Option – ERO) (EFSA, 2013). This is at least questionable according to the monitored spray series where an average field faced more than 1 application per month during our stream monitoring period from April to mid-July (Figure 19). In 30% (n = 266) and

75% (n = 670) of analysed spray series, a follow-up application was sprayed less than 7 or 24 days after the previous application. Especially for crops with high application frequency such as apple (mean = 20 times per season, see SI Table 1), potato (10), and vine (8), it can be assumed that application intervals are too short to allow non-target organisms to fully recover or for pesticide residues to degrade. The agricultural streams also encountered a mean of 2.5 and up to 10 exposure pulses resulting in RAC exceedances during the sampling period (Figure 19). In 88% of spray series and 53% of streams, such pulse intervals were, at least once, shorter than 8 weeks – the time period after exposure in which recovery renders adverse effects acceptable under the ERO in the ERA (EFSA, 2013).

Especially vulnerable species are often characterised by generation times of six months or longer clearly exceeding exposure pulse intervals (Liess and Ohe, 2005). Individual-, population-, and community-level effects can accumulate within a single generation (Wiberg-Larsen et al., 2020) and culminate over multiple generations (Liess et al., 2013). Indirect effects (e.g. competition) further increase pesticide sensitivity and can delay recovery from pulse exposure (Dolciotti et al., 2014; Foit et al., 2012; Knillmann et al., 2012). Conversely, species and whole communities have been seen to recover from single pulses and even acquire tolerance to toxic pressure to a certain degree (Beketov et al., 2008; Shahid et al., 2018). Hence, complex and partly contradictory processes determine the effect of sequential exposure and its prediction is therefore challenging. This in turn complicates risk assessment, where no general concept has yet been identified to account for sequential exposure and this uncertainty is translated into assessment factors that lack robust validation.

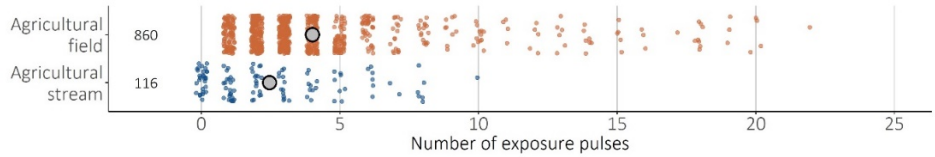


Figure 19: Number of exposure pulses from April to mid-July (stream monitoring period) for agricultural fields and streams. Orange points reflect the number of applications per field (n = 860), blue points reflect number of samples showing a RAC exceedance ($RQ_{max} > 1$) per stream (n = 116). Grey points depict respective means.

4.4 Conclusion

While PPP are considered mostly individually in the process of authorisation, we found them to occur almost exclusively as a mixture in the environment. 73% of PPP applications already featured a mixture of multiple PPP and stream water samples exhibiting the pesticide use footprint of an entire catchment revealed a mean of 30 detected pesticides. However, we revealed that environmental pesticide mixtures are mostly dominated by one, but alternating, pesticide. Assuming additive effects of mixture components and realistic worst-case conditions, the simultaneous pesticide mixture risk in the environment exceeds the estimated single PPP toxicity by a factor of 3.2. However, uncertainties remain concerning the validity of the additive effect of mixtures under environmental conditions disregarding any potential synergistic interactions. The proposed factor also does not account for the observed sequential pesticide exposure, where the high frequency of pesticide applications and recurring inputs into surface waters most likely exacerbate the ecological risk. Our findings imply that both the simultaneous mixture risk as well as the sequential pesticide exposure represent typical field conditions and hereby confirm concerns described by EFSA's aquatic guidance document stating that "assessing risks for individual PPPs for their use in crop protection programmes characterised by intensive PPP use (e.g. simultaneous use of PPPs with similar mode of action in tank mixtures or their repeated use)" may be "uncertain". The ERA of pesticides thus needs to consider simultaneous and sequential exposure. Further research is needed to estimate the environmental

relevance of mixture component interactions (synergism and antagonism) under realistic conditions and to elaborate concepts enabling a quantification of the additional ecological risk due to sequential exposure. This study therefore provides one piece of the puzzle to narrow the gap between prospective single PPP-oriented risk assessment and reality.

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5 Assessing the Mixture Effects in In Vitro Bioassays of Chemicals Occurring in Small Agricultural Streams during Rain Events

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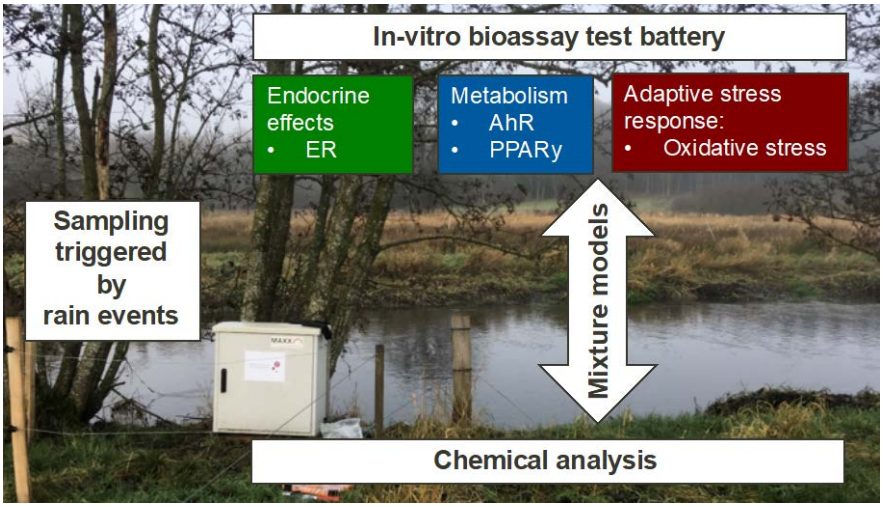
Abstract

Rain events may impact the chemical pollution burden in rivers. Forty-four small streams in Germany were profiled during several rain events for the presence of 395 chemicals and five types of mixture effects in in-vitro bioassays (cytotoxicity, activation of the estrogen, aryl hydrocarbon and peroxisome proliferator-activated receptors and oxidative stress response). While these streams were selected to cover a wide range of agricultural impacts, in addition to the expected pesticides, wastewater-derived chemicals and chemicals typical for street run-off were detected. The unexpectedly high estrogenic effects in many samples indicated impact by wastewater or overflow of combined sewer systems. The 128 water samples exhibited a high diversity of chemical and effect patterns, even for different rain events at the same site. The detected 290 chemicals explained only a small fraction (<8 %) of the measured effects. The experimental effects of designed mixtures of detected chemicals that were expected to dominate the mixture effects of detected chemicals were consistent with predictions for concentration addition by a factor of two for 94 % of the mixtures. Overall, the burden of chemicals and effects were much higher than previously detected in surface water during dry weather with the effects often exceeding effect-based trigger values.



5.1 Introduction

Surface waters can be impacted by a large number of organic micropollutants, including pesticides, pharmaceuticals and industrial compounds, which can enter the aquatic environment from both point sources, such as wastewater effluent discharge, and non-point sources, such as agricultural run-off. Small streams have large lotic biodiversity, but, in comparison to larger systems, can be disproportionally affected by chemical pollution due to smaller dilution ratios (Lorenz et al., 2017). Pesticides from agricultural run-off reduced invertebrate biodiversity in streams in Australia and Europe (Beketov et al., 2013; Liess and Ohe, 2005) and wastewater treatment plant (WWTP) effluents may also impact invertebrates (Münze et al., 2017). Further, the ecological effects of pesticides on small streams generally increase after rainfall events due to run-off from agricultural areas (Szöcs et al., 2017).

Several studies that have evaluated the risk posed by organic chemicals in small streams have focused on chemical analysis (Le et al., 2017; Spycher et al., 2018). Targeted chemical analysis is traditionally applied to monitor chemical water quality, but lacks information on effects of non-target chemicals or chemicals at concentrations below analytical detection limits. Still, these may contribute to the overall effect. In-vitro bioassays can be applied for water quality monitoring to detect the mixture effects of chemicals present in a sample. Combinations of in-vitro bioassays and chemical analysis have been applied mainly to larger water bodies (Creusot et al., 2014; König et al., 2017; Scott et al., 2014; Tousova et al., 2017), with fewer studies addressing smaller streams and mainly under low flow conditions in dry weather (Müller et al., 2018; Neale et al., 2017b). In contrast, during rainfall events, concentrations of pesticides and their transformation products have been observed to peak in small rivers (Kern et al., 2011; Leu et al., 2004). Given that substantial effects in in-vitro assays have been observed in collected stormwater (Kayhanian et al., 2008; Tang et al., 2013), it is timely to ask the question how chemicals and their mixtures assessed by an in-vitro test battery fare during rain events in small streams.

We assessed the chemical burden in small agricultural streams during rainfall events using a battery of in-vitro bioassays to identify which mixture effects exceed acceptable levels and which types of chemicals are driving the observed mixture effects. Water extracts were collected from 44 sites throughout Germany, with multiple samples collected during different rain events at most sites. The studied bioassays covered different stages of cellular toxicity pathways, including induction of xenobiotic metabolism, hormone receptor-mediated effects and adaptive stress responses. Specifically, this included assays indicative of activation of the aryl hydrocarbon receptor (AhR), binding to the peroxisome proliferator-activated receptor gamma (PPARγ), activation of the estrogen receptor (ER) and oxidative stress response. These bioassays were responsive in surface water and wastewater (Escher et al., 2014; König et al., 2017; Nivala et al., 2018), with the endpoints also identified as most the responsive and therefore priority endpoints for surface water using the multiplexed Attagene assays that cover 69 endpoints (Blackwell et al., 2019; Corsi et al., 2019; Escher et al., 2014). The effect in the water extracts were compared with bioassay specific effect-based trigger values (EBTs) derived from Environmental Quality Standards (EQS) from the European Union Water Framework Directive (WFD) (Escher et al., 2018a). In addition to bioanalysis, chemical analysis of 395 chemicals including pesticides, pharmaceuticals and industrial chemicals was undertaken.

Iceberg modelling using the bioanalytical equivalent concentration (BEQ) approach was applied in the current study to determine the contribution of detected chemicals to the observed effect (Neale et al., 2018). Bioanalytical equivalent concentrations from bioanalysis ($BEQ_{bio,iceberg}$) relates the effect of the sample to the effect induced by the assay reference compound, whereas bioanalytical equivalent concentrations from chemical analysis (BEQ_{chem}) are determined based on the concentration of a chemical in a sample and its relative effect potency (REP_i). BEQ_{chem} is similar to the toxic unit (TU) approach (Beckers et al., 2018; Kuzmanović et al., 2015) or exposure-activity ratio (EAR) approach (Corsi et al., 2019), and the different measures can be converted into each other (Villeneuve et al., 2019).

The BEQ concept is based on the assumption that the many chemicals in a mixture act in a concentration additive manner, which was appropriate to predict mixture toxicity in assays indicative of receptor-mediated effects, adaptive stress responses and cytotoxicity (Escher et al., 2013; Tang et al., 2013; Tang et al., 2014). In the field, stress can exacerbate the mixture effects and lead to more-than additive effects (Shahid et al., 2019), but for large number of chemicals, as in our study, additive mixture models are considered as broadly applicable also in in-vivo assays (Belden et al., 2007). $BEQ_{bio,iceberg}$ and BEQ_{chem} can be compared to determine how much of the effect is explained by detected chemicals. In previous studies only a small fraction of the sample's effect in assays indicative of xenobiotic metabolism and adaptive stress responses could be explained by the quantified chemicals (Belden et al., 2007; Creusot et al., 2014; Escher et al., 2014; König et al., 2017; Neale et al., 2015; Neale et al., 2017a). This is likely due to the thousands of non-quantified chemicals expected to be present in water samples (Escher et al., 2020b) that may trigger these bioassays. To further explore which and how chemicals contribute to the known effect (i.e., the "tip of the iceberg") (Tang et al., 2014), more than 200 synthetic mixtures of detected chemicals were run in the bioassays indicative of activation of AhR, binding to PPARγ and oxidative stress response. In contrast, for hormonal effects, a small number of potent hormone receptor agonists can typically explain the majority of effects (Könemann et al., 2018), and therefore no synthetic mixtures were measured in the assay for the activation of ER.

5.2 Materials and Methods

5.2.1 Sampling and Sample Processing

128 water samples were collected from 44 sites in eleven German states from April to September 2018 (Table S1 of the Supporting Information) using a modified sampling device based on the technology introduced by Schulze et al. (2017) Rain events causing water levels to rise by at least 5 cm in the streams triggered sampling. Two different sampling devices were used. One autosampler (Maxx Mess- und Probenahmetechnik GmbH,

Rangendingen, Germany) collected forty subsamples of 50 mL over a time period of 3 hours 20 minutes during the rain event with each subsample collected every 5 min (duration of sampling approximately 45 sec). The other sampling device was also triggered by rising water levels and collected up to 1 L of water in one bottle as described by Liess and Ohe (2005). The combined water samples of each rain event yielded a volume of up to 1 L or 2 L (less if the sampling device clogged), which was enriched after filtration using solid-phase extraction (SPE) with HR-X sorbent (Välitalo et al., 2017) with SPE process blanks run in parallel. For details on sampling sites, sampling and sample processing, see SI, Section S1.

5.2.2 Chemical Analysis

395 compounds (Table S2) were analyzed by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) by direct injection as described in Section S2.

5.2.3 Bioanalysis

The extracts were run in four bioassays, AhR CALUX, PPARγ GeneBLAzer, ERα GeneBLAzer and AREc32 (see Table S4). All studied bioassays are mammalian reporter gene assays and were run in 384-well plates, with detailed methods provided in Neale et al. (2017a) and König et al. (2017). In addition to the environmental extracts, individual chemicals found at high concentrations or expected to contribute to the effect were also run in the AhR CALUX (78 chemicals), PPARγ GeneBLAzer (43 chemicals) and AREc32 (87 chemicals) assays (all fingerprinted chemicals listed in Table S5). For all assays, cell viability in the mammalian cell lines was assessed in parallel to induction based on cell confluency using an IncuCyte S3 live cell imaging system (Essen BioScience, Ann Arbor, Michigan, USA) (Nivala et al., 2018). Any concentrations that reduced cell viability by 10% or more (i.e., caused 10% or more cytotoxicity) were excluded from further data evaluation.

5.2.4 Data Evaluation

Linear concentration-effect curves at effect levels up to 30% were used for data evaluation, with the concentration causing 10% effect (EC_{10}) derived

for AhR CALUX, PPARγ GeneBLAzer and ERα GeneBLAzer and the concentration causing an induction ratio of 1.5 ($EC_{IR1.5}$) determined for AREc32. The concentration causing 10% inhibition (IC_{10}) was also evaluated using linear concentration-effect curves. Detailed information about the applied data evaluation approach is available in Escher et al. (2018b). The EC_{10} and $EC_{IR1.5}$ values were expressed as a relative enrichment factor (REF) in units of $L_{water}/L_{bioassay}$, while the EC_{10} and $EC_{IR1.5}$ values for the individual chemicals were given in molar units.

5.2.5 Iceberg Modelling

Iceberg modelling using both the BEQ and TU approaches was applied in the current study to determine how much of the observed effect can be explained by quantified chemicals and how much is due to unknown chemicals (Figure 20). Sample EC values were converted to $BEQ_{bio,\ iceberg}$ using the EC value of the reference compound (Equation 11). BEQ_{chem} was calculated using Equation 12 by summing the BEQ_i of each quantified and bioanalytically characterized chemical. BEQ_i is the product of the concentration of the detected chemical (C_i) in molar units and its REP_i. REP_i was calculated using Equation 13 using the EC value of the detected chemical i and the EC value of the reference compound. Note that $BEQ_{bio,\ iceberg}$ was based on the effect of SPE extracts, whereas BEQ_{chem} was calculated from C_i using direct injection into the LC-HRMS, which is acceptable because generally good chemical recovery was observed previously for HR-X sorbent (Neale et al., 2018). Hydrophilic compounds are likely to be poorly recovered by the HR-X sorbent, but these chemicals were not expected to contribute significantly to the observed mixture effect due to their typically much lower potency (Table S5). The EC values for the detected chemicals were either measured as part of this study or collected from the literature and the US EPA Tox21 database (Escher et al., 2020a). BEQ was expressed as benzo[a]pyrene equivalent concentrations (B[a]P-EQ) for AhR CALUX, rosiglitazone-EQ for PPARγ GeneBLAzer, 17β-estradiol equivalent concentrations (EEQ) for ERα GeneBLAzer and dichlorvos-EQ for AREc32.

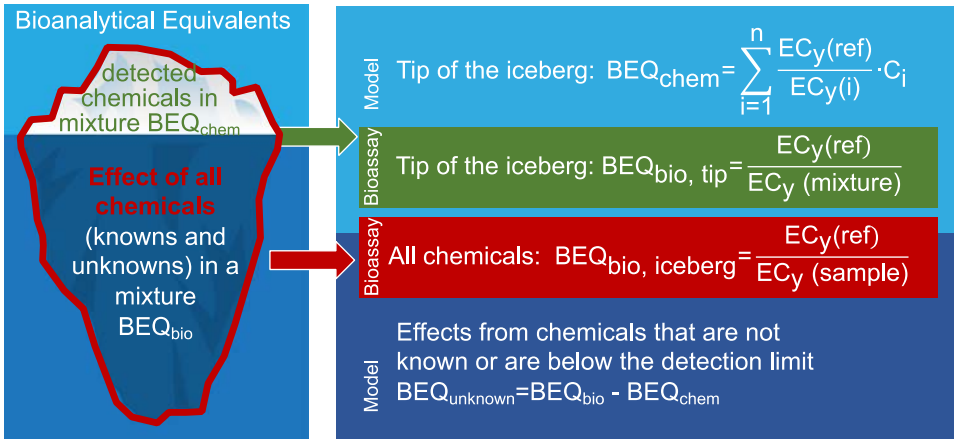


Figure 20: Bioanalytical equivalent concentrations from chemical analysis (BEQ_{chem}) are compared to the bioanalytical equivalent concentrations from bioanalysis ($BEQ_{bio,\ iceberg}$) using iceberg modelling. The contribution of detected chemicals to BEQ_{chem} (e.g., "tip of the iceberg") is determined both by modelling and using designed mixture experiments ($BEQ_{bio,\ tip}$). Y stands for the effect measure, e.g., y=10 for 10%, EC_{10}, or IR1.5 for $EC_{IR1.5}$.

$$BEQ_{bio,iceberg} = \frac{EC_y\ (ref)}{EC_y\ (sample)} \qquad 11$$

$$BEQ_{chem} = \sum_{i=1}^{n} BEQ_i = \sum_{i=1}^{n} REP_i \cdot C_i \qquad 12$$

$$REP_i = \frac{EC_y\ (ref)}{EC_y\ (i)} \qquad 13$$

The sample IC_{10} values were converted to $TU_{cytotoxicity(bio,\ iceberg)}$ using Equation 14 based on (Müller et al., 2018) TU based on chemical analysis ($TU_{cytotoxicity(chem)}$) was calculated using the detected chemical concentration and the IC_{10} value of the detected chemical i (Equation 15). IC_{10} values for analyzed chemicals were measured in the current study or collected from the US EPA Tox21 database (Escher et al., 2020a). While not commonly applied for in-vitro bioassays, TUs from chemical analysis are

often calculated for whole organisms, such as algae, daphnia and fish (Kuzmanović et al., 2015).

$$TU_{cytotoxicity(bio,\ iceberg)} = \frac{1}{IC_{10}\ (sample)} \quad (14)$$

$$TU_{cytotoxicity(chem)} = \sum_{i=1}^{n} \frac{C_i}{IC_{10\ (i)}} \quad (15)$$

The percent contribution of individual detected chemicals i to the known fraction of effect (e.g., BEQ_{chem} or $TU_{cytotoxicity(chem)}$) was calculated using Equations 16 and 17.

$$\%\ \text{contribution of i to } BEQ_{known} = \frac{REP_i \cdot C_i}{BEQ_{chem}} \cdot 100\% \quad (16)$$

$$\%\ \text{contribution of i to } TU_{known} = \left(\frac{C_i}{IC_{10(i)}} \cdot \frac{1}{TU_{cytotoxicity(chem)}} \right) \cdot 100\% \quad (17)$$

5.2.6 Tip of the Iceberg Mixtures

Chemicals that dominated BEQ_{chem} were mixed in the ratios of concentrations they were detected in the samples. For activation of AhR 17 chemicals (1H-benzotriazole, 2-benzothiazolesulfonic acid, 2-hydroxybenzothiazole, 2,6-dichlorbenzamide, 5-methyl-1H-benzotriazole, 7-diethylamino-4-methylcoumarin, chlorotoluron, diflufenican, diuron, epoxiconazole, genistein, iminostilbene, isoproturon, MCPA, metamitron, pindolol, propylparaben) were mixed in 107 combinations of detected concentrations. Pindolol and 2,6-dichlorbenzamide were added because they had shown a positive response in the Tox21 database but our experiments showed no activity. Logistic reasons prohibited preparing matching mixtures for all water samples, but 107 of 128 mixtures were prepared. For PPARγ, we mixed 17 other chemicals (2-

benzothiazolesulfonic acid, 2-hydroxybenzothiazole, 2,4-dichlorophenoxyacetic acid, 3,5,6-trichloro-2-pyridinol, 7-diethylamino-4-methylcoumarin, bezafibrate, chloridazon, desethylterbutylazine, diclofenac, losartan, MCPA, naproxen, prosulfocarb, prothioconazole-desthio, quinoxyfen, thiacloprid amide, triphenylphosphate) in 76 mixtures ratios as they were detected and one chemical (prothioconazole-desthio) turned out to be inactive during mixture experiments. For AREc32, 16 chemicals (2-benzothiazolesulfonic acid, 2-hydroxybenzothiazole, 2,4-dinitrophenol, 7-diethylamino-4-methylcoumarin, benalaxyl, desphenyl-chloridazon, dimethenamid, ethofumesate, flufenacet, genistein, iminostilbene, metazachlor, metolachlor, pethoxamid, propylparaben, triphenylphosphine oxide), one of which (benalaxyl) turned out to be inactive, were mixed in 44 mixture ratios. In addition, an equipotent mixture was prepared for all assays.

The stock solutions of the mixtures were prepared in DMSO from DMSO stocks of single compounds using a Tecan D300e Digital Dispenser (Tecan, Crailsheim, Germany). The effect concentrations of the mixtures EC_y(mixture) were reported in total molar concentration (of all 17 or 16 chemicals including the inactive ones) and converted to simulated REF by dividing by the total molar concentrations of these compounds in the water samples to yield EC_y(mixture) in units of REF. The $BEQ_{bio,tip}$ of the designed mixtures (Equation 18) were then compared with BEQ_{chem} and $BEQ_{bio,iceberg}$.

$$BEQ_{bio,\,tip} = \frac{EC_y(ref)}{EC_y(mixture)} \qquad 18$$

The index on prediction quality (IPQ, Equations 19 and 20) serves as a measure of how well experimental ($BEQ_{bio,\,tip}$) and predicted mixture effect ($BEQ_{chem,\,tip}$) agree, with an IPQ of 0 indicating optimal agreement (Altenburger et al., 1996; Escher et al., 2013).

$$\text{For } BEQ_{bio,tip} > BEQ_{chem,tip}: IPQ = \frac{BEQ_{chem,tip}}{BEQ_{bio,tip}} - 1 \quad (19)$$

$$\text{For } BEQ_{chem,tip} > BEQ_{bio,tip}: IPQ = 1 - \frac{BEQ_{chem,tip}}{BEQ_{bio,tip}} \quad (20)$$

5.3 Results and Discussion

5.3.1 Chemical Analysis

290 of the analyzed 395 chemicals were detected in at least one water sample (Table S2), with 10 to 144 chemicals detected per site. The industrial compound 2-benzothiazolesulfonic acid was most frequently detected and was found in 124 of the 128 samples (97% detection frequency). It is used in the production of rubber, is also a transformation product of mercaptobenzothiazole and its derivatives and has been previously detected in wastewater and surface water (Hug et al., 2015; König et al., 2017). It was also one of the most commonly detected chemicals in the Danube River (Neale et al., 2015). In street run-off the concentrations of 2-benzothiazolesulfonic acid were up to 50 µg/L and thus 10 times higher than in wastewater or surface water, where it was present in similar concentration ranges as in the current study (Kloepfer et al., 2005). The chemical found at the highest concentration, with up to 126.2 µg/L (average concentration 11.2 µg/L), was oxypurinol, which is the pharmaceutical metabolite of the anti-gout pharmaceutical allopurinol, and has previously been found at concentrations up to 22.6 µg/L in German surface water (Funke et al., 2015). The chemical profile also varied between sites and over time, with some sites dominated by pesticides and others containing higher concentrations of pharmaceuticals and personal care products (PPCPs) (Figure 21 & S1). A thorough evaluation of the chemical analysis is beyond the scope of the present study, which focuses on bioassays.

5.3.2 Bioanalysis

The observed effect in the activation of AhR, binding to PPARγ, activation of ER, oxidative stress response and cytotoxicity varied both between sites and within the same site over time (Figure 21 & S2, see Table S6 for all EC values). For example, estrogenic activity varied by almost a factor of one hundred in Site 22 between different rain events (Figure 21). Activation of ER was often the most responsive endpoint, followed by the responses of assays indicative of xenobiotic metabolism, activation of AhR and binding to PPARγ. The oxidative stress response assay was in many sites the least responsive.

While the studied small streams were in agricultural areas, five of the 44 sites (5, 26, 29, 35, 37) were directly impacted by municipal WWTP effluents and three others (sites 21, 22, 23) by industrial WWTPs (Table S1). Several other sites showed typical markers of wastewater, including the pharmaceutical carbamazepine and artificial sweeteners sucralose and saccharin (Table S1). A subset of these sites often had EC_{10} values less than one (i.e., effect observed after dilution) in the activation of ER assay pointing towards wastewater discharge (e.g., sites 13, 31 and 36). This suggests that water from water retention basins or combined sewer systems, where capacities were exceeded during rainfall events, entered streams or diffuse effluents from small upstream urban areas (Table S1) contributed to the effects.

The level of activation of AhR and binding to PPARγ was similar to that previously observed in the German Ammer River, with EC_{10} REF values ranging between 2.0 to 35 and 1.1 to 90, respectively (Müller et al., 2018). In contrast, estrogenic activity in the small streams was often higher than the observed effect in the Ammer River (Müller et al., 2018), with many of the samples showing activity similar to wastewater effluent (Escher et al., 2014; König et al., 2017). The oxidative stress response was in a similar range as detected previously in streams and rivers in Australia, Germany and Switzerland (Escher et al., 2014; Müller et al., 2018; Neale et al., 2017b).

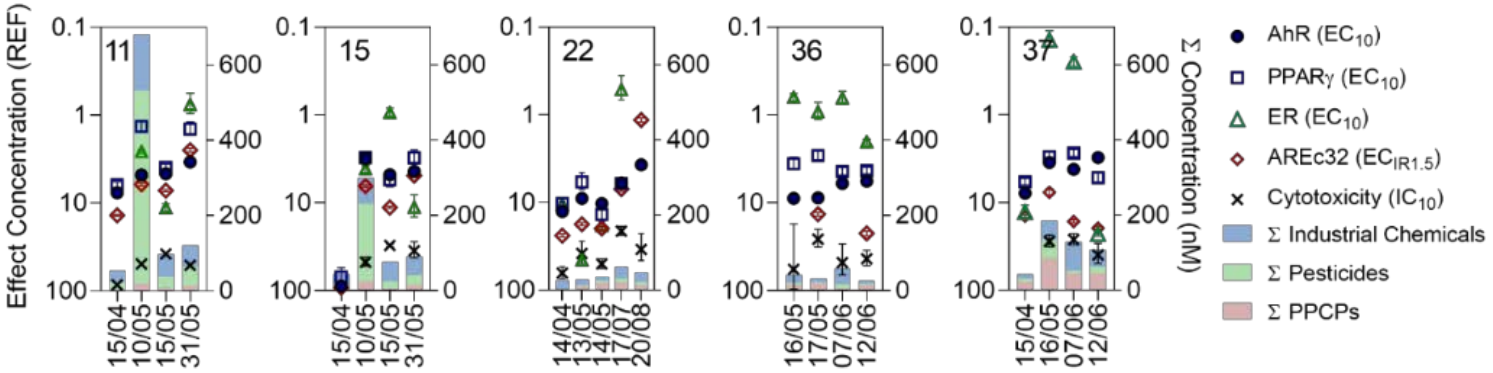


Figure 21: EC values for activation of AhR, binding to PPARγ, activation of ER and oxidative stress response (AREc32) for selected sites (11, 15, 22, 36, 37), with sum concentration of industrial compounds, pesticides and pharmaceuticals and personal care products (PPCPs) (nM). Cytotoxicity IC_{10} values are for the AhR CALUX, with IC_{10} values for the other assays provided in Table S6.

5.3.3 Comparison of Measured Effects in the Water Samples with Effect-Based Triggers

The surface water extract EC values were converted to $BEQ_{bio,\ iceberg}$ values in units of ng or µg of reference compound per litre and were compared with preliminary surface water effect-based triggers (EBTs) derived from the EU Water Framework Directive (Escher et al., 2018a). The preliminary EBTs, which were derived by reading across from the current environmental quality standards in the Water Framework Directive and applying a mixture factor where necessary, were updated with the newly available single chemical effect data (Table S5, no update of EBT for ERα GeneBLAzer) using the template provided by Escher et al. (2018a).

The EEQ of 79% of samples (Table S6) exceeded EEQ-EBT of 0.34 ng_{E2}/L for ERα GeneBLAzer (Escher et al., 2018a) (Figure 22A), which was an unexpectedly high percentage, given that the sampling sites were selected with a focus on agricultural impact. However, chemicals usually associated with treated or untreated wastewater were detected at several sites (Table S1), which is consistent with the high EEQs. Previously, the EBT-EEQ had been able to differentiate clearly between wastewater and surface water with surface water rarely exceeding the EBT-EEQ (Escher et al., 2018a). The elevated estrogenic activity could be related to lower retention times in the WWTP and thus lower treatment efficacy and diffuse input of urban stormwater contamination from combined sewer systems. Rain events can also lead to dilution but since we only sampled during rain events, not the periods before and after the event, we cannot judge if dilutions by rain occurred. For example, sites 5, 21, 26, 29 and 35 were impacted by wastewater (Table S1) and all exceeded the activation of EBT-EEQ. In contrast, sites 22 and 37 also had WWTPs upstream of the respective sampling sites, but only exceeded the EBT during some rainfall events.

The EBT-B[a]P-EQ for AhR CALUX was published as 6.4 $ng_{B[a]P}$/L (Leu et al., 2004) but this value was only based on four experimental EC_{10} values. Using nine additional EC_{10} values (Table S5) brought the EBT-B[a]P-EQ to 4.3 $ng_{B[a]P}$/L, indicating the robustness of the initial derivation. The EC_{10} in

Table S6 were converted to B[a]P-EQ with Equation 11 using the EC_{10} for B[a]P of 212 $ng_{B[a]P}$/L. 98% of the samples' B[a]P-EQ exceeded this EBT-B[a]P-EQ (Figure 22B). Prior experience with AhR CALUX in water samples is limited, but WWTP effluents (Nivala et al., 2018) and wastewater-impacted rivers had similarly high B[a]P-EQ values as many of the present water samples, while small streams unimpacted by wastewater had lower B[a]P-EQ levels (Müller et al., 2018).

The EBT-rosiglitazone-EQ for PPARγ GeneBLAzer was previously 36 $ng_{rosiglitazone}$/L (Escher et al., 2018a), but was only based on data for three chemicals. With now six active chemicals the revised EBT-rosiglitazone-EQ amounted to 19 $ng_{rosiglitazone}$/L. Only 13% of the samples (Table S6) were compliant, with the remainder exceeding this EBT (Figure 22C). This is in contrast to a previous study, where only untreated wastewater exceeded the preliminary EBT for PPARγ, whereas surface water samples from the Danube River were compliant (König et al., 2017). In another small stream, this revised EBT-rosiglitazone-EQ was able to clearly differentiate between unimpacted stretches and tributaries of the river and WWTP effluent or thereby impacted stretches of the river (Müller et al., 2018).

The EBT-dichlorvos-EQ for AREc32 remained virtually constant with 140 $ng_{dichlorvos}$/L despite the database increasing from 11 to 21 chemicals. 60% of the samples exceeded this EBT-dichlorvos-EQ (Table S6, Figure 22D). Again, this EBT had previously differentiated well between more polluted water (wastewater and urban stormwater) and river water (Escher et al., 2018a) and in another small stream study during dry weather, all sites, including those impacted by WWTP effluent were below the EBT-dichlorvos-EQ (Müller et al., 2018).

This comparison with EBT-BEQs as well as with previous samples from wastewater and surface water suggests that many of the sites have a high chemical mixture burden, particularly concerning chemicals that activate AhR and ER.

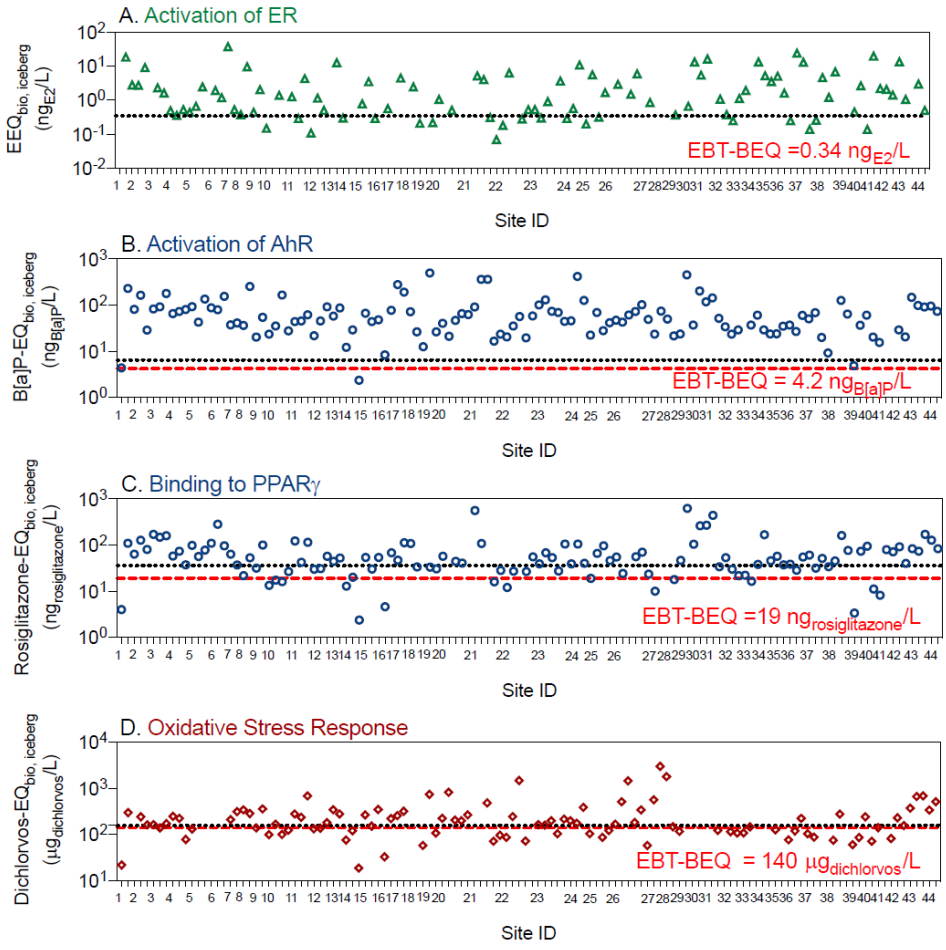


Figure 22: Comparison of water extract $BEQ_{bio,\ iceberg}$ values (ordered by site ID (Table S1)) with the preliminary effect-based trigger values (EBTs) from Escher et al. (2018a) (dotted black lines) and the revised EBTs (red dashed lines).

5.3.4 Which Chemicals Are Driving the Effects in the Water Extracts?

To better understand which chemicals are driving the observed effects, chemicals detected in the water extracts at high concentrations or expected to contribute to the effect in assays indicative of activation of AhR, binding to PPARγ and the oxidative stress response were fingerprinted. We omitted fingerprinting of single chemicals in the activation of ER assay because a small number of potent chemicals, namely natural and synthetic steroidal hormones, typically explain most of the effect in this endpoint (Conley et al., 2017; Rutishauser et al., 2004).

Bioanalysis is sufficient to characterize estrogenicity in water samples as the ratio of bioactive estrogens is typically fairly constant in surface waters (Könemann et al., 2018). A wider range of chemicals are active in assays indicative of induction of xenobiotic metabolism and adaptive stress responses (Martin et al., 2010). The IC_{10} and EC values for all chemicals measured in the current study or taken from literature are provided in Table S5.

For activation of AhR, effect measurements were available for 316 of the 395 analyzed chemicals (80%) using both experimental data and the Tox21 database. Of the 290 detected chemicals, effect data was available for 236 chemicals (81%), but most were not active (Table S5, Figure S3). EC_{10} values were available for 40 chemicals detected in the water extracts for the activation of the AhR assay. Nineteen of these values were from the Tox21 database, which used a different activation of AhR assay (rat cell line in the current study versus human cell line in Tox21 database). However, EC_{10} values for common chemicals run in both assays were generally within one order of magnitude (Figure S4), so both datasets were used to determine the effect based on chemical analysis, BEQ_{chem} (Table S7).

On average, 2-benzothiazolesulfonic acid explained 29.2% of the B[a]P-EQ_{chem} in the water extracts (between 0 to 98.2% explained), followed by the herbicide diuron (average 14.9%) (Figure 23A). The average contribution to B[a]P-EQ_{chem} is presented in Figure 23, but the contribution of each chemical to B[a]P-EQ_{chem} varied greatly for the different water extracts because the presence and concentrations of the individual chemicals varied considerably (Table S2) resulting in a wide range of B[a]P-EQ_i (Figure S5). For example, the industrial compound 7-diethylamino-4-methylcoumarin explained on average 4.8% of B[a]P-EQ_{chem} but contributed to over 95% of B[a]P-EQ_{chem} in all water extracts from the wastewater-impacted Site 37. 2-Benzothiazolesulfonic acid was one of the least potent chemicals in AhR CALUX (REP_i 5.67×10^{-6}), but it was present in all but two of the water extracts and was found at high concentrations (up to 6.4 µg/L). Therefore, not only highly potent chemicals but also chemicals present at high concentrations will contribute to the effect.

When comparing B[a]P-EQ_{chem} to B[a]P-$EQ_{bio,iceberg}$, only between 0.0004 to 2.79% of the effect could be explained by detected chemicals (Table S7). Previous studies have found between 0.2 to 71% of activation of AhR that could be explained by the quantified chemicals in surface water (Neale et al., 2015; Neale et al., 2017b). These studies only had EC values for three to four of the detected chemicals, compared to 40 detected bioactive chemicals in the current study. AhR is mainly activated by hydrophobic organics such as polycyclic aromatic hydrocarbons. These bind to suspended particulate matter and would not be expected in the water sample filtered with a 0.7 µm filter but residual smaller particles and colloids may pass and be enriched by SPE, contributing to the unknown fraction of B[a]P-$EQ_{bio,iceberg}$. For these particles, a source in addition to road run-off, agricultural run-off and WWTP effluent will also be atmospheric deposition (Mesquita et al., 2016).

Effect measurements were available for 310 out of the 395 analyzed chemicals for PPARγ GeneBLAzer, with data available for 232 of the detected chemicals (80%) (Table S5). However, only 9% of the detected chemicals tested in PPARγ GeneBLAzer were active, with REP_i values available for 20 chemicals (Figure S3). Diclofenac explained on average around a third (35.4%) of rosiglitazone-$EQ_{chem,}$ followed by 2-benzothiazolesulfonic acid (average 25.3%) and the herbicide MCPA (average 12.4%) (Figure 23B & S6). Diclofenac was among the most potent chemicals measured in the PPARγ GeneBLAzer assay in the current study (REP_i 5.42×10^{-4}) and was also found at high concentrations (up to 1.3 µg/L). However, rosiglitazone-EQ_{chem} could only explain up to 1.66% of rosiglitazone-$EQ_{bio,iceberg}$ (average 0.18%) (Table S8). Detected chemicals have previously shown to explain a low fraction of the effect (<1%) in the PPARγ GeneBLAzer assay in surface water and wastewater (König et al., 2017) and spiked surface water (Neale et al., 2018).
Bioassay data were available for either the AREc32 or ARE GeneBLAzer assays for 309 of the 395 chemicals analyzed. If both were available, only AREc32 was reported. Of the 290 detected chemicals, effect data was available for 233 chemicals (80%), with 52 of the detected chemicals active

in the AREc32 (29 chemicals) or ARE GeneBLAzer assays (23 chemicals) (Table S5, Figure S3). The ARE GeneBLAzer data were collected from the US EPA Tox21 database and was expressed as an EC_{10} rather than an $EC_{IR1.5}$. The $EC_{IR1.5}$ and EC_{10} values for common chemicals were generally within an order of magnitude (Figure S7), but the REP_i values for chemicals run in ARE GeneBLAzer were calculated using the dichlorvos EC_{10} value from the Tox21 database.

2-Benzothiazolesulfonic acid explained 35.4% of dichlorvos-EQ_{chem} on average, followed by industrial compound 2,4-dinitrophenol (average 12.0%) and herbicide metolachlor (average 7.2%) (Figure 23C & Figure S8). Metolachlor was previously found to contribute to dichlorvos-EQ_{chem} for the oxidative stress response in wastewater effluent and surface water downstream of a WWTP in Switzerland (Neale et al., 2017b). On average, only 0.28% of dichlorvos-$EQ_{bio,iceberg}$ could be explained by dichlorvos-EQ_{chem} (Table S9). This is similar to previously observed for surface water and wastewater (Escher et al., 2013; Neale et al., 2015; Neale et al., 2017b). In one sample, 8b, 8% of dichlorvos-$EQ_{bio,iceberg}$ was explained by the potent herbicide pethoxamid (REP_i 2.66), which was detected at 13.1 µg/L.

While many different chemicals contributed to the BEQ_{chem} in the three assays, 2-benzothiazolesulfonic acid explained between 25.3 to 35.4% of BEQ_{chem} on average in the three assays. While 2-benzothiazolesulfonic acid was not particularly potent in any of the assays, the widespread presence and high concentrations (average concentration 1.1 µg/L) meant it was a dominant contributor to BEQ_{chem}. This suggests that future water quality monitoring studies should include 2-benzothiazolesulfonic acid, especially as it is also a marker of street run-off and as such complements the traditional wastewater markers such as estrogenic hormones or pesticides as markers for agricultural inputs.

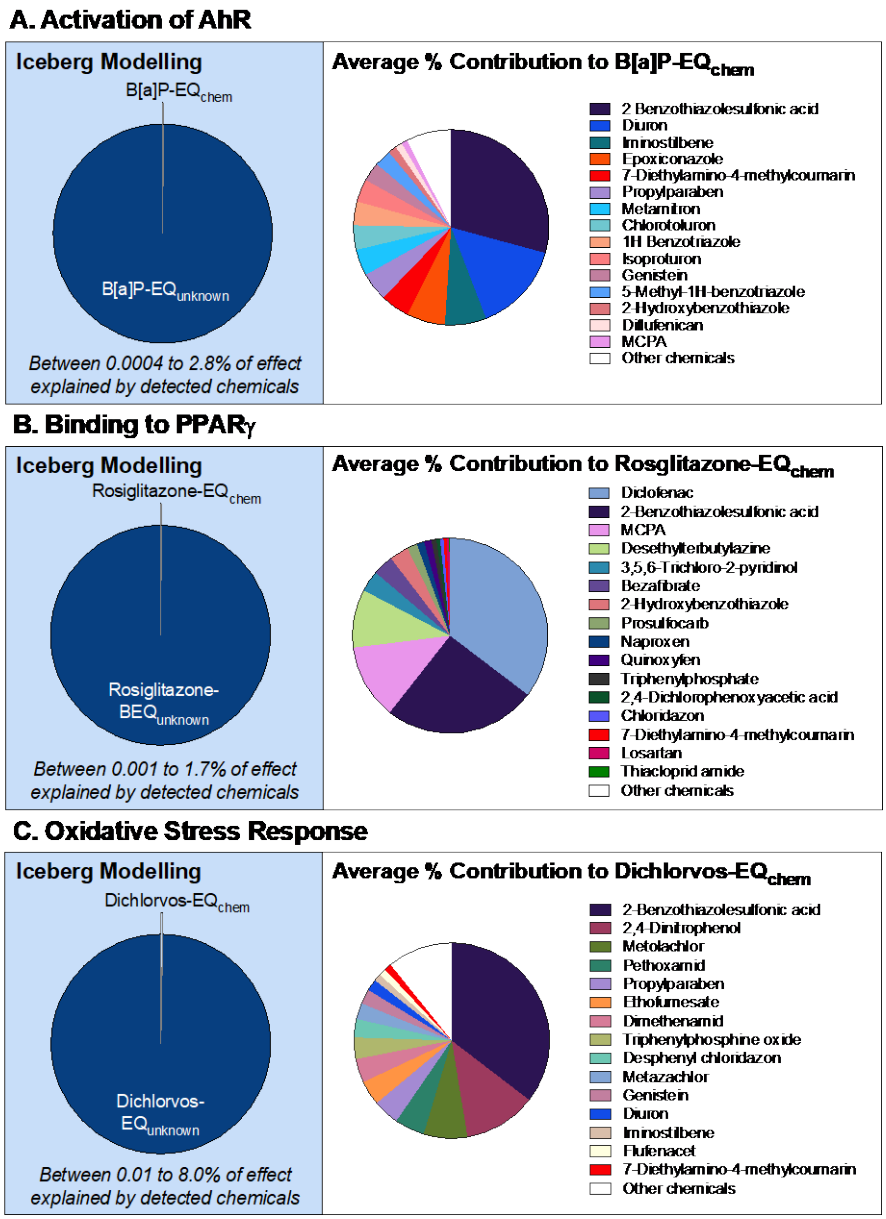


Figure 23: Average fraction of BEQ_{chem} that explained $BEQ_{bio,iceberg}$ (left) and top 15 to 16 chemicals contributing on average to BEQ_{chem} (right) for assays indicative of activation of (A) AhR, (B) binding to PPARγ and (C) oxidative stress response.

Other studies have also used in-vitro or in-vivo data to prioritize chemicals of concern. Focusing on assays included in the US EPA Tox21 database, Corsi et al. (2019) found that the industrial compounds 4-nonylphenol and

bisphenol A and the herbicides metolachlor and atrazine were among the chemicals identified as of greatest concern in water samples collected from the Great Lakes tributary. Metolachlor was also identified as a contributor to dichlorvos-EQ_{chem} for oxidative stress response in the current study. Further, many of the chemicals contributing to BEQ_{chem}, including the pharmaceuticals bezafibrate and diclofenac and the herbicides prosulfocarb and metolachlor, also ranked highly in a list of 214 chemicals present in European surface waters that potentially pose an acute hazard to fish, algae or crustaceans (Busch et al., 2016).

Iceberg modeling of cytotoxicity is described and discussed in the SI, Section S5. Overall, a substantially higher fraction of cytotoxicity than of activation of specific effects could be explained because a larger number, i.e., 102, detected chemicals had experimental cytotoxicity IC_{10}: 0.2 to 122% for AhR CALUX, 0.2 to 22% for PPARγ GeneBLAzer and 0.02 to 8.8 % for AREc32 (Figure S10).

5.3.5 Equipotent Mixtures of the Detected Chemicals

The concentration-response curve for activation of AhR of the equipotent mixture of the 15 chemicals that contributed most to the BEQ_{chem} agreed well with the prediction for concentration addition (Figure S11A) with an index of prediction quality (IPQ) of -0.11. This means that the chemicals detected are acting according to the mixture concept of concentration addition in mixtures. The equipotent mixture of PPARγ GeneBLAzer (Figure S11B) was much more potent than predicted for concentration addition with an IPQ of 3.69. This is especially surprising because the mixtures with the concentration ratios as detected in the water samples were generally much closer to IPQ 0. The equipotent mixture of AREc32 (Figure S11C) had an IPQ of 0.46, which means that the experimental effect was higher than the predicted mixture effect. Various 5- to 10-component equipotent mixtures run in the AREc32 assay had IPQs around 0 confirming concentration addition but some mixtures had IPQ up to 1 indicating some variability (Escher et al., 2013).

5.3.6 Tip of the Iceberg Mixtures

Since B[a]P-EQ_{chem} explained only a very small fraction of the B[a]P-EQ_{bio} (Figure S12A), it was checked by designed mixture experiments of chemicals in the detected concentration ratios of water samples if the detected chemicals act together according to concentration addition. The 107 reconstituted mixtures in AhR contained between 3 and 14 components in the detected concentration ratios. The selected 17 chemicals explained on average 93% of the overall BEQ_{chem} (min 26 %, max 99.9%). The concentration-response curves for activation of AhR are depicted in Figure S13 together with the predictions for CA. The EC_{10} values were converted to $BEQ_{bio,tip}$ and compared with $BEQ_{chem,tip}$ (Table S7, Figure 24A & S12B). With few exceptions, the agreement was within a factor of two, which is also reflected by the IPQ values (Table S7), which had a mean of 0.24 (95% CI 0.14 to 0.33, Figure S12C), indicating a slightly higher effect of the experimental mixture $BEQ_{bio,tip}$ than of the predicted $BEQ_{chem,tip}$. This small systematic deviation may be caused by the two chemicals that were inactive in the mixture experiments, whereas they had been reported active in Tox21. They may have been below their threshold of effect alone but contributed to the mixture effect.

The 76 mixtures of the 17 chemicals with the highest predicted rosiglitazone-EQ_{chem} in concentration ratios of the water samples (Table S8, concentration-response curves (CRCs) in Figure S14) yielded IPQs ranging from -6.9 to 5.4, with a mean of 0.32 but the 95% CI only ranged from -0.04 to 0.70, which indicates that the majority of IPQs is above 0, indicating more potent mixtures than expected (Figure S15C). The relationship between rosiglitazone-$EQ_{chem,tip}$ and rosiglitazone-$EQ_{bio,tip}$ showed more variability than in AhR CALUX but the values are within a factor of two around the one-to-one line (Figure 24B). The higher variability between prediction and measurement is caused by the generally higher variability of individual data points in the CRCs of this assays, which is due to a larger background signal and hence lower signal-to-noise ratio.

The deviation from the relationship between dichlorvos-$EQ_{bio,tip}$ and dichlorvos-$EQ_{chem,tip}$ was well within a factor of two (CRCs in Figure S16 & C, Table S9) but directed towards higher experimental effects similar to

AhR. Hence the deviation towards higher potency experimentally as compared to the mixture model of concentration addition appears to be small but consistent and might be caused by some imprecision of the single chemicals' EC_{10} values or the one inactive chemical benalaxyl. The IPQ values of the 44 mixtures (Table S9) ranged from -0.69 to 3.5 with a mean of 0.51 (95% CI 0.30 to 0.59, Figure S17C).

In summary, over all the 227 mixtures the mixture components appeared to act fairly close to concentration-additive in all three in-vitro bioassays, confirming that the BEQ concept is applicable to these bioassays and types of samples. The IPQs were close to 0 with a tendency to positive values for AhR CALUX (Figure S12C) and AREc32 (Figure S17C), even more for PPARγ GeneBLAzer (Figure S15C), which points to experimental effects being slightly higher than predicted, but the IPQ values did not shown any correlation to the composition of any of the mixtures.

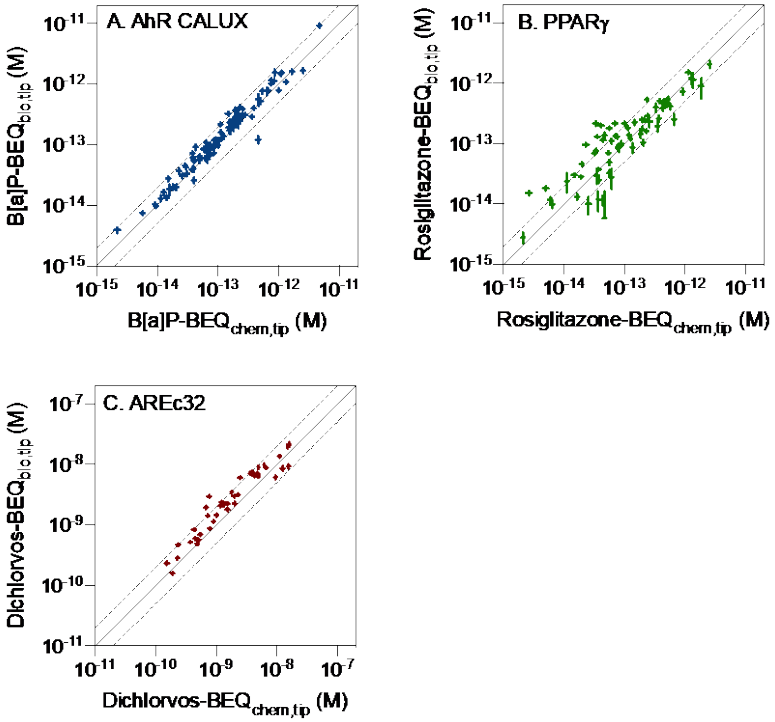


Figure 24: Agreement between $BEQ_{bio,tip}$ and $BEQ_{chem,tip}$ for (A) activation of AhR, (B) binding to PPARγ and (C) oxidative stress response. No symbols are shown, the lines at the points are the error bars (standard error), the full line is the 1:1 relationship and the dashed lines indicate 2:1 and 1:2 ratios.

5.4 Outlook

It has been demonstrated previously that a complete pesticide screening is required to estimate the surface water quality of small streams (Moschet et al., 2014) and, while individual pesticides might exceed chemical-specific water quality criteria, it is really the mixture effect that needs to be considered to understand ecological effects (Schäfer et al., 2013) and risk (Spycher et al., 2018). Pesticides drive the risk predicted with the method of multi substance potentially affected fraction (msPAF) even in wastewater impacted streams at low-flow conditions (Munz et al., 2017). But the situation might change dramatically during rain events as described here, where we recorded a high spatial and temporal variability. While further studies on exceedance of chemical-specific water quality criteria and the ecological impact and in-vivo toxicity of the described rain events are forthcoming, the focus on present study was on the in-vitro assays and biological endpoints most commonly impacted by water-borne pollutants. We demonstrated that non-pesticide chemicals and even typical wastewater-derived chemicals were found at sites assumed prior to the study to be largely free from wastewater effects. All observed in-vitro effects were dominated by street run-off chemicals such as 2-benzothiazolesulfonic acid. Previous effect studies on stormwater demonstrated that effect levels were similarly high as WWTP effluent and all urban stormwater samples investigated showed estrogenic effects (Tang et al., 2013). Rain events clearly pose a threat to water quality in small streams and analysis of pesticides alone cannot adequately judge the toxicological impact unless analytical monitoring is complemented by bioassays.

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6 Three Reasons Why the Water Framework Directive (WFD) Fails to Identify Pesticide Risks

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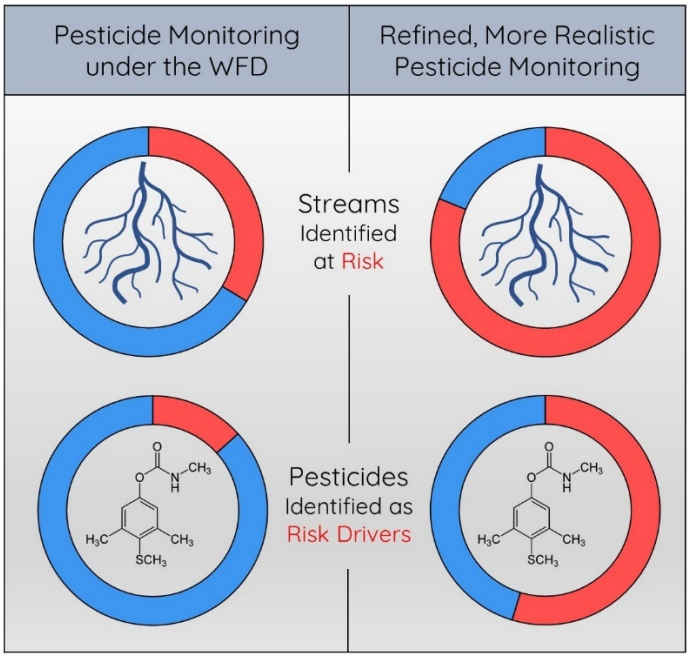
Abstract

The Water Framework Directive (WFD) demands that good status is to be achieved for all European water bodies. While governmental monitoring under the WFD mostly concludes a good status with regard to pesticide pollution, numerous scientific studies have demonstrated widespread negative ecological impacts of pesticide exposure in surface waters. To identify reasons for this discrepancy, we analysed pesticide concentrations measured in a monitoring campaign of 91 agricultural streams in 2018 and 2019 using methodologies that exceed the requirements of the WFD. This included a sampling strategy that takes into account the periodic occurrence of pesticides and a different analyte spectrum designed to reflect current pesticide use. We found that regulatory acceptable concentrations (RACs) were exceeded for 39 different pesticides at 81% of monitoring sites. In comparison, WFD-compliant monitoring of the same sites would have detected only eleven pesticides as exceeding the WFD-based environmental quality standards (EQS) at 35% of monitoring sites. We suggest three reasons for this underestimation of pesticide risk under the WFD-compliant monitoring: (1) The sampling approach - the timing and site selection are unable to adequately capture the periodic occurrence of pesticides and investigate surface waters particularly susceptible to pesticide risks; (2) the measuring method - a too narrow analyte spectrum (6% of pesticides currently approved in Germany) and insufficient analytical capacities result in risk drivers being overlooked; (3) the assessment method for measured concentrations - the protectivity and availability of regulatory thresholds are not sufficient to ensure a good ecological status. We therefore propose practical and legal refinements to improve the WFD's monitoring and assessment strategy in order to gain a more realistic picture of pesticide surface water pollution. This will enable more rapid identification of risk drivers and suitable risk management measures to ultimately improve the status of European surface waters.



6.1 Introduction

Since its implementation in the year 2000, the Water Framework Directive (WFD, 2000/60/EG) has served as the legal basis for EU member states to protect their surface waters (European Union, 2000). It requires member states to achieve and maintain a good status of all lentic and lotic waters. To have good status, a surface water must exhibit both a good chemical and a good ecological status. However, the latest results on the status of European surface waters submitted by the member states reveal that at least 35% of surface waters fail to achieve a good chemical status and 51% show an insufficient ecological status (moderate, poor or bad) (EEA, 2018).

The drivers made responsible for this poor status mainly include the occurrence of ubiquitous, persistent, bioaccumulative and toxic substances (uPBTs), morphological degradation and high nutrient loads (BMUB/UBA, 2016; EEA, 2018). Pesticides, on the contrary, are broadly represented in the WFD list of analytes but cause only 0.4% of surface waters to fail to achieve a good chemical status according to the monitoring data from the

2nd river basin management plan (Mohaupt et al., 2020). This contradicts numerous studies which observed that pesticides frequently exceed regulatory acceptable concentrations (RACs) (Stehle and Schulz, 2015b; Szöcs et al., 2017) and even pose a greater threat to European surface water ecology than any other pollutant class (Malaj et al., 2014; Wolfram et al., 2021). Pesticides have been shown to impair surface water fauna and flora within Europe (Beketov et al., 2013; Larras et al., 2017; Liess et al., 2021a; Liess and Ohe, 2005; Schäfer et al., 2011), but also worldwide, for example in Africa (Ganatra et al., 2021), Australia (Burgert et al., 2011; Wood et al., 2019) and North and South America (Chiu et al., 2016; Hunt et al., 2017). These contrasting results suggest that the current monitoring and assessment methods used in compliance with the WFD result in an underestimation of the actual pesticide risk.

The WFD surface water monitoring strategy focuses on larger rivers while catchments are surveyed less frequently if <100 km^2 or only in exceptional cases if <10 km^2 (Szöcs et al., 2017; Wick et al., 2019). The chemical and ecological status of European small streams is therefore largely unknown. This is problematic because small headwater streams play a decisive role in large-scale overall ecological condition and biodiversity, as they make up two thirds of the entire river network (BfN, 2021; Meyer et al., 2007). Small stream ecosystems are considered biodiversity hotspots, offering diversified habitats for numerous animal, plant, algae and fungi species, and act as recolonization sources for impaired downstream reaches (Liess and Ohe, 2005; Orlinskiy et al., 2015). Such streams have also been shown to be particularly susceptible to agricultural diffuse pesticide pollution, often being located in direct proximity to agricultural fields while lacking the capacity of larger waters to dilute pesticide inputs (Schulz, 2004; Stehle and Schulz, 2015b; Szöcs et al., 2017). These inputs are mostly due to rainfall-induced surface runoff transporting pesticide residues from fields into adjacent streams, resulting in short-term concentration peaks (Liess et al., 1999). For these reasons, there is growing global concern about the chemical and ecological quality of small rivers, which is also reflected in more recent monitoring programmes focusing on small streams such as

the Regional Stream Quality Assessment (RSQA) in the US (https://webapps.usgs.gov/RSQA/#!/) or the NAWA SPEZ in Switzerland (https://www.eawag.ch/en/research/water-for-ecosystem/pollutants/nawaspez).

Among other objectives, the German National Action Plan (NAP) for the Sustainable Use of Plant Protection Products addressed this blind spot in WFD monitoring, specifically requiring representative monitoring of small surface waters in agricultural catchments with an area of <10 km^2 (BMEL, 2013). Consequently, a uniquely comprehensive monitoring campaign of 124 small streams designed to adequately characterise pesticide pollution was carried out in 2018 and 2019 throughout Germany, Central Europe (see project homepage under www.ufz.de/kgm). Apart from the focus on small streams, its strategy comprised (i) event-driven sampling (EDS) to capture transient pesticide peak concentrations in addition to WFD-compliant regular grab sampling, (ii) an analyte spectrum based on current pesticide use statistics, which differs from the WFD pesticide analytes, and (iii) the consideration of additional pesticide surface water thresholds beyond those listed for the purposes of the WFD. On the basis of this stream monitoring, Liess et al. (2021a) confirmed the frequent occurrence of pesticides in ecologically harmful concentrations generally exceeding regulatory thresholds. Additionally, they linked ecological status to pesticide pressure and proposed protective pesticide thresholds relying on field observations. Further, Halbach et al. (2021) quantified the periodic occurrence of pesticides following rain events in these streams and compared measured concentrations with those recorded during the routine WFD monitoring of two German federal states. The present study now uses this stream monitoring data to evaluate the WFD's pesticide monitoring strategy. Therefore, we compared the results of the surface water assessment of our refined stream monitoring approach against a WFD-compliant approach of the same monitoring sites. In this way, we aim to evaluate the WFD's ability to detect pesticide risks in surface waters, identify reasons for divergent results where they exist, and propose refinements to improve the WFD's pesticide monitoring strategy.

6.2 Material and Methods

6.2.1 Pesticide Monitoring under the WFD – the Current Situation

Under the WFD, EU member states monitor three different categories of sites: (i) Surveillance monitoring sites, where all the WFD quality elements (ecological, hydromorphological, chemical and physico-chemical) are normally assessed. In Germany, the extensive surveillance monitoring network comprises about 260 sites mostly located in larger rivers. (ii) Operational monitoring sites are more abundant (>13,000 in Germany), but require a limited monitoring effort restricted to the assessment of quality elements known to react most sensitively in a water body. This operational monitoring therefore depends on the locally specific pressure situation. (iii) Investigative monitoring sites to locate and assess causes of water pollution that make a surface water fail to achieve a good status (Arle et al., 2016).

The WFD monitoring of pesticides is involved in both the chemical and the ecological status assessment. To classify a surface water's chemical status, all EU member states regularly measure 45 priority substances (PS) or substance groups listed in the WFD and implemented in German law by the Surface Water Ordinance (BGBl, 2016 Annex 8). The list of PS contains 23 pesticides (see supplementary information - SI Table 1). As part of the ecological classification, each EU member state is also obliged to identify pollutants of regional or local importance, the river basin-specific pollutants (RBSP). In Germany, the list of RBSP comprises 67 substances, 44 of which are pesticides (BGBl, 2016 Annex 6). Both PS and RBSP are assigned legally binding environmental quality standards (EQS) reflecting concentration levels below which it is assumed that the aquatic environment and human health are protected. If a single PS or RBSP exceeds an EQS, the chemical status is classified as "not good" or the ecological status is downgraded to less than "good" (at most "moderate"), respectively. In contrast to PS, RBSP must be monitored if "discharged in significant quantities". Monitoring frequencies are legally defined in that

PS are measured twelve times per year at least once every three years (operational monitoring) or six years (surveillance monitoring), while RBSP require monitoring four to 13 times per year at least once every three years (operational monitoring) or six years (surveillance monitoring) (BGBl, 2016 Annex 10).

6.2.2 Monitoring Design Used in this Study

The information on stream water pesticide concentrations was collected as part of a Germany-wide monitoring campaign of 124 small lowland streams in 2018 and 2019. The monitoring strategy was described in detail by Liess et al. (2021a) and only a short summary is provided here.

This study focused on a subset of the complete monitoring dataset by considering lowland streams (i) within agricultural catchments, i.e. those with > 20% agricultural land cover within the catchment (Copernicus Land Monitoring Service, 2019) and (ii) where rainfall event-driven sampling (EDS, see below) could be carried out. This subset comprised 91 agricultural streams, of which ten were monitored in both 2018 and 2019. These ten streams are analysed individually for each year, as weather conditions and/or crop types in the catchments differed between the years. The hydrological catchments of these small streams were mostly <30 km^2 (mean = 19 km^2) with an agricultural land cover ranging from 22% to 100% (mean = 75%). Although the selection of agricultural stream monitoring sites and respective catchments showed a higher percentage of agricultural land cover than average German small stream catchments, we estimate the level of pesticide pollution to be representative for German agricultural streams in general (see SI – Representativity analysis). Urban land cover accounted for less than 5% in the majority of stream catchments (see SI Figures 1 & 2).

The streams were sampled from the beginning of April to mid-July, covering the intense application period of pesticides in early summer (Szöcs et al., 2017; Weisner et al., 2021). The samplings were carried out in two different ways: (i) Grab samples (n = 450) were taken on a regular, three-week cycle comparable to the monthly samplings performed under

the WFD. Grab sampling was thus carried out irrespective of weather and discharge conditions. (ii) Additionally, the streams were sampled directly after rainfall assumed to cause surface runoff (EDS, n = 312) using automatic sampling devices collecting time-integrated composite samples triggered by a significant water level increase (for details see SI). In total, an average of 4.5 grab samples and 3.1 EDS samples was collected per site.

All water samples were cooled below 4°C during sampling and transport and analysed within four days for 75 pesticides and 33 pesticide metabolites using LC-MS/MS (see SI for substance list and Halbach et al., 2021 for the analytical method). The selection of pesticide analytes was compiled from a prior study by Wick et al. (2019), taking into account (i) a pesticide's current use statistics in relation to its toxicity, (ii) measured concentrations in previous monitoring programmes and (iii) its compatibility with a multi-substance method for chemical analysis. The selected analyte spectrum overlapped with the list of PS and RBSP for two and 22 pesticides, respectively (see SI Table 2). Pyrethroid insecticides and the herbicide glyphosate are expected potential risk drivers for aquatic ecosystems that were omitted due to analytical limitations. Nonetheless, we consider that the analyte spectrum covered the majority of ecotoxicologically relevant pesticides at the time.

6.2.3 Pesticide Surface Water Thresholds

We applied three different types of pesticide surface water thresholds to assess the ecological relevance of measured concentrations: the WFD-based EQS, the regulatory acceptable concentrations (RAC) derived during the authorisation of plant protection products containing the pesticides (UBA, 2019) and the field-based acceptable concentrations (AC_{field}) (Liess et al., 2021a).

The EQS values were taken from the list of PS and German RBSP according to the Surface Water Ordinance (BGBl, 2016 Annex 6/8). To account for the duration of exposure, there are two different EQS under the WFD: (i) the annual average-EQS (AA-EQS) covering long-term effects normally derived on the basis of chronic toxicity data, and (ii) maximum acceptable

concentration-EQS (MAC-EQS), which covers short-term effects normally derived on the basis of acute toxicity data (European Commission, 2018). AA-EQS are therefore used to assess time-averaged, long-term concentration levels, while MAC-EQS are used to assess short-term peak concentrations. AA-EQS were available for 24 pesticides (three insecticides, three fungicides, 18 herbicides) and MAC-EQS were available for ten of these 24 pesticides (two insecticides, one fungicide, seven herbicides). When comparing MAC-EQS to the RAC and AC_{field}, we also considered pesticides that are listed as RBSP in other EU member states (see SI Table 2, EEA, 2021) and/or were not included in the stream monitoring analyte spectrum (see SI Table 3).

The RACs as thresholds derived within the environmental risk assessment of plant protection products were obtained from UBA (2019). As each plant protection product containing a specific pesticide (= active ingredient) requires (re-)authorisation prior to use, RACs were available for all pesticides analysed (n = 75, eleven insecticides, 25 fungicides, 39 herbicides). The metabolites methiocarb sulfoxide and prothioconazole-desthio are also assigned a RAC due to their elevated ecotoxicological potential. The RACs applied in this study reflect the regulatory status when monitoring was carried out in 2018 and 2019. Individual RACs may have been adjusted in the meantime as the plant protection products may have been reauthorised taking new scientific knowledge into account. Both RAC and MAC-EQS assess concentration maxima but originate from different legal frameworks and differ in terms of the definition of the protection goal and the precise derivation approach. If a MAC-EQS is exceeded then counteractive measures must be initiated, while compliance with RACs is not legally required.

The AC_{field} was derived on the basis of field observations by Liess et al. (2021a) by linking a stream's peak exposure to its ecological status as reflected by the invertebrate community. This threshold aims for 95% of streams to show a good or high ecological status in terms of the invertebrate-based indicator $SPEAR_{pesticides}$, which responds specifically to

pesticide pressure. An AC_{field} was only assigned to the 22 pesticides (eleven insecticides, eight fungicides, three herbicides) for which freshwater invertebrates were considered the most sensitive organism group according to UBA (2019) (referred to in this article as primarily invertebrate-toxic pesticides from here). In contrast to the EQS or RAC, this threshold incorporates other environmental stresses present in the field that interact with pesticide toxicity (e.g. other pesticides, nutrients, temperature or competition). All thresholds are listed in SI Table 2.

6.2.4 Evaluation of Risk Indicated by Threshold Exceedances

Exceedances of the RAC, MAC-EQS and AC_{field} were determined by comparing the measured concentration c_i of pesticide or pesticide metabolite *i* to the relevant threshold. A threshold exceedance is indicated by a risk quotient (*RQ*) greater than 1:

$$RQ = \frac{c_i}{Threshold_i} \qquad 21$$

To determine exceedances for the AA-EQS, the average of all measured concentrations of the pesticide *i* is divided by the threshold:

$$RQ_{AA-EQS} = \frac{mean\ c_i}{AA\text{-}EQS_i} \qquad 22$$

In the WFD-compliant assessment, the monthly sampled concentrations are commonly averaged over an entire year and then compared to the AA-EQS (LAWA-AO, 2019). Since the stream samples of this study were taken only during the period of intense pesticide application, our averaging period only ranged from April to July. This limited averaging period may result in a higher risk than if considering the year as a whole, which would include months with no or reduced pesticide application, particularly in winter (Weisner et al., 2021). However, unlike in practice, the WFD guidance document also explicitly advises that averaging periods should be shorter than a year when episodic exposure is known, which will also be discussed below (see chapter 3.1) (European Commission, 2018).

Therefore, we also considered a best-case scenario including hypothetical measurements in which no pesticides were detected in the months when no samplings took place and calculated annual average concentrations following the German guidance (see SI and LAWA-AO, 2019). All calculations were performed using the statistical software R (version 3.5.1) and all plots were created using the R package "ggplot2" (version 3.2.0) (R Core Team, 2018; Wickham, 2009a).

6.3 Results and Discussion

6.3.1 Reason #1 – Sampling Pesticides

Here we discuss the time and sites to sample surface waters for pesticides. Firstly, the WFD sampling frequencies and intervals must be regarded as unsuitable with respect to the seasonal application of pesticides and their event-related input. The rainfall event-driven sampling (EDS) used in our refined monitoring approach captured on average 8.3 times higher pesticide concentration peaks (95^{th} percentile) compared to common grab sampling as performed under the WFD (see Figure 25 and SI Table 2) (Halbach et al., 2021; Liess et al., 2021a). For the metabolites analysed, EDS concentration peaks exceeded the relevant grab sample concentration on average by a factor of 3.8. EDS detected higher total pesticide concentrations compared to grab sampling in 80% of streams (n = 81).

As a consequence, EDS increased the probability that an exceedance of the maximum acceptable concentration environmental quality standard (MAC-EQS) would be detected by a factor of four: respective exceedances were identified in 3% (n = 16) of grab samples and 12% (n = 35) of EDS samples. Restricting our analysis to grab sampling caused 16 of the 30 streams with MAC-EQS exceedances to go unnoticed. EDS was thus indispensable to adequately monitor pesticide toxicity peaks as shown in multiple studies (Bundschuh et al., 2014; Lorenz et al., 2017; Rasmussen et al., 2017). It is these peak concentrations that were shown to determine the ecological status of a surface water (Liess et al., 2021a; Ohe et al., 2011; Schäfer et al., 2012).

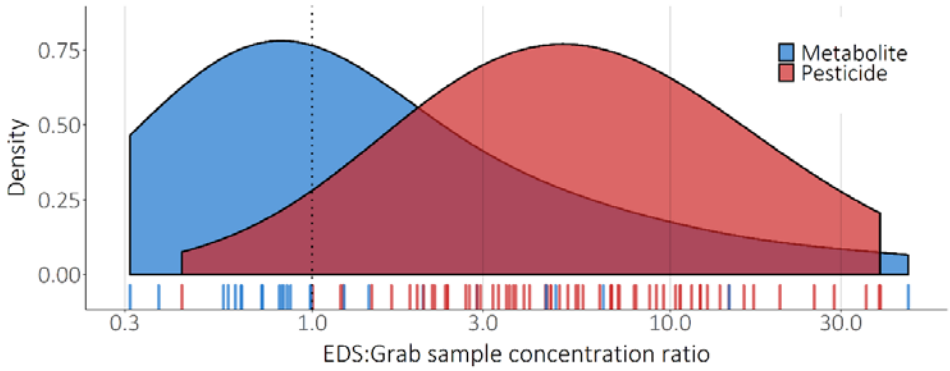


Figure 25: Smoothed distribution of ratios of measured concentration peaks (95th percentile) from event-driven sampling (EDS) and grab sampling for monitored pesticides (red, n = 63 substances) and pesticide metabolites (blue, n = 25) on a logarithmic scale. Vertical lines at the bottom show the single, compound-specific ratios. Pesticides and metabolites not shown revealed 95th percentiles of 0 in EDS (n = 3), grab samples (n = 8) or both (n = 9, see SI Table 2).

By investigating the concentration differences depending on weather conditions, Szöcs et al. (2017) and Halbach et al. (2021) confirmed the periodic occurrence of pesticides in surface waters on runoff-relevant days. However, WFD-compliant grab sampling following a regular schedule coincided with such runoff-relevant days in only 7% of samplings, minimizing the likelihood of capturing relevant concentration peaks (rainfall >10 mm/d). Norman et al. (2020) and Spycher et al. (2018) found that regular grab sampling needed to be performed at a high frequency of 12 – 24 hours to capture transient peaks adequately. For optimal cost benefit, we therefore recommend supplementing the usual grab sampling with EDS sampling during the main period of pesticide application and following rainfall events. This can also be performed with less elaborate methods than automated sampling devices, for example simple bottle samplers (Liess and Ohe, 2005).

The monthly WFD samplings also cover periods outside the growing season when no relevant pesticide inputs are expected. Accordingly, the assessment of chronic exposure through compliance with annual average-EQS (AA-EQS) involves averaging all monthly measurements for the entire year (LAWA-AO, 2019). However, pesticide application frequencies peaking in April-May (Weisner et al., 2021) were shown to directly relate to

measured toxicity peaks in streams in April-June (Liess et al., 1999; Spycher et al., 2018). The current AA-EQS assessment under the WFD thus causes a downscaling of time-averaged concentrations which conceals exceedances of AA-EQS. This is in contrast to the WFD guidance explicitly stating that "when the exposure pattern for a substance is known to be episodic e.g. many pesticides, the averaging period may be a shorter period than a year" (European Commission, 2018). So far, this guidance has been disregarded in practical implementation. The scheduling of sampling and the corresponding averaging period for the AA-EQS assessment thus need to account for the substance-specific, periodic occurrence of pesticides. For larger rivers, the timing of sampling may be of less relevance as pesticide exposure may occur in flattened peaks as inputs from different tributaries arrive successively.

Secondly, the selection of sampling sites currently monitored under the WFD is biased, resulting in unrepresentative estimations of the status of surface waters and contributing to the underestimation of pesticide risk. Wolfram et al. (2021) estimated a median catchment area of 238 km^2 of European surface waters monitored under the WFD, while the median catchment area of the natural river network is less than 20 km^2. Small streams are thus underrepresented in the WFD monitoring site selection while being particularly susceptible to pesticide pollution (Lorenz et al., 2017; Schulz, 2004; Stehle and Schulz, 2015b; Szöcs et al., 2017). This especially concerns small waters with catchments of <10 km^2, which are completely omitted from regular WFD monitoring and are not required to achieve good status despite making up approximately two thirds of the entire river network (BfN, 2021). For these, we observed the same concerning level of pesticide pollution: the number of RAC exceedances detected between streams with catchments of >10 km^2 ($n = 65$) and <10 km^2 was comparable ($n = 36$, Wilcoxon rank sum test, $p = 0.6$). We therefore recommend that the current monitoring performed in the context of the WFD be shifted more towards small water bodies (30-100 km^2) and even include smaller waters with catchments of <10 km^2.

6.3.2 Reason #2 – Measuring Pesticide Contamination

In this section, we discuss issues related to the chemical analysis following water sampling. Firstly, we found the spectrum of pesticide analytes to be measured under the WFD to be outdated and inconsistent. All 108 pesticides and metabolites detected in this study were chosen on the basis of their expected environmental relevance (see chapter 2.2). However, only 24 of the 75 detected pesticides are subject to mandatory monitoring under the WFD and assigned an EQS (two priority substances (PS) and 22 river basin-specific pollutants (RBSP), see SI Table 2). Accordingly, WFD-compliant monitoring of the 101 streams identified eleven pesticides that exceeded their EQS if only grab samples were counted, or 16 if EDS were included. We also found that pesticides not listed in the WFD occurred in ecologically relevant concentrations, with 31 pesticides and one metabolite (grab samples only) or 37 pesticides and two metabolites (EDS included) exceeding the regulatory acceptable concentrations (RACs, see SI Table 2). For aclonifen and metazachlor, the EQS but not the RAC was exceeded. By contrast, 31 RAC exceeding pesticides were identified that would have gone unnoticed in WFD monitoring (see SI Figure 4). Of the ten pesticides most frequently found in concentrations exceeding their RAC, only three are included in the WFD spectrum of analytes. None of the four pesticides that most frequently caused RAC exceedances - thiacloprid, clothianidin, methiocarb and fipronil ($\sum$ = 54% of RAC exceedances) - are listed as a PS or RBSP. These results are supported by Tsaboula et al. (2016) who identified 71 pesticides that required monitoring based on a multi-criteria prioritisation in a large Greek river basin while only small fractions of 13 and 6 pesticides were PS and RBPS, respectively. Accordingly, Moschet et al. (2014) found that when measurements were restricted to pesticides listed as PS in a Swiss stream monitoring campaign, 80% of threshold exceedances remained undetected.

This significantly influences the status classification of surface waters. WFD-compliant pesticide monitoring would yield a good status for 65% (n = 66) of the streams investigated in this study (see Figure 26). Only in 12% (n = 12) of streams, more than one pesticide exceeding the EQS would

have been detected. By including EDS samples and RACs to assess additional pesticide analytes, only 19% (n = 19) of streams were found to achieve good status with respect to pesticides. Almost two thirds of the streams (64%, n = 65) exhibited at least two RAC-exceeding pesticides. WFD-compliant monitoring and assessment therefore failed to detect the unacceptable pesticide risk (RAC exceedance) for 57% of agricultural streams and 72% of the pesticides. Consequently, the list of analytes to be monitored under the WFD by far does not include the majority of environmentally relevant pesticides.

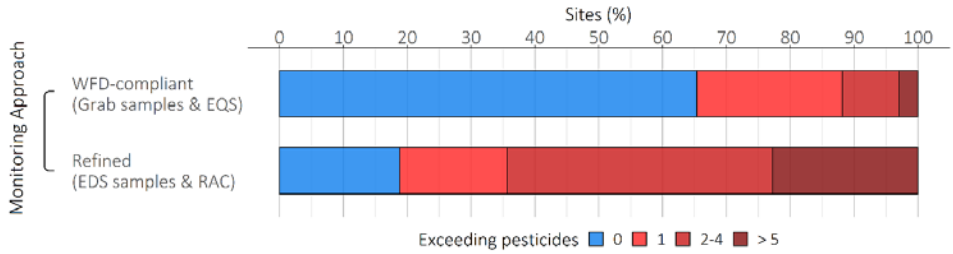


Figure 26: Fraction of sites with good status (blue = no threshold exceedance) and failing to achieve good status (shades of red = threshold exceedances) due to pesticides depending on the type of assessment. The WFD-compliant assessment is limited to grab samples and pesticides with an assigned EQS and found 65% of agricultural streams to have a good status with respect to pesticides. When EDS samples and a wider spectrum of pesticides were included, only 19% of streams were found to have a good status with respect to pesticides.

At the same time, we found that approximately three quarters (n = 49) of pesticides considered under the WFD were no longer approved for use in Germany (EU Pesticides Database of the European Commission, as of July 2021). In contrast, of the 301 different pesticides currently approved for use in Germany, only 6% (n=18) are subject to mandatory analysis under the WFD. Previous investigations have already emphasized that prioritization, monitoring and assessment mostly cover long-known substances while those of emerging concern remain disregarded (Brack et al., 2017; Heiss and Küster, 2015). Thiacloprid, for example, was responsible for 25% of RAC exceedances showing the highest rate of exceedances in our study. Thiacloprid, along with other neonicotinoids, was placed on the so-called Watch List, which brings together candidates

for an updated list of PS, in 2015. In 2020, however, its use for plant protection was banned across the EU (European Commission, 2020). Not yet listed as a PS, thiacloprid has probably already peaked in terms of environmental relevance. In the stream monitoring campaign, clothianidin, methiocarb and fipronil were also often measured in concentrations exceeding the RAC. These substances have not been monitored under the WFD and were also banned for plant protection in recent years. Nevertheless, substitutes (e.g. anthranilic diamides like chlorantraniliprole (Schmidt-Jeffris and Nault, 2016)) will fill the emerging gap, and if the aim is to avoid unexpected ecological consequences environmental concentrations must be monitored directly when a compound is used in significant amounts. The list of WFD pesticide analytes and the corresponding EQS must therefore respond more rapidly to the continuously changing spectrum of pesticides applied and relevant in the environment. The Watch List needs to be updated before the candidate substance's environmental relevance peaks. This could be achieved by monitoring a wide range of pesticides in a representative selection of agricultural surface waters and through regular dialogue with pesticide regulators familiar with the dynamics of the current-use pesticide spectrum. For now, we recommend that environmental authorities in charge of monitoring extend the mandatory analyte spectrum to include pesticides currently used (e.g. on the basis of sales quantities as published by the BVL for Germany) or identified as drivers of risk in this study (see SI Table 2). To classify measured concentrations when EQS are not available, we suggest using the AC_{field} (for invertebrate toxic pesticides, Liess et al., 2021a) and the RAC (for pesticides primarily affecting other organism groups, UBA, 2019) to assess concentration maxima. The Swiss Ecotox Centre has also derived chronic and acute quality standards for many pesticides not assigned an AA- or MAC-EQS following the official guidance (Oekotoxzentrum, 2021), that may not provide sufficient protection, though (see Reason #3 below).

Furthermore, the spectrum of RBSP to be measured by an EU member state involves two deficiencies: (i) Increasing the monitoring effort and

extending the RBSP spectrum involves additional costs for monitoring and possible risk mitigation measures. By providing less monitoring data, the obligation to initiate such measures can be circumvented, thus penalising ambitions to protect the environment. (ii) Under the WFD, RBSP are monitored in a certain surface water if they were considered beforehand to be "discharged in significant quantities". Whether an RBSP is "discharged in significant quantities" in a specific water body and needs to be integrated in routine WFD monitoring is difficult to evaluate reliably as long as the RBSP is not measured. Monitoring capacities for almost 10,000 WFD water bodies in Germany alone are limited and do not allow all pollutants "discharged in significant quantities" to be precisely identified in advance. Meanwhile, continuous changes in agricultural use and pesticide application schemes make it more difficult to monitor relevant RBSP (Arle et al., 2016). Moreover, the WFD does not define what "significant quantities" are, with the result that different interpretations prevail in the EU member states. We therefore support the integration of RBSP monitoring into the chemical status assessment as proposed by Brack et al. (2017). The separate assessment of PS for chemical status and RBSP for determining ecological status unjustifiably implies different monitoring intensities and complicates the interpretation of the effect of chemicals on the ecological status. The proposed integration would also have the positive side effect of harmonizing monitoring ambitions, as all EU member states would monitor the same list of RBSP assigned harmonized EQS. To take into account regional differences in pollution patterns and risk drivers, EU member states might omit analytes of negligible concern for their region or river basin. Such a negligible concern would have to be convincingly demonstrated on a regular basis by representative measurements, pesticide sales and application quantities or exposure modelling.

In addition to the insufficient analyte spectrum, analytical capacities hinder measuring the pesticide contamination. Several pesticides are so toxic for aquatic organisms that their acceptable concentrations in the water phase are below common analytical limits of detection. This partly concerns

legacy compounds like heptachlor and dichlorvos, but also current-use neonicotinoid and pyrethroid insecticides. The AA-EQS for imidacloprid and cypermethrin, for example, are only 2 ng/L and 80 pg/L – concentrations too low to be quantified by the commissioned laboratories in the WFD monitoring (Jarosch, 2018; Moschet et al., 2014; Rösch et al., 2019; Weißbach and Stricker, 2020). EQS exceedances may therefore remain unmeasured, raising the question of how to adequately monitor such toxic compounds and whether their use is generally justifiable when the resulting risk cannot be reliably assessed.

6.3.3 Reason #3 – Assessing Pesticide Effects

Here, we address the assessment of potential ecological consequences of measured concentrations by applying regulatory thresholds. Firstly, we raise concerns regarding the capacity of current regulatory thresholds to adequately assess pesticide risk. We compared the absolute values of MAC-EQS (including other member states' RBSP) with the German RACs and the field-based acceptable concentrations (AC_{field}, Liess et al., 2021a), all of which aim to assess acute pesticide risks.

RAC and MAC-EQS values differed for 29 of the 31 analysed pesticides that are assigned both thresholds, but were on average comparable (log-transformed paired t-test, $p = 0.4$). All four pesticides that are assigned MAC-EQS and AC_{field} values, Imidacloprid, Dimethoate, Pirimicarb and Ethofumesate, exhibit a MAC-EQS greater than the respective AC_{field} by a mean factor of 16 (geometric mean, min = 2.4, max = 195, see Figure 27). The RAC exceeded the corresponding AC_{field} values for 90% ($n = 20$) of compared pesticides. RACs were significantly higher than AC_{field} values (log-transformed paired t-test, $p<0.001$) by a mean factor of 4.2 (geometric mean, $n = 22$, min = 0.04, max = 56.5). Consequently, applying the mostly lower AC_{field} classified more streams as being at risk than the EQS or RAC, showing 96% ($n = 97$) of agricultural streams as failing to achieve good status (see SI Figure 4).

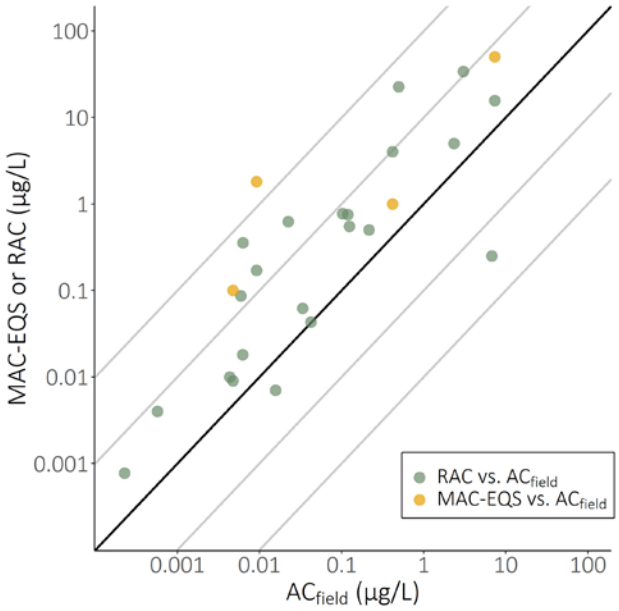


Figure 27: Comparison of the acceptable concentrations for pesticides derived from field observations (AC_{field}, Liess et al., 2021a) with those provided in the WFD (maximum acceptable concentration environmental quality standard - MAC-EQS) or from pesticide risk assessment (regulatory acceptable concentration - RAC). Each dot represents one pesticide for which the AC_{field} and either the MAC-EQS (orange) or the RAC (olive) is available. A dot on the black bisectrix indicates equal values for the AC_{field} and the MAC-EQS/RAC. Dots above or below the black bisectrix indicate a lower or higher AC_{field} compared to the MAC-EQS/RAC, respectively. Grey lines indicate value differences in orders of magnitude. The average deviation from the AC_{field} was 16 for the four MAC-EQS and 4.2 for the 22 RACs (geometric mean).

The general comparability of absolute values of MAC-EQS and RAC and the divergence from the AC_{field} are largely due to the differing assessment factors (AFs) applied in the respective threshold derivation. MAC-EQS and RAC rely on comparable or partly equal AFs aiming to account for the uncertainties relating to the transferability of effects from artificial test systems to the field. To extrapolate from acute toxicity tests to the field for invertebrates for example, the guidance for the derivation of MAC-EQS and RAC propose AFs of 100 (EFSA, 2013; European Commission, 2018). While AFs of MAC-EQS and RAC are generally based on estimations derived from artificial test systems, the AF determined for the AC_{field} is calibrated to pesticide effects observed in the field. Following this approach, Liess et al.

(2021a) determined an AF for acute toxicity tests of almost 2,000 required to protect vulnerable species in the field, resulting in the mostly lower AC_{field} values. This insufficiency of current AFs is supported by several other studies relating pesticide concentrations to effects on invertebrates under field conditions. Significant shifts in stream invertebrate communities were demonstrated at concentrations of one 100^{th} of the concentration causing 50% of organisms to display effects in acute toxicity tests (Knillmann et al., 2018; Liess and Ohe, 2005; Münze et al., 2017; Ohe et al., 2011). Schäfer et al. (2012) found that the relative abundance of sensitive species decreased by 27% - 61% with an AF of 100 and estimated that an AF of 1,000 - 10,000 was required to avoid pesticide-related effects. In addition, the richness of invertebrate families was found to decrease in the field when concentration maxima exceeded levels equalling one tenth of regulatory thresholds (Beketov et al., 2013; Stehle and Schulz, 2015a). In contrast to these field investigations, an AF of ten to 100 was estimated as sufficient to extrapolate from single species acute toxicity tests to multi-species micro- and mesocosms (Brock and van Wijngaarden, 2012; van Wijngaarden et al., 2015). These test systems, however, fail to realistically represent environmental conditions and to account for factors that increase the sensitivity of organisms in the field. These include the joint toxicity of co-occurring pesticides (Weisner et al., 2021), additional environmental stress (Beermann et al., 2018), complex trophic interactions leading to indirect effects (Miller et al., 2020), delayed effects appearing after the runtime of the test (Rasmussen et al., 2017), sequential pesticide exposure (Wiberg-Larsen et al., 2020) and the insensitivity of commonly studied biological metrics (Liess and Beketov, 2011).

All these investigations indicate that regulatory thresholds are too high to protect aquatic ecosystems. This inadequacy of regulatory thresholds is also supported by an extreme variability between EQS for a single RBSP in different EU member states (when national RBSP overlap) with divergences amounting to as much as a factor of 100,000 despite a common guideline for the derivation of thresholds (Arle et al., 2016). This is despite an absence of evidence that effect thresholds vary by such magnitude across

geographic regions. Instead, this underlines the regulatory uncertainty when predicting effect thresholds from experimental data. Further efforts are therefore needed to validate regulatory thresholds based on field observations – also for AA-EQS and considering groups of organisms other than invertebrates. For many pesticides, algae, plants or fish are the first organism groups to show effects (Leblanc, 1984) but still lack a suitable bioindicator for pesticide stress, which is required to validate the relevant regulatory thresholds and AFs.

Besides the question whether EQS are protective enough, we raise concerns regarding the availability of MAC-EQS to assess concentration maxima. For the 24 pesticides to be analysed both under the WFD in Germany and in our study, only ten are assigned a MAC-EQS. However, the remaining fourteen pesticides also showed a periodically increased occurrence following rain events (mean EDS:Grab sample concentration ratio = 9.5, see Figure 25). The guideline theoretically requires that exposure duration be taken into account, since "exposure may also occur intermittently for short periods e.g. coinciding with storm events" (European Commission, 2018), but once again, the implementation has so far disregarded this requirement.

In conclusion, there is strong evidence that compliance with current regulatory thresholds does not ensure a good ecological status in the field. We therefore recommend the use of AC_{field} values validated by field observations for invertebrate-toxic pesticides. However, a field-based validation of MAC-EQS for pesticides primarily affecting organism groups other than invertebrates as well as AA-EQS in general is lacking. The comparability of status assessments throughout the EU and the coherence of initiation of risk-reducing strategies requires an EU-wide harmonization of EQS for pesticides and other RBSP. Furthermore, there is no logical reason to separately define divergent pesticide thresholds for acceptable concentration maxima, as for the RAC under Regulation (EC) No 1107/2009 and the EQS under the WFD. Following the recommendations of Brack et al. (2017) and Schäfer et al. (2019), coexisting legal frameworks should

thus be more interconnected where their scopes overlap in order to harmonize protection goals.

6.3.4 Our Findings in the Light of EU-Wide Results

Even if pesticide risk drivers are expected to vary locally due to differing cropping patterns and pest pressures, pesticide pressure and related ecological risks were found to be comparable for surface waters across European regions despite differences in agricultural use intensities (Schreiner et al., 2021; Stehle and Schulz, 2015b; Wolfram et al., 2021). We thus assume that our findings quantifying pesticide risk are generally transferable to other regions beyond our German study area. However, our results differ distinctly from EU-wide WFD-compliant assessments. By applying the RAC, we found 81% of the streams investigated to be at risk due to pesticides (see Figure 26). RACs were exceeded in 38% (n = 38) of streams by herbicides and in 75% (n = 76) of streams by insecticides. An EU-wide assessment of WFD monitoring data covering the period 2007 to 2017 found only 5% to 15% and 3% to 8% of surface waters failing to achieve a good status due to herbicides and insecticides, respectively (Mohaupt et al., 2020). This discrepancy is partly rooted in our focus on surface waters in the agricultural landscape. More importantly, we conclude that the discrepancy in results is due to the issues associated with the WFD monitoring strategy as outlined above, which apply to all EU member states.

6.4 Conclusions

- WFD sampling, chemical analysis and assessment of measured concentrations are insufficient to identify pesticide risks in surface waters. As a consequence, the chemical status of surface waters is overestimated and the contribution of pesticides to the ecological status is underestimated under the WFD.
- We propose legal and practical adjustments that would enable refined and more realistic WFD pesticide monitoring. This will (i) help explain and narrow the gap between the chemical and ecological status of surface water bodies also requiring the

consideration of suitable ecological indicators that respond to pesticide pressure and (ii) implement an adequate pesticide post-registration monitoring that enables a shift in the prospective pesticide risk assessment from non-validated exposure and effect predictions to actual environmental exposure and protective thresholds. As shown in this study, current governmental monitoring under the WFD is only of very limited use for such validation as critical pesticides and threatened surface waters remain undetected. Following the polluter pays principle, the European Parliament has already suggested in the plant protection products regulation that the additional costs for specific pesticide monitoring could be (co-)financed by plant protection product manufacturers.

- Early identification of risk drivers and immediate feedback to pesticide regulators is key to reducing the proportion of surface waters that fail to achieve a good chemical and ecological status. 20 years after the implementation of the WFD, the failure to come closer to meeting the envisaged good status for European surface water bodies underlines the necessity to substantially improve the monitoring and assessment strategy.

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7 Discussion

7.1 Pesticide Exposure

Knowledge gap: Previous or existing monitoring programmes, scientific or governmental, left multiple blind spots when investigating pesticide exposure in surface waters. Most prominently, monitoring programmes are locally very restricted, neglect small streams, consider an outdated analyte spectrum or fail to record the periodic occurrence of pesticide exposure peaks.

The KgM monitoring results enable a general and representative description of pesticide exposure in small agricultural streams. The chemical analysis of more than 1,000 stream water samples for 75 pesticides (11 insecticides, 25 fungicides, 39 herbicides) and 33 pesticide metabolites revealed the dynamic and complex patterns of pesticide surface water pollution. All of the 108 analytes were detected at least in one sample. Frequencies of detection exceeded 40% for multiple compounds (Halbach et al., 2021). Total metabolite concentrations exceeded the total pesticide concentrations on average by a factor of 13 despite the limited number of metabolite analytes. The mean concentrations of some metabolites were even two to three orders of magnitude higher than the respective parent pesticide concentrations. Confirming the findings of previous studies (Liess et al., 1999; Rabiet et al., 2010; Spycher et al., 2018; Stehle et al., 2013) on a broader scale, in-stream concentrations of pesticides were found to increase after rainfall. For parent compounds, concentration peaks after rainfall were higher by an average factor of ten compared to dry weather conditions. This pattern was less distinct but still observable for the majority of metabolites. Generally, the tendency to occur periodically after rainfall strongly varied for the different pesticides investigated and was partly explained by the physico-chemical properties aqueous half-life and polarity.

The sampled stream ecosystems were exposed to complex pesticide mixtures. An average water sample featured 17 and 30 detected pesticides in grab samples and EDS samples taken after rainfall (Weisner et al.,

2021b). The maximum number of pesticides detected within one sample mounted up to 57. When estimating the toxicity of measured concentrations for aquatic risk indicators (e.g. invertebrates, algae) applying Toxic Units, there was a significant toxic imbalance in mixture components. This means that the estimated toxicity of the water samples was mostly dominated by one to few pesticides while the other mixture components only contributed marginally. However, the toxicity driving pesticides were found to alternate for the different streams, but also within the successive samples taken at one stream site. As supported by other studies on other geographic regions (Gustavsson et al., 2017; Schreiner et al., 2016; Vallotton and Price, 2016), in-stream pesticide exposure patterns are thus temporally and spatially highly variable. Besides weather conditions and the timing and amount of pesticide application, this variability is due to a multitude of catchment characteristics known to drive pesticide exposure including land use and cultivated crops (Szöcs et al., 2017), slope facilitating runoff (Holvoet et al., 2007), vegetation and soil type determining leaching as well as subsurface drainage and the presence of buffer strips (Reichenberger et al., 2007).

The pesticide concentrations measured in the KgM often reached levels exceeding regulatory threshold levels. In more than 80% of agricultural streams at least one pesticide exceeded its RAC during the sampling period (Liess et al., 2021). 37 pesticides and two metabolites exceeded their RAC at least once. Insecticides, and particularly the insecticide class of neonicotinoids including thiacloprid, clothianidin, imidacloprid, thiamethoxam and acetamiprid, most often exceeded their RAC. Yet, herbicides (especially terbuthylazin, nicosulfuron and lenacil) and strobilurin fungicides (dimoxystrobin and azoxystrobin) also often failed to comply with the RACs. Factors of RAC exceedance amounted to more than 10 or even 100 in some extreme cases. More than 60% of EDS samples collected from agricultural streams revealed at least one RAC exceedance. That is far beyond the goal that Germany set itself in the NAP by aiming for a RAC exceedance rate of less than 1% for EDS samples by the year 2023 (BMEL, 2013). In view of several previous studies, however, this is not so

much due to surprisingly high pesticide concentrations than to an extremely optimistic goal of the NAP: Stehle and Schulz (2015) performed a comprehensive meta-analysis of surface water insecticide contamination on a global scale and found 41% of measurements to exceed regulatory threshold levels. The limitation of the analysis to insecticides and the limited availability of EDS measurements are likely to cause the moderate decrease in the exceedance rate. Only considering grab samples (n>24,000) but also catchments less dominated by agricultural land use, Szöcs et al. (2017) determined about 26% of German streams to exhibit RAC exceedances on the basis of monitoring data from 2005 to 2015. While the RAC exceedance rate for the samples was at a low level of 7% compared to Liess et al. (2021), the exceedance rate for the measurements of 22 insecticides mounted up to 67%. These studies further suggest that the frequent exceedances of regulatory thresholds detected in the KgM do not represent a local or temporal peculiarity, but rather the common situation in the field.

Investigating the causes for the frequent RAC exceedances observed, Liess et al. (2021) analysed whether the ERA performed during the PPP authorisation process underestimates environmental concentrations of the 20 pesticides with the highest RAC exceedances. The publication hence compared the environmental concentrations measured for a specific pesticide with a PEC derived for the respective PPP. As one pesticide is often sold and used in form of various PPPs, this analysis involves the uncertainty of not knowing exactly which PPP(s) were applied that led to the measured pesticide concentration and therefore which PEC to consider. As a conservative approach, the analysis selected the highest PEC available for a specific pesticide assuming the implementation of risk mitigation measures prescribed by the PPP. These PECs were exceeded in at least 1% of EDS samples for 11 of the 20 pesticides. Part of the RAC exceedances can thus be traced back to an underestimation of environmental concentrations in the ERA. The ecological effects related to the widespread and excessive occurrence of pesticides overserved are addressed in the next section.

Contribution to narrowing the knowledge gap: This thesis draws a detailed picture of pesticide surface water contamination in 2018 and 2019 resulting from agricultural nonpoint source pollution. It reveals risk drivers, common contamination levels, occurrence dynamics and environmental pesticide mixture patterns.

7.2 Ecological Pesticide Effects

Knowledge gap: The impact of agricultural pesticide use on stream ecosystems has not been investigated on broader scale as linking the chemical and ecological status requires monitoring efforts exceeding those of common large-scale monitoring programmes. Therefore, the relevance of pesticide pressure for ecological stream quality among other stressors was unknown.

7.2.1 Aquatic Invertebrates

The analysis of the invertebrate community composition and the subsequent application of the $SPEAR_{pesticides}$ indicator was shown to indicate pesticide-related effects in numerous studies (Burgert et al., 2011; Ganatra et al., 2021; Hunt et al., 2017; Liess and Ohe, 2005; Schäfer et al., 2007) and was chosen as a central risk indicator for pesticide pollution in small waters in the NAP (BMEL, 2013). According to $SPEAR_{pesticides}$, 83% of the agricultural streams investigated in the KgM failed to achieve a good status (Liess et al., 2021). This means a significant reduction of taxa classified as SPEcies At Risk in the vast majority of water bodies, concerning for example ephemeropterans, plecopterans, trichopterans or odonates. That is consistent with the fact that more than half of the EDS samples revealed RAC exceedances by pesticides primarily affecting invertebrates (Weisner et al., 2021b). The investigation of other invertebrate-based ecological endpoints such as biomass or taxa richness was less or not at all associated with pesticide pressure. Summarising these findings, pesticide pressure induced a shift in community composition from communities shaped by a large fraction of pesticide-sensitive and relatively long-living species towards communities mostly comprising insensitive and partly short-living species. However, such shifts remain undetected when a pesticide-insensitive indicator or ecological endpoint is selected. Many of

the established invertebrate-based metrics used to assess the ecological status did not respond to pesticide pressure, such as the Shannon diversity index, the German fauna index or the ecological status class according to the WFD. This suggests that pesticide risks cannot be identified using these indices generally questioning current ecological assessment practices, at least for small streams.

The ecological effects in terms of $SPEAR_{pesticides}$ were best explained by the maximum pesticide toxicity measured at a stream site (Liess et al., 2021). On the one hand, this confirms the selectivity of the $SPEAR_{pesticides}$ indicator, largely responding to pesticide pressure. Hydromorphological and bed habitat structure were also shown to influence $SPEAR_{pesticides}$, but to a minor extent. On the other hand, acute toxicity as a consequence of short-term concentration peaks shapes the invertebrate community rather than the continuous background exposure level. In line with previous studies, these concentration peaks mostly occurred in EDS samples collected after rainfall underlining the ecological relevance of pesticide inputs via runoff (Holvoet et al., 2007; Liess et al., 1999; Spycher et al., 2018). However, accounting for rarely measured extreme toxicity peaks that are exceptionally high in a given stream relative to exposure measured at other times weakened the relationship between pesticide exposure and $SPEAR_{pesticides}$. Specifying an ecologically relevant measure for pesticide exposure would thus need to better integrate the levels of peak and background exposure in combination with the frequency of occurrence of such peak exposures.

The comprehensive data on measured pesticide concentrations in a large number of streams in combination with the $SPEAR_{pesticides}$ as an index fairly specifically responding to pesticide pressure allowed the identification of effect thresholds for invertebrates in the field and the validation of regulatory threshold levels. By defining a benchmark for $SPEAR_{pesticides}$ (60% of the $SPEAR_{pesticides}$ reference value representing no or only slightly disturbed conditions), Liess et al. (2021) derived field-based acceptable concentrations (AC_{field}) for 22 pesticides, for which invertebrates

represented the most sensitive organism group. The AC_{field} involves an AF of almost 2,000 required to extrapolate from acute toxicity test data to the field in order to comply with the defined benchmark. This AF exceeds AFs commonly applied for the regulatory thresholds RAC and MAC-EQS, where respective guidance documents propose an AF of 100 (EFSA, 2013; European Commission, 2018). That is why the AC_{field} is lower than 90% (n = 20) of RAC values and all four MAC-EQS when comparing pesticides that were assigned an AC_{field} and a RAC/MAC-EQS (Weisner et al., 2021a). These findings are supported by several other studies assessing effects on invertebrates under field conditions: Significant shifts in stream invertebrate communities were demonstrated at concentrations that equal acute toxicity endpoints including the established AF of 100 (Knillmann et al., 2018; Liess and Ohe, 2005; Münze et al., 2017; Ohe et al., 2011). Schäfer et al. (2012) found that the relative abundance of sensitive species decreased by 27% - 61% when applying an AF of 100 and estimated that an AF of 1,000-10,000 was required to avoid such pesticide-related effects.

These results suggest that pesticide effects in the field occur at concentrations more than three orders of magnitude below concentration levels causing effects in standard toxicity tests. This is likely due to the simplified experimental designs of standard tests that disregard

- the co-occurrence of multiple pesticides in the field. Toxicity testing and the ERA commonly only consider the exposure and effects of single pesticides. In reality, PPP applications mostly involve multiple pesticides at a time and mixtures in stream water are highly complex featuring a mean of 30 detected pesticides in EDS stream water samples (Weisner et al., 2021b). These pesticide mixtures may result in additive, antagonistic or synergistic effects (Cedergreen, 2014; Siviter et al., 2021).
- the presence of a multitude of other compounds such as biocides, pharmaceuticals, industrial and household chemicals. Even if their contribution to the mixture toxicity for invertebrates estimated for

KgM water samples was minor (Liess et al., 2021), Neale et al. (2020) found only a small fraction of effects to be explained by the broad spectrum of 395 suspect screening compounds using several bioassays. This suggests that a multitude of not measured or not detected compounds may contribute to the ecological effect. Similar results were observed for algae, where reconstituted stream water samples revealed effect concentrations one order of magnitude greater than those derived by testing the actual stream water samples (Stenström et al., 2021).

- the presence of other environmental stressors and their potential interactions. Examples include nutrients, intra- and interspecific competition, food scarcity and temperature, which may act synergistically in combination with pesticides (Shahid et al., 2019) or each other (Piggott et al., 2015).
- frequent, repeated exposure to pesticide pulses that may result in an increased effect compared to the single pesticide pulses applied in standard test systems. Accordingly, individual-, population-, and community-level effects were shown to accumulate within a single generation (Wiberg-Larsen et al., 2020) and culminate over multiple generations (Liess et al., 2013).

7.2.2 Aquatic Plants and Algae

No investigation of the aquatic flora community was performed within the realm of this thesis. However, the widespread occurrence of herbicides led to exposure levels estimated to also harm aquatic photosynthetic organisms including macrophytes and algae. 18% of EDS samples revealed RAC exceedances by pesticides primarily affecting macrophytes or algae (Weisner et al., 2021b). Whether the RACs for macrophytes and algae are sufficiently protective cannot be assessed at that stage as no pesticide-specific bioindicator is available. Benthic diatoms seem promising for this purpose as respective communities are highly diverse and sensitive to a wide range of natural and anthropogenic stressors (Larras et al., 2017). There are already approaches to develop a pesticide-specific indicator based on diatoms (Wood et al., 2019), but these require further research

to strengthen the indicative power. On the basis of EU-wide WFD monitoring data, Malaj et al. (2014) and Wolfram et al. (2021) confirmed the exceedance of safe concentration levels for aquatic plants and algae mostly driven by herbicides in surface waters on a broader scale.

Contribution to narrowing the knowledge gap: This thesis revealed the presence of pesticides in ecologically relevant concentrations in small agricultural streams throughout Germany. The toxic pressure exerted by pesticides was found to affect the community composition of invertebrates and represents the major stressor for vulnerable insects compared to other stressors.

7.3 Implications for the Environmental Risk Assessment of Pesticides

Knowledge gap: The limited amount of useful information regarding pesticide exposure and related ecological effects in streams on a broad scale has impeded an adequate validation of the concepts of current ERA of pesticides.

The European parliament requires that no authorisation for pesticides shall be granted "unless it is clearly established through an appropriate risk assessment that under field conditions no unacceptable impact on the viability of exposed species […] occurs" (European Commission, 2011). The environmental status observed for the agricultural streams investigated within the KgM, however, revealed an impairment of vulnerable populations, represented by a decline of invertebrate taxa classified as "SPEcies At Risk" (Liess et al., 2021). This situation does neither fulfil the requirements of Regulation (EU) 546/2011 stating that "Member States shall ensure that use of plant protection products does not have any long-term repercussions for the abundance and diversity of non-target species" (European Commission, 2011), nor comply with EU regulation 1107/2009 according to which pesticides must not cause "unacceptable effects on the environment". (European Union, 2009). In the context of aquatic risk assessment, "unacceptable effects" are defined as "negligible population-level effects" on the "most sensitive populations" (so-called ecological

threshold option - ETO) or “some population effects if ecological recovery takes place within an acceptable time-period (ecological recovery option – ERO) (EFSA, 2013).

On the basis of the presented publications, the failure to meet the self-imposed targets for the aquatic ERA of pesticides can be attributed to 3 main causes:

1. Underestimation of pesticide exposure

The measured environmental pesticide concentrations exceeded those predicted by the exposure model “Exposit” used in the German ERA during the authorisation of plant protection products (Liess et al., 2021). This is consistent with Knäbel et al. (2012), who found that the corresponding exposure model used in other EU member states, FOCUS, underestimates insecticide concentrations in surface water. Reasons for this underestimation of exposure are expected to be (i) incorrect assumptions made in exposure modelling (e.g. no runoff-driven inputs assumed for Methiocarb), (ii) a false exposure model parameterisation, (iii) the disregard of multiple applications of a pesticide within a single stream catchment, (iv) the overestimation of the effectiveness of risk mitigation measures (e.g. buffer strips), or/and (v) unauthorised application rates. However, the occurrence of PEC exceedances in numerous streams argues against single unauthorised application rates and suggests the systematic causes (i)-(iv). Besides the unexpectedly high environmental concentrations of single pesticides, the ERA underestimates the real exposure by its restriction to single applications of single plant protection products. It therefore fails to consider the presence of other pesticides on top of the single pesticide exposure, which results in an underestimation of the additive risk by a factor of 3.2 under realistic worst-case conditions (Weisner et al., 2021b). Finally, the ERA largely disregards that non-target organisms face repeated exposures during their lifetime and lacks a concept to account for the respective ecological effects.

2. Insufficiency of regulatory thresholds

EFSA's Aquatic Guidance states that "it should be realised that, in the first tier, an AF of 100 for acute and 10 for chronic toxicity may not be protective in 100% of the cases. Consequently, this will also be the case for all effect tiers" (EFSA, 2013). Liess et al. (2021) confirmed this assumption by having observed distinct reductions of vulnerable species at concentration levels below the RACs. For the pesticides driving toxicity in the KgM, an additional factor of 5.3 - 40 to lower the RACs was determined to ensure a high or good $SPEAR_{pesticides}$ in 95% and 99% of agricultural streams, respectively. In principle, the exceedance of RACs were well associated with the ecological effects in terms of $SPEAR_{pesticides}$ (Liess et al., 2021) and RAC values correlated with the AC_{field} (Weisner et al., 2021a), supporting the approach for deriving RACs in general but demonstrating a systematic underestimation of effects in the field. Building up on the results of Beketov et al. (2013), Stehle and Schulz (2015) support this by attributing the decrease in invertebrate family richness to concentration maxima exceeding levels equalling one tenth of regulatory thresholds. Even the compliance of regulatory thresholds derived in the ERA may therefore not ensure the achievement of corresponding ecological targets.

3. Inertia of the authorisation process

At the time of the KgM in 2018 and 2019, eight of the 20 pesticides most often exceeding the RAC would not have gained approval for use anymore (Liess et al., 2021). Due to relevant new scientific evidence concerning these pesticides, the effect assessment of the ERA was carried out again under the consideration of the newly gained knowledge. As this evidence revealed that earlier RACs were too high to avoid "unacceptable effects on the environment", this resulted in a lowering of RACs. The PECs derived in the exposure assessment remain the same, though. A new comparison of the PEC and RAC would have indicated a risk, as the PEC now exceeded the lowered RAC. However,

the re-evaluation involving an updated comparison of PEC and RAC was not performed at that time as it is generally intended only every ten to fifteen years. Even if new knowledge finds its way into the ERA, the inertia of the authorisation impedes its timely incorporation into practice, allowing pesticide use known to cause unintended environmental risks. The findings of the KgM are the first to reveal this fundamental problem in the authorisation of pesticides.

The assessment of the ERA of pesticides using the results of the KgM monitoring campaigns in 2018 and 2019 in Germany reflects a temporal and spatial snapshot. Nevertheless, it can be expected that the problems identified apply equally in many other parts of the world and will continue to be of relevance after the monitoring period. On the one hand, the ERA of pesticides builds on the same principles of comparing expected exposure with expected effects in countries where pesticides regulation is similarly advanced. The implications are therefore transferable to other regions including, among others, fellow EU member states (European Union, 2009), Great Britain (HSE, 2021), North America (United States Congress, 1910) and Australia (Australian Government, 1992). On the other hand, the history of pesticide regulation reveals that banning pesticides of concern has not led to a reduction of overall environmental impacts so far (Boyd, 2018; Schulz et al., 2021). Banned substances were replaced by even more toxic substitutes, which sooner or later turned out to be no less of a concern (e.g. neonicotinoid effects on pollinators). The spectrum of pesticides used is continuously changing and risk drivers identified in the KgM may have already been replaced by others. Especially the neonicotinoid insecticides thiacloprid, clothianidin, imidacloprid and thiamethoxam caused a large fraction of RAC exceedances and were banned in recent years. Other substance groups such as pyrethroids or anthranilic diamides are already being considered as potential successors, though (Schmidt-Jeffris and Nault, 2016; Werner and Young, 2018). The above-mentioned problems in the ERA need to be addressed so that risks are not discovered after the ecological damage is done.

More and more studies claim a gap between the ERA's intentions and self-imposed protection goals and emphasised the need to refine or overhaul the ERA of pesticides (Brühl and Zaller, 2019; Frische et al., 2018; Schäfer et al., 2019; Topping et al., 2020). Boyd (2018) concludes that "uncertainty will always be a problem when making judgements about diffuse environmental impacts, but we can be much more certain about the fact that these diffuse effects are part of a farming system which needs overhaul and thorough reform". The results of the KgM confirmed this need. Moreover, it was possible to attribute ecological impacts to pesticide exposure, thus minimising the "uncertainty in diffuse environmental impacts" referred to. Finally, the KgM findings imply that the ERA of pesticides requires continuous post-authorisation monitoring to validate exposure and effect predictions, representing the only way to survey the actual environmental risk. The next chapter addresses what the KgM results entail for the monitoring of pesticides in surface waters.

Contribution to narrowing the knowledge gap: The thesis provided additional evidence supporting assumptions drawn from previous small-scale studies, but also identified and quantified factors so far unknown to cause the gap between intended protection level and reality. This includes the underestimation of exposure and the insufficiency of ecological thresholds predicted in the ERA of pesticides as well as the inertia of the risk assessment process.

7.4 Implications for the Monitoring of Pesticides in Surface Waters

Knowledge gap: Common monitoring strategies were suspected to underestimate pesticide risks. It was unclear, how significant this underestimation was and what adjustments were needed most urgently to monitor pesticide risk more realistically.

As already observed through previous studies on a smaller scale (Kreuger, 1998; Liess et al., 1999; Lorenz et al., 2017; Spycher et al., 2018), Halbach et al. (2021) confirmed the periodically increased concentrations of pesticides in streams during or after rainfall compared to dry weather

conditions. Peaks of pesticide concentrations are thus likely to occur after rainfall and EDS sampling is considered indispensable to adequately quantify surface water pesticide pollution. Particularly when it comes to assessing the acute risk, as for the surface water status assessment under the WFD applying the MAC-EQS, common grab sampling is unlikely to capture the relevant concentration peaks (Weisner et al., 2021a). It is this acute toxicity of exposure peaks that was found to explain the ecological status reflected by the invertebrate community in multiple studies (Knillmann et al., 2018; Liess et al., 2021; Liess and Schulz, 1999; Schäfer et al., 2012). It remains to be clarified to what extent the short-term exposure peaks are also determining the ecological effect for other organism groups such as diatoms or fish. However, the use of grab samples alone may reason, at least partially, the weaker correlation between pesticide pressure and observed ecological status in other studies (Birk et al., 2020; Rico et al., 2016; Schäfer, 2019). The automatic samplers used in the KgM are one method of approximating actual peak exposure. Also triggered by a water level rise, simple bottle samplers as used by Liess and Ohe (2005) allow for taking EDS samples in a similar way. The pesticide load in passive samplers can be used to approximate peak concentrations in the water phase to some extent (Schreiner et al., 2020). Best coverage is yielded by automated permanent high-frequency sampling (Spycher et al., 2018), where the large number of samples and the related financial, time and personnel effort limit the number of monitoring sites at which such a detailed sampling design is feasible.

The large number of pesticides measured in environmentally relevant concentrations demonstrates that realistic pesticide monitoring requires a broad spectrum of analytes on the one hand. This is aggravated by a high spatio-temporal variability regarding the occurrence of pesticides (Weisner et al., 2021b), where a single stream may feature multiple toxicity drivers in the successive samples taken throughout the monitoring campaign or where streams of neighbouring catchments feature different toxicity drivers at the same time. In the light of the permanently changing spectrum of pesticides approved and used, the analyte spectrum to be monitored

needs to account for current-use patterns on the other hand (Moschet et al., 2014).

Governmental monitoring under the WFD represents the major routine monitoring programme for pesticides and other contaminant groups in surface waters in Germany and the EU. However, it underestimates pesticide risks in surface waters mostly due to its limitation to grab sampling, an incomplete analyte spectrum (Weisner et al., 2021a) and the use of biological metrics unsuitable to indicate pesticide pressure (Liess et al., 2021). This underestimation of pesticide risk hampers the determination of causes for the often-observed insufficient ecological status of surface waters and the rapid identification of pesticides adversely affecting the environment more than predicted by the ERA. The WFD monitoring would profit from refinements perpetuating part of the KgM methods to effectively lower pesticide risks in streams in the long term.

Contribution to narrowing the knowledge gap: The thesis underlines the need for monitoring strategies to account for the periodic occurrence of current-use pesticides in surface waters and the increased vulnerability of small streams. Current governmental monitoring under the WFD significantly underestimates pesticide risks and requires refinements with respect to sampling, analysing and assessing pesticides in surface waters.

8 Conclusions

From the findings of this thesis it can be concluded that the majority of small agricultural streams and their ecological integrity are at risk due to pesticide exposure. This contrasts the widespread assumption that pesticide use is environmentally safe causing no or only negligible ecological effects supported by the prevailing perception of highly elaborate standards in the ERA. In this thesis, these standards were shown to be insufficient to comply with legally defined protection goals. One example of these protection goals, anchored in the NAP, requires that a maximum of 1% of EDS samples exceeds the RAC by the year 2023. However, 60% of EDS samples taken within the KgM in 2018 and 2019 featured at least one RAC exceedance. Closing this gap between intended and actual level of pesticide risk requires fundamental adjustments in the regulation of pesticide use.

This thesis both confirmed already known and identified undiscovered deficiencies in the ERA forming the basis of pesticide regulation. To remedy these deficiencies both exposure and effect threshold predictions need to be more conservative. Further research is needed to validate applied exposure models and the efficacy of risk mitigation measures in more detail, e.g. via a combined analysis of pesticide application and in-stream concentration data originating from the same catchment. Assessment factors need to be increased to derive acceptable concentration thresholds that comply with the premise to prevent unacceptable effects on the environment (Liess et al., 2021). In line with EU's pesticide regulation, the reassessment of a pesticide's risk needs to be carried out more immediately when new knowledge impacting the predicted exposure and effect becomes available (European Union, 2009). The implementation of "landscape laboratories" to assess environmental fate and ecological effects on a small scale under real-world conditions prior to broad use would prolong the authorisation process but decrease the probability of "negative surprises" (Schäfer et al., 2019). This should be complemented by an adequate post-authorisation monitoring permanently reviewing

regulation decisions under a continuously changing spectrum of pesticides in use.

Refining pesticide risk assessment is vital as trends in biodiversity decline aggravate while global pesticide use is expected to further increase in the upcoming decades. The rapid increase in the world's population forecasted to reach 11 billion by 2100 requires a maximisation of yields to ensure food production (Tilman et al., 2001). Moreover, climate change favours the expansion of pests and is expected to further promote pesticide use (Chen and McCarl, 2001; Koleva and Schneider, 2009). On the other side, biodiversity decline and initiatives to ban Glyphosate have gained considerable media coverage, brought pesticides and their ecological effects further into the public eye and initiated a broad debate in recent years. A rising awareness and more profound understanding of pesticide effects have initiated a steady expansion of organic farming (Seufert et al., 2012; Willer and Lernoud, 2019) and the development of farming strategies that combine more eco-friendly pest control measures only drawing on pesticides as a second-choice tool (Ehler, 2006). Moreover, technical advances on the basis of digitalised farming enable the limit pesticide use to pest-affected hotspots (King, 2017). Promoting these developments, governments have also started to commit themselves to reducing pesticide use. The recently published “Farm to Fork” strategy by the European Commission intends an expansion of organic farming to 25% and a 50% reduction of pesticide use and risks by 2030 for EU member states (European Commission, 2020). This involves a huge potential, but what sounds ambitious now will have to be measured in terms of actual implementation and achievements. The holistic transformation of today’s agriculture required to reverse environmental impacts and secure ecosystem services reflects one of the key challenges of this century. Luckily, however, “we know enough to act now” (Forister et al., 2019).

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9 Declaration

I, the author of this work, certify that this work contains no material which has been accepted or submitted for the award of any other degree at any university or other tertiary institution. The work has been interdependently prepared. All aids and sources have been clearly specified and the contribution of other authors have been documented and reference lists given.

Leipzig, November 15th 2021

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Oliver Weisner

10 Contributions to Publications

1 Halbach, K.; Möder, M.; Schrader, S.; Liebmann, L.; Schäfer, R. B.; Schneeweiss, A.; Schreiner, V.C.; Vormeier, P.; **Weisner, O.**; Liess, M.; Reemtsma, T. (2021): Small Streams – Large Concentrations? Pesticide Monitoring in Small Agricultural Streams in Germany during Dry Weather and Rainfall. In: *Water Research.*

15% - Investigation, data curation, formal analysis, writing (review & editing)

2 Liess, M.; Liebmann, L.; Vormeier, P.; **Weisner, O.**; Altenburger, R.; Borchardt, D.; Brack, W.; Chatzinotas, A.; Escher, B.; Foit, K.; Gunold, R.; Henz, S.; Hitzfeld, K.L.; Schmitt-Jansen, M.; Kamjunke, N.; Kaske, O.; Knillmann, S.; Krauss, M.; Küster, E.; Link, M.; Lück, M.; Möder, M.; Müller, A.; Paschke, A.; Schäfer, R.B.; Schneeweiss, A.; Schreiner, V.C.; Schulze, T.; Schüürmann, G.; von Tümpling, W.; Weitere, M.; Wogram, J.; Reemtsma, T. (2021): Pesticides are the dominant stressors for vulnerable insects in lowland streams. In: *Water Research.*

20% - Investigation, data curation, formal analysis, visualisation, writing (review & editing)

3 **Weisner, O.**; Frische, T.; Liebmann, L.; Reemtsma, T.; Roß-Nickoll, M.; Schäfer, R.B.; Schäffer, A.; Scholz-Starke, B.; Vormeier, P.; Knillmann, S.; Liess, M. (2021): Risk from Pesticide Mixtures – the Gap between Risk Assessment and Reality. In: *Science of the Total Environment.*

60% - Sampling, conceptualization, methodology, investigation, data curation, formal analysis, visualization, writing (original draft)

4 Neale, P.A.; Braun, G.; Brack, W.; Carmona, E.; Gunold, R.; König, M.; Krauss, M.; Liebmann, L.; Liess, M.; Link, M.; Schäfer, R.B.; Schlichting, R.; Schreiner, V.C.; Schulze, T.; Vormeier, P.; **Weisner, O.**; Escher, B.I. (2020): Assessing the Mixture Effects in In Vitro Bioassays of Chemicals Occurring in Small Agricultural Streams during Rain Events. In: *Environmental Science & Technology*.

10% - Investigation, data curation, writing (review & editing)

5 **Weisner, O.**; Arle, J.; Liebmann, L.; Link, M.; Schäfer, R.B.; Schneeweiss, A.; Schreiner, V.C.; Vormeier, P.; Liess, M. (2021): Three Reasons Why the Water Framework Directive (WFD) Fails to Identify Pesticide Risks. In: *Water Research*.

60% - Sampling, conceptualization, methodology, investigation, data curation, formal analysis, visualization, writing (original draft)

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12 Curriculum Vitae

Personal Information

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Scientific Education & Career

Since 04/2018	*Helmholtz Centre for Environmental Research – UFZ Leipzig,* Germany **PhD study** in the department for System Ecotoxicology: "The Kleingewässer-Monitoring (KgM) – A Monitoring of German Small Streams and Its Implications for the Environmental Risk Assessment of Pesticides"
10/2015 – 03/2018	*University of Koblenz-Landau*, Germany **MSc Ecotoxicology** Thesis: "Linking Agricultural and Urban Land Use to Nonpoint Source Pesticide Pollution in Streams: A GIS Based Analysis of the U.S. Water Quality Portal Data"
01/2014 – 06/2014	*Université Jean Monnet Saint Etienne*, France **BSc Science de la terre et de l'environnement** ERASMUS semester abroad
04/2012 – 01/2016	*TU Bergakademie Freiberg*, Germany **BSc Geoökologie** Thesis: "Determination of pH-Dependent Hydrolysis Rate Constants of Two Organophosphate Esters" at the Helmholtz Centre for Environmental Research – UFZ Leipzig, Germany
09/2002 – 05/2011	*Staffelsee-Gymnasium Murnau*, Germany

13 Annex - Supporting Information of Publications

13.1 Small Streams – Large Concentrations? Pesticide Monitoring in Small Agricultural Streams in Germany during Dry Weather and Rainfall – Supporting Information

The amount and/or format of the Supporting Information is not suitable to be presented here. Please see the homepage related to the publication to view this supporting information: (https://www.sciencedirect.com/science/article/abs/pii/S0043135421007314)

13.2 Pesticides Are the Dominant Stressors for Vulnerable Insects in Lowland Streams – Supporting Information

The amount and/or format of the Supporting Information is not suitable to be presented here. Please see the homepage related to the publication to view this supporting information: (https://www.sciencedirect.com/science/article/abs/pii/S0043135421004607)

13.3 Risk from Pesticide Mixtures – The Gap between Risk Assessment and Reality – Supporting Information

The amount and/or format of the Supporting Information is not suitable to be presented here. Please see the homepage related to the publication to view this supporting information: (https://www.sciencedirect.com/science/article/abs/pii/S0048969721040894)

13.4 Assessing the Mixture Effects in In Vitro Bioassays of Chemicals Occurring in Small Agricultural Streams during Rain Events – Supporting Information

The amount and/or format of the Supporting Information is not suitable to be presented here. Please see the homepage related to the publication to view this supporting information: (https://pubs.acs.org/doi/10.1021/acs.est.0c02235)

13.5 Three Reasons Why the Water Framework Directive (WFD) Fails to Identify Pesticide Risks – Supporting Information

The amount and/or format of the Supporting Information is not suitable to be presented here. Please see the homepage related to the publication to view this supporting information: (https://www.sciencedirect.com/science/article/pii/S0043135421010423)