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
Sci. Total Environ. **796** , art. 149017

http://dx.doi.org/10.1016/j.scitotenv.2021.149017

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, 3446 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.

Keywords

- Environmental risk assessment
- Plant protection products
- Spray series
- Pesticide exposure
- Aquatic ecotoxicology

- Ecological effects

List of abbreviations

PPP	Plant protection product
MCR	Maximum cumulative ratio
ERA	Environmental risk assessment
EDS	Event-driven sampling
TU	Toxic unit
CA	Concentration addition
RAC	Regulatory acceptable concentration
RQ	Risk quotient
AI	Aquatic invertebrates
AP	Aquatic plants/algae

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 (German Environment Agency - 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. 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 Parliament and Council of the 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 (European Food Safety Authority (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 (Dutch Board for the Authorisation of Plant Protection Products and Biocides (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.

2 Material and methods

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.

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, S. & Scholz-Starke, B. et al., 2021). The dataset included 889 real-world spray series from the years 2007-2015 (see Supporting Information/SI Fig. 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 = 3446) 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 (European Food Safety Authority (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.

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).

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, 1969). 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

$$TU_i = \frac{c_i}{EC_{50_i}} \quad (1)$$

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 Parliament and Council of the European Union, 2009; Umweltbundesamt (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 ($RQ > 1$).

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

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

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):

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

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

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

Application

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

Water samples

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

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

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, 2009).

3 Results & Discussion

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 1). 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 1 - 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 1).

Single application - The PPP applications (n = 3446) 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 1 - 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 1 - 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. 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.

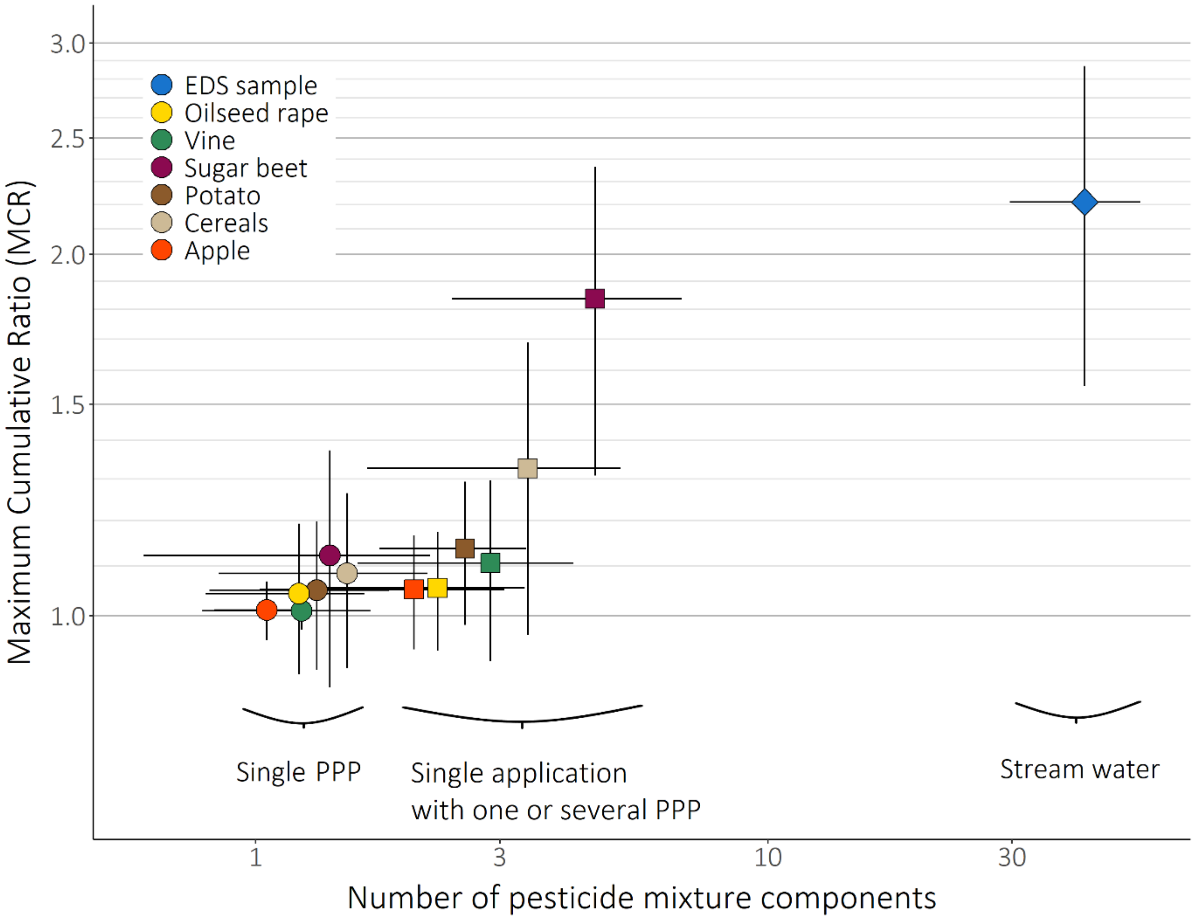

Figure 1: *MCR*s of different mixture categories against the number of pesticide mixture components: Culture-specific Plant Protection Products (PPP) applied (MCR_{PPP}, circle), applications (MCR_{app}, square) and EDS stream water samples (MCR_{sample}, diamond, including metabolites). Data points represent mean values and bars display the respective standard deviation.

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.

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 2). 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.

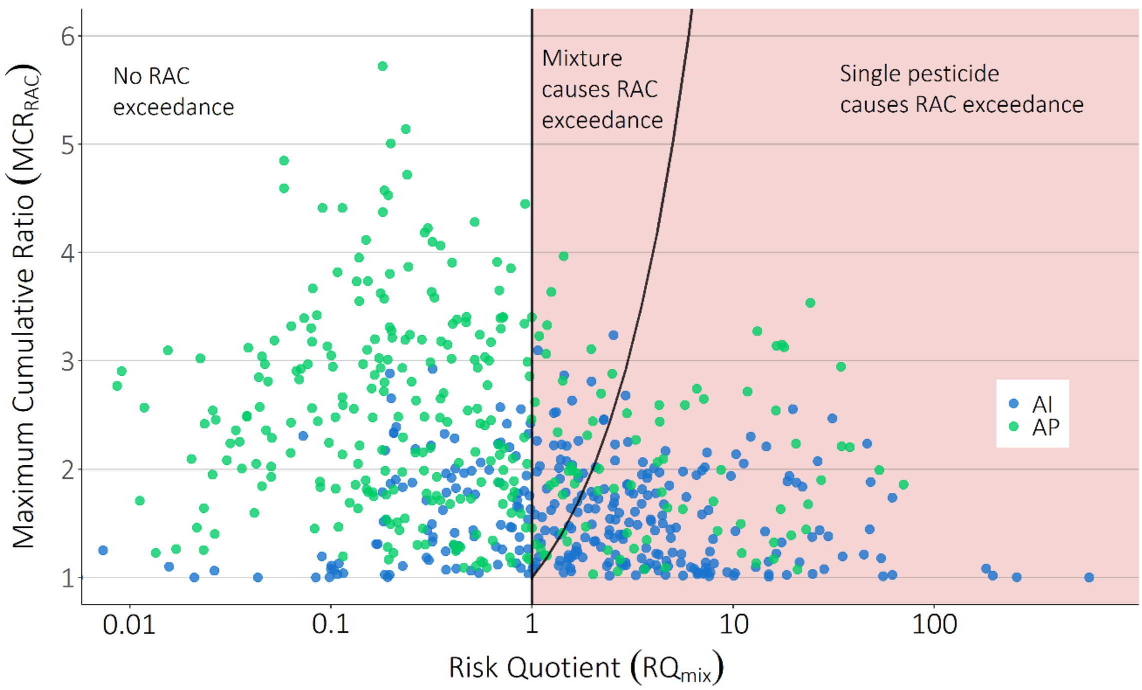

Figure 2: The additive concentration-RAC quotient (RQ_{mix}) indicating regulatory threshold exceedance and respective Maximum Cumulative Ratio (MCR_{RAC}) derived separately for aquatic invertebrates (AI, blue dots) and aquatic plants/algae (AP, green dots) of each event-driven stream water sample (n=312). Log RQ_{mix} values ≤ 0 represent samples not exceeding the RAC (34% for AI, 74% for AP). Log RQ_{mix} 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 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.

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; Rydh 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.

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) (European Food Safety Authority (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 3). 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 3). 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 (European Food Safety Authority (EFSA), 2013).

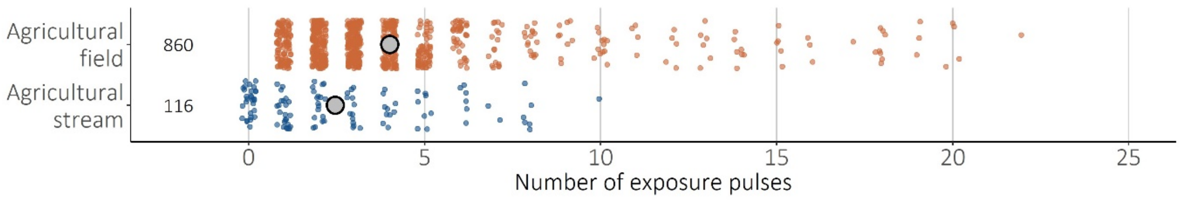

Figure 3: Number of exposure pulses from April to mid-July (stream monitoring period) for agricultural fields and streams. Orange points reflect the number of applications per field (n = 860), blue points reflect number of samples showing a RAC exceedance ($RQ_{max} > 1$) per stream (n=116). Grey points depict respective means.

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.

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.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The spray series data that support the findings of this study were provided by the INL – "Privates Institut für Nachhaltige Landbewirtschaftung" Halle, Germany, are not publicly available and only presented in aggregated form (see Supporting Information). The stream monitoring data that support the findings of this study are presented in further detail in the Supporting Information, Liess et al. 2021 and available via the PANGAEA data publisher (https://doi.org/10.1594/PANGAEA.931673) (Liess et al., 2021b). Measured pesticide concentrations can be explored via the "Kleingewässermonitoring" project homepage under https://www.ufz.de/kgm/index.php?en=48130.

Competing of interests

The authors declare that they have no competing interests

Funding

The COMBITOX project including the monitoring of spray series was funded by the German Federal Ministry for the Environment, Nature Conservation and Nuclear Safety (FKZ 3715 63 407 0). The stream monitoring was supported by the German Helmholtz long-range strategic research funding and the "Pilotstudie zur Ermittlung der Belastung von Kleingewässern in der Agrarlandschaft mit Pflanzenschutzmittel-Rückständen" funded by the German Federal Ministry for the Environment, Nature Conservation and Nuclear Safety (FKZ 3717 63 403 0). Additional funding was supplied by the MOSES (Modular Observation Solutions for Earth Systems) and TERENO (Terrestrial Environmental Observations) projects.

Acknowledgements

We thank Maren Lück, Oliver Kaske, Monika Möder and Steffi Schrader from the Helmholtz-Centre for Environmental Research (UFZ), Anke Schneeweiss, Verena C. Schreiner and Moritz Link from the Institute for Environmental Sciences, University of Koblenz-Landau as well as all involved students for assisting in the stream monitoring. We further thank Susanne Bär and Christian Ullrich for their contribution through their involvement in the COMBITOX project.

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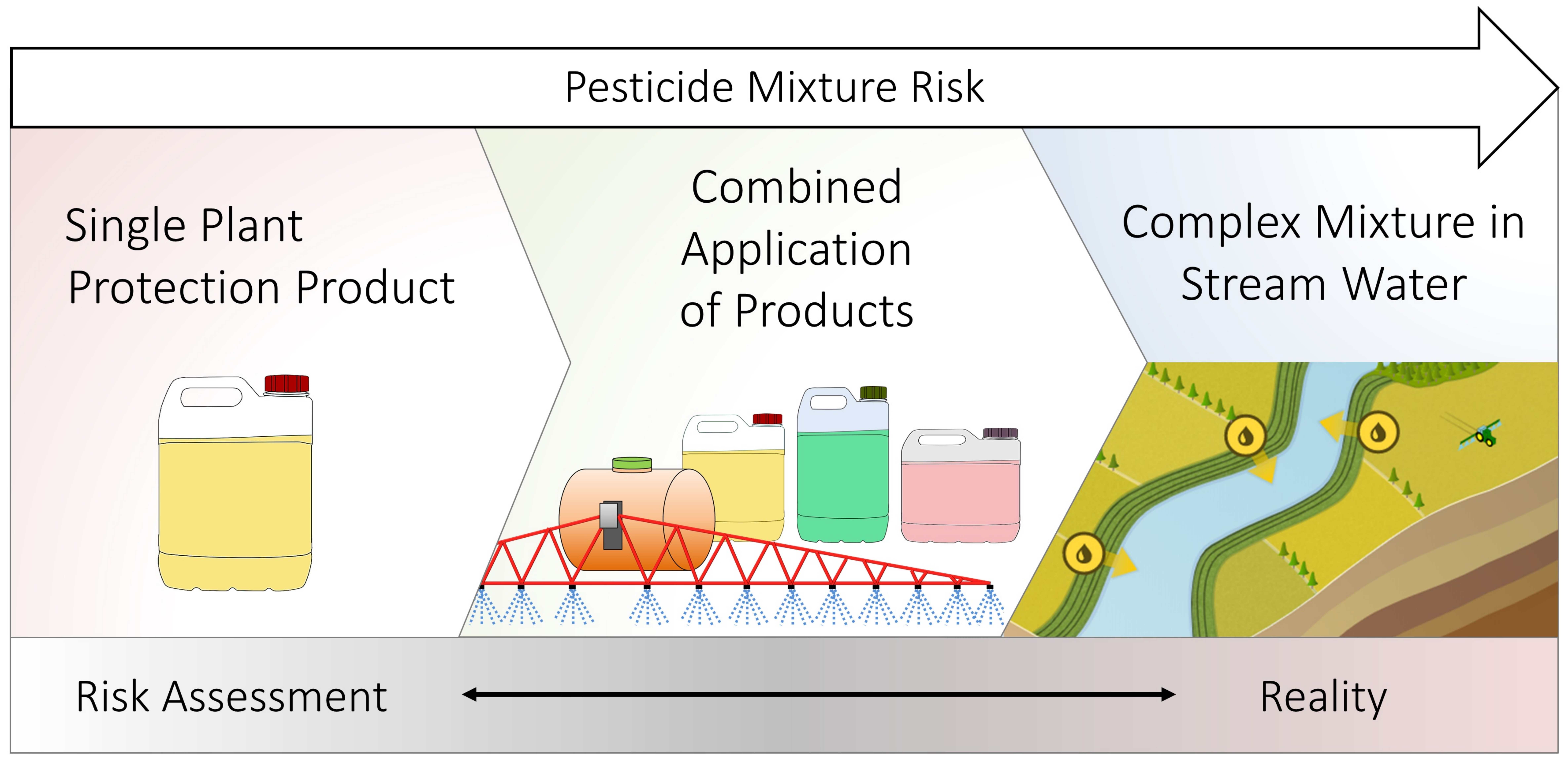
Highlights

- Comprehensive analysis of reported pesticide applications and stream water samples
- Mixture risk compared in plant protection products, applications and stream water
- Mixture risk and regulatory threshold exceedances driven by single pesticides
- Assessment factor of 3.2 required to account for simultaneous pesticide mixtures
- Frequency of pesticide exposure higher than considered in risk assessment


Pesticide Mixture Risk
Single Plant Protection Product
Combined Application of Products
Complex Mixture in Stream Water
Risk Assessment
Reality