Indirect effects of chemical stream pollution on the riparian food web

by

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I dedicate this thesis to my grandparents Franz and Christa, my parents Ron and Christine, and my brother Max, for supporting and inspiring me throughout my journeys

"By their very nature chemical controls are self-defeating, for they have been devised and applied without taking into account the complex biological systems against which they have been blindly hurled."

Rachel Carson, Silent Spring

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Abstract

Chemical pollution is a ubiquitous stressor affecting streams and their linkages to riparian forests. Contaminants act by altering the emergence of aquatic insects from streams. Emergent insects can also take up contaminants and transfer them into the terrestrial ecosystem. Emergent insects are an important source of prey for riparian insectivores and changes in the emergence flux or contamination of insects can affect the riparian food web. However, little is known about the implications of emerging contaminants such as agricultural pesticides and wastewater effluent on the terrestrial food web. In this dissertation, I address possible consequences of agricultural and wastewater stream pollution for riparian insectivores, namely bats and spiders.

The contribution of aquatic prey to riparian spider diets has mainly been determined by stable isotope analysis, but DNA metabarcoding, a highly sensitive method of identifying consumed prey using DNA, promises to further detangle changes in these trophic interactions. In Chapter 2, we tested a bleaching decontamination protocol to determine the suitability of using metabarcoding on spiders contaminated during sampling. We confirmed the applicability of metabarcoding, but also found that the wolf spiders (Lyocsidae) collected in riparian areas did not appear to rely strongly on aquatic prey. This informed our choice of *Tetragnatha montana*, which are highly reliant on aquatic prey, for the field study in Chapter 3.

We then conducted three field studies. Chapters 3 and 4 evaluate indirect trophic effects of chemical stream pollution on spiders and bats, respectively. Chapter 5 quantifies the accumulation of pesticides from the stream to riparian spiders via emergent insects. We found that riparian bats foraged more and that spiders consumed more Chironomidae at more polluted sites, indicating that there was no overall decrease in emergence due to chemical pollution. We also found that certain pesticides accumulated in emergent insects and riparian spiders. Together, this suggests that chemical stream pollution resulted in an increased dietary exposure of riparian insectivores to contaminants, rather than a decrease in prey availability.

These results demonstrate the role of streams and aquatic-terrestrial linkages in propagating stressors across ecosystem boundaries. They also show the benefit of using sensitive methods like DNA metabarcoding to unveil trophic effects of chemical pollution. Future studies should focus on quantifying the risk of contaminant uptake and potential effects for riparian bats, as well as considering how the observed drivers change in different contamination scenarios and ecosystems. This knowledge is important to protect the functionality of the riparian ecosystem and its inhabitants.

Chapter 1: General Introduction

Maike Huszarik

Chemical contaminants as stressors in streams

Environmental pollution with anthropogenic chemicals is one of the strongest drivers of global change (Bernhardt et al. 2017). Chemical pollution is associated with global biodiversity loss (Sigmund et al. 2023), and considered to be related to recently reported insect declines (Hallmann et al. 2017; Kehoe et al. 2021). It has also become a problem for surface waters, where chemical inputs from agricultural, urban, industrial and mining sources threaten the integrity of freshwater ecosystems (Malaj et al. 2014; Stehle and Schulz 2015). These pollutants enter waters via runoff from urban or agricultural surfaces, atmospheric deposition or spray drift, or wastewater from municipal and industrial sources. Lotic systems, such as streams, play important roles in chemical pollution as they not only receive, but can also transport chemical contaminants to downstream areas (Barber et al. 2013), enabling contaminants to reach much larger areas than where they were emitted.

Streams are one of the ecosystems which have been most altered by human activity (Carpenter et al. 2011; Albert et al. 2021; Brauns et al. 2022). However, streams and the adjacent terrestrial ecosystems, known as riparian areas (Gregory et al. 1991), are also important habitats for both freshwater and terrestrial organisms (Sabo et al. 2005; Ramey and Richardson 2017). These ecotones act as an interface of aquatic and terrestrial nutrient transfers that tightly link water and land. While the quantity of organic material flowing from the land into streams is much higher than *vice versa*, the high quality of aquatic to terrestrial inputs makes these flows equally important for the recipient system (Bartels et al. 2012). A classic example of this are the salmon runs on the Pacific coast of North America, where the carcasses of spawning salmon are a vital pulse of nutrients for many terrestrial organisms (Gende et al. 2002). A more widespread form of aquatic-to-terrestrial transfer is the emergence of aquatic insects from streams into the surrounding terrestrial ecosystems (Baxter et al. 2005). These nutrient inputs may act as subsidies for the recipient system (Polis et al. 1997), enabling a higher density of consumers to be supported in riparian areas (Ballinger and Lake 2006). However, streams are flowing systems and import dynamics and stressors from upstream areas, which can alter the flux of insect emergence and affect the riparian consumers dependent on them.

Chemical contaminants are one of the major stressors of streams (Malaj et al. 2014; Liess et al. 2021; Brauns et al. 2022), especially as they can be transported downstream into ecosystems which may not be otherwise exposed (Wolfram et al. 2023). Chemical contaminants in streams may have a range of effects on insect emergence, and consequently, the riparian food web.

Depending on the nature of the contaminants, they can affect insect emergence directly through mortality or sublethal effects reducing emergence success, or shift the stream community to more tolerant species. Increasing evidence also shows that emergent insects can accumulate and export a variety of contaminants from streams (Daley et al. 2011; Koch et al. 2021; Kraus et al. 2021a; Previšić et al. 2021). Kraus (2019) has proposed a heuristic model to describe and predict possible outcomes of chemical pollution on both the flux of emergent insects and the flux of contaminants from aquatic to terrestrial systems. They present four outcomes, which depend on the toxicity and potential for bioaccumulation of the contaminant: (1) a reduction in insect emergence due to toxic effects of chemical pollution ("exposure driving subsidies") without bioaccumulation, (2) no reduction in emergence but an increased export of contaminants due to bioaccumulation in emergent insects ("subsidies driving exposure"), (3) both a reduction in emergence and high contaminant accumulation resulting in contaminant export, (4) no significant effect: neither a reduction in emergence nor accumulation and export of the contaminant in emergent insects (Kraus 2019; Kraus et al. 2021b). Depending on which of these effects are caused by chemical contaminants in a stream, riparian insectivores may suffer from a lack of aquatic prey and/or an increased dietary exposure to chemical contaminants. However, as noted by Bundschuh et al. (2022), few studies have evaluated the implications of these hypotheses on predators of emergent insects and overall effects of chemical stream pollution on the riparian food web have rarely been investigated.

Agricultural and wastewater pollution

Agricultural pesticides and contaminants in municipal wastewater effluent, such as pharmaceuticals, are a major concern for streams due to their ubiquitous presence in surface waters and high potential toxicity (Schäfer et al. 2011; Richmond et al. 2018; Schulz et al. 2021). These contaminants enter streams from a variety of sources. Point sources are well-defined entry points such as wastewater effluent emitted from a municipal treatment plant outlet. Non-point sources are undefined, entering the stream through spray drift or rainwater runoff, such as runoff from an agricultural field (Neumann et al. 2002). Agricultural pollutants in streams include pesticides, but also are associated with nutrient input from fertilizer use. Wastewater inputs, which may be treated by wastewater treatment plants or untreated, are more diverse, also containing pesticides (Le et al. 2017; Burdon et al. 2019), in addition to pharmaceuticals, personal care products, and illicit drugs, among other pollutants (Lee et al. 2016; Richmond et al. 2018). Although wastewater treatment plants are able to remove some synthetic chemicals, many compounds are difficult to remove from wastewater completely and

are emitted into streams (Nelson et al. 2011; Stalter et al. 2013; Čelić et al. 2019). Both pesticides and wastewater effluent have been found to directly affect the flux of emergent insects (Kalcounis-Rueppell et al. 2007; Barmentlo et al. 2021; Kraus et al. 2021a; Marshall et al. 2022), though less is known about the indirect effects of agricultural and wastewater contaminants on the riparian food web.

The riparian food web and known effects of chemical pollution

Emergent aquatic insects

Emergent aquatic insects (hereafter "emergent insects") link aquatic and terrestrial food webs as they are important sources of prey for terrestrial insectivores in riparian areas (Baxter et al. 2005). They are characterized by an aquatic larval stage, which matures to emerge as a flying adult (Figure 1). These adults can then be consumed by predators such as lizards, beetles, birds, spiders, and bats (Gray 1993; Baxter et al. 2005; Paetzold et al. 2005; Fukui et al. 2006). Emergent insects are valuable prey due to their high nutritious quality. They are rich in long-chain poly-unsaturated fatty acids (PUFAs) which are not normally found in terrestrial prey (Hixson et al. 2015; Parmar et al. 2022). Many terrestrial organisms are able to produce PUFAs from precursors in their diet, but riparian consumers may rely less on this ability and use emergent insects as a PUFA source (Twining et al. 2019, 2021). In addition to their quality, some insects emerge in temporal pulses, which coincide with important life history periods for predators, as well as with periods where less terrestrial prey is available (Nakano and Murakami 2001; Fukui et al. 2006; Marczak and Richardson 2008). Thus, due to their nutritious and temporal value, riparian predators benefit from emergent insects. The distribution and density of many riparian insectivores along streams often reflect that of insect emergence (Henschel et al. 2001; Kato et al. 2003; Fukui et al. 2006; Hagen and Sabo 2011), highlighting the importance of this resource.

Figure 1: Examples of emergent aquatic insects. **Left:** The larval stage of caddisflies (Trichoptera), which mature in streams. **Right:** An emerged damselfly (Odonata) resting on vegetation.

Due to their aquatic larval development, the flux of emergent insects is affected by the conditions of their aquatic habitat. Emergence abundance or biodiversity can change depending on the quality of the stream habitat that emergent insect larvae are exposed to (Larsen et al. 2016; Serra et al. 2017, Raitif et al. 2018; Manning and Sullivan 2021). Several highly sensitive groups exist, including insects from the orders Ephemeroptera, Plecoptera, and Trichoptera (EPT; Chang et al. 2014; Rico and Van den Brink 2015). Others, such as some Chironomidae (Diptera) tend to be tolerant to stressful conditions, either due to adaptations or quick reproduction cycles (Serra et al. 2017).

Pesticides, particularly insecticides, can be highly toxic to aquatic invertebrates. They can reduce the flux of insect emergence by direct mortality of emergent insect larvae (Miller et al. 2020; Roodt et al. 2023), or through sublethal effects causing temporal shifts in emergence (Cavallaro et al. 2018; Monteiro et al. 2019). In addition, communities present in polluted streams can shift to more tolerant groups (Cuffney et al. 1984; Pallottini et al. 2017). Pharmaceuticals and other contaminants found in wastewater effluent are also associated with changes in insect emergence, either through a reduction in emergence or a change in the emergent insect community (Let et al. 2022). In addition, both agricultural and wastewater pollutants have been found to accumulate in emergent insects (Kraus et al. 2021a; Previšić et al. 2021). Thus, agricultural pesticides and wastewater pollutants have the potential to result in several of the outcomes for riparian insectivores predicted by Kraus (2019).

Spiders

Spiders living in riparian areas are one of the main consumers of emergent insects. Most spider species are generalist predators and are able to switch their diet based on available prey, including emerging insects (Kato et al. 2004; Ishijima et al. 2006; Radermacher et al. 2020; Twining et al. 2021). Some spiders, such as web-building tetragnathids (*Tetragnatha* spp.; Figure 2), consume mainly aquatic prey (up to 100%; Krell et al. 2015). Wolf spiders (Lycosidae) living along the stream shore also prey on emergent insects, but have a more variable reliance on aquatic prey (Paetzold et al. 2005; Krell et al. 2015; Siebers et al. 2021), possibly due to their increased dietary flexibility as free-hunters. Nevertheless, riparian spiders are able to take advantage of the nutritional value of emergent insects to assimilate high levels of PUFAs rather than needing to synthesize them from terrestrial sources (Kowarik et al. 2021; Twining et al. 2021). The strength of the link between riparian spiders and emergent insects is demonstrated by changes in spider density and community in the absence of insect emergence from streams (Kato et al. 2003). Not only do spiders act as consumers in the riparian food web, but they are also important prey for other terrestrial predators such as birds and bats (Recalde et al. 2020). As such, spiders play a role as emergent insect consumers and in passing on the nutrients obtained from emergent insects into the terrestrial food web.

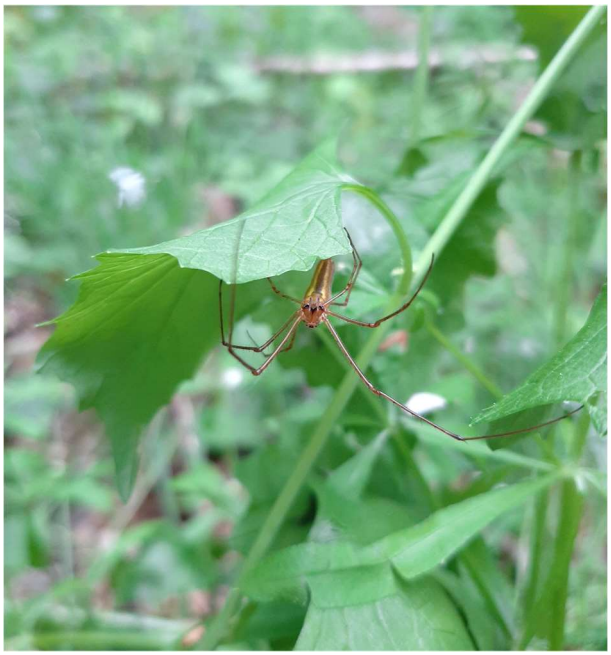

Figure 1: A female *Tetragnatha montana*. This species is commonly found in riparian areas and consume emergent aquatic insects. Photo by Maike Huszarik

Although riparian spiders are thought to consume mainly aquatic prey, specific diet information is not available for many species. Spider predation is difficult to document due to their liquid feeding mode, and studies evaluating spiders' dependence on aquatic prey have often been limited to visual observation of spiders feeding or stable isotope analysis (SIA). SIA is very effective at determining the source of prey consumed by a predator and quantifying the contributions of sources to the diet but it cannot give detailed taxonomic information of prey captured by the spiders (Nielsen et al. 2018). Recently, molecular gut content analysis with DNA metabarcoding has emerged as a highly sensitive and informative approach for diet analysis (Hambäck et al. 2016, Liu et al. 2020). It is especially useful for spiders and should provide a wealth of information to detangle spider food webs

Few studies have looked at the indirect effects of chemical stream pollution on riparian spiders. However, as consumers of emergent insects, it can be expected that riparian spiders are affected by a change in emergence. As riparian spiders are not obligate consumers of aquatic insects, they may switch their diets to consume more terrestrial prey if aquatic insects become less available (Briers et al. 2005; Graf et al. 2017). Alternatively, they may leave or suffer population declines in these areas, resulting in a density decrease (Paetzold et al. 2011). Graf et al. (2019) found that increased pesticide toxicity in streams resulted in a change in spider community composition and a lower species richness. In addition, the contribution of aquatic prey in the diet of wolf spiders and tetragnathids increased along a gradient of agricultural stream pollution, although the dietary changes were specific to the spider groups and shifts in consumed prey responsible for this change remain unclear (Graf et al. 2020). It appears that little, if any, research has evaluated indirect trophic effects of wastewater effluent on riparian spider diet. However, there is evidence that spiders bioaccumulate pharmaceuticals (Richmond et al. 2018) and other chemicals from streams and can act as "sentinels" of stream pollution (Chumchal et al. 2022). Most studies evaluating changes in spider diets due to stressors have used SIA to examine the difference in aquatic contribution to the spider diet. Including DNA metabarcoding as a tool for investigating changes in spider diet would be advantageous.

Bats

Streams are important habitats for many bat species as a water source, travelling route, and foraging area. Insectivorous bats foraging in riparian areas are also one of the main consumers of emergent insects (Figure 3). Bats are highly vulnerable organisms, with all European species strictly protected following past population declines (Barova and Streit 2018). They continue to be threatened by habitat loss, chemical pollutants, and insect decline, among other threats

(Voigt and Kingston 2016; Frick et al. 2020; Browning et al. 2021). Bats have long lifespans, low reproduction rates, and energetically demanding lifestyles. They must consume large quantities of high-quality prey to support their daily energy requirements, as well as accumulating energy reserves for reproduction, lactation, and migration or hibernation. This life history makes bats highly vulnerable to stressors such as prey loss or pollutants, but also means that they are good bioindicators for the health of their habitats (Jones et al. 2009). Emergent insects, particularly Diptera, are an important food source for riparian bats due to their high nutritious value as well as their temporal pulses (Vesterinen et al. 2016, 2018; Andriollo et al. 2021). Indeed, riparian bats are known to track areas of high emergence along streams, demonstrating the importance of this resource (Fukui et al. 2006; Akasaka et al. 2009; Hagen and Sabo 2011). Furthermore, emergent insect peaks may be an important food source in the spring after hibernation, when fewer terrestrial insects are present, and during reproduction periods (Encarnação and Dietz 2006).

Figure 3: Three European bat species associated with streams and forested riparian areas in Germany. **Top:** Daubenton's bat, *Myotis daubentonii*, is a specialist adapted to hunting directly above water surfaces, using their tail membranes and feet to trawl prey from the water. **Bottom left:** Brandt's bat, *Myotis brandtii*, is not a stream specialist but is associated with riparian forest habitats as foraging areas. **Bottom right:** The common pipistrelle, *Pipistrellus pipistrellus*, is a generalist species which is also found foraging in riparian forests. Photos made and provided by Christian Giese.

Chemical pollution in freshwaters has been identified as one of the major, yet understudied, threats to bats (Frick et al. 2020; Browning et al. 2021). Nevertheless, effects of pesticides, especially current-use pesticides, are not well-studied in bats and are an area of European (EFSA PPR et al. 2019) and global concern (Mineau and Callaghan 2018; Torquetti et al.

2020). Bats may be affected by either direct exposure to contaminants, or through a change in aquatic prey availability, though none of these risks have been thoroughly investigated. Riparian specialists such as Daubenton's bat (*Myotis daubentonii*, Kuhl 1817) are likely to be especially vulnerable due to their dependence on aquatic prey. A handful of studies have evaluated indirect effects of wastewater effluent on bats by comparing bat foraging activity up- and downstream of wastewater treatment plants. Bat activity was associated with changes in insect emergence in affected streams, though both decreases and increases in emergence and bat foraging have been reported (Vaughan et al. 1996; Kalcounis-Rueppell et al. 2007; Abbott et al. 2009). As far as I am aware, no study has evaluated the indirect or direct effects of pesticides in streams on bats. Dietary uptake of chemical pollutants may be a risk to bats if they consume contaminated emergent insects, although this has also rarely been addressed. Quantifying the effects of chemical stream pollution and risks for riparian bats remains an important goal to aid in their conservation.

Research Questions

Although there are clear effects of pesticide and wastewater pollution on insect emergence, little is known about how these effects propagate further into the riparian food web. In particular, the mechanisms driving effects for riparian consumers remain unclear. To address this, I focused on riparian spiders and bats as important insectivores in riparian forests (Figure 4). I evaluated how pesticide and wastewater pollution in streams affected them through changes in prey availability, and aimed to address the following research question within my dissertation:

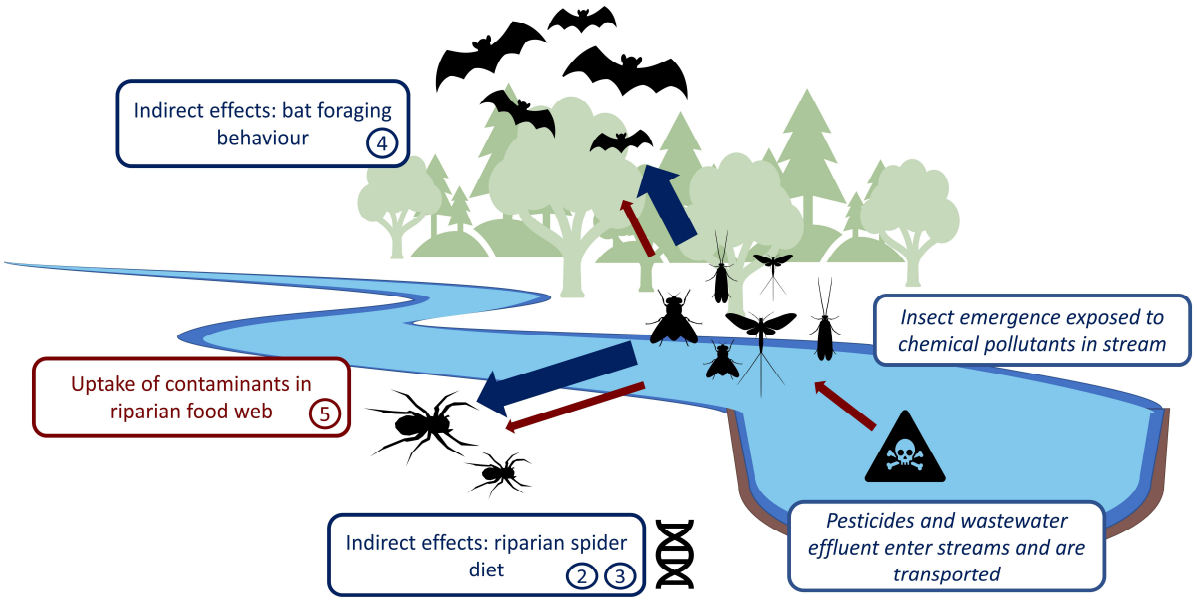

Figure 4: Graphical summary of topics covered in the dissertation, represented by the studied elements of the riparian forest food web. Emergent insects leave the stream as flying adults and are consumed by riparian spiders and bats (dark blue arrows). Chemical contaminants in the form of pesticides and pharmaceuticals from agricultural and wastewater inputs are present as stressors in streams and can affect the riparian food web by changing the insect emergence flux and by being transported by emergent insects (red arrows). Numbers in circles refer to the chapter corresponding to each topic. The strand of DNA indicates where genetic methods were used.

Are riparian insectivores foraging at polluted streams limited by reduced prey availability or at a higher risk of dietary exposure to contaminants?

Based on the possible combinations of the "exposure driving subsidy" and "subsidy driving exposure" hypotheses put forward by Kraus (2019), riparian consumers may be affected by stream pollution in several ways, depending on the properties of the contaminants. There is an overall lack of data describing how chemical pollution may affect riparian bats (Browning et al. 2021), especially in regards to pesticides. The connections between chemical contaminants and effects on riparian spiders also require in-depth investigation (Graf et al. 2020).

Together with my coauthors, I conducted field studies at forested stream sites using bats and spiders both as representatives and individual cases of riparian insectivores. With these studies we mainly aimed to identify food web effects of chemical contaminants in the streams by comparing sites along a gradient of chemical pollution. We also quantified the bioaccumulation of pesticides in emergent insects and riparian spiders in a second set of field studies to evaluate the transfer of contaminants across the aquatic-terrestrial boundary. Thus, the overall research question is divided into three subsections, which are illustrated above in Figure 4.

1. *Does chemical stream pollution indirectly affect riparian spiders through a dietary shift in consumed taxa?* (Chapters 2 and 3)

Spiders are important and widespread predators of emergent insects, and may shift their diet to other prey when aquatic prey are less available. To evaluate how riparian spiders responded to chemical stream pollution, we used DNA metabarcoding as a tool for the analysis of prey DNA in the spider gut contents, i.e., the prey taxa which had been consumed by spiders. In addition, due to the sensitivity of this method, we addressed the issue of sample contamination to aid in the use of DNA metabarcoding with a wider variety of sampling methods. Prior to the field study, we conducted a method development study (Chapter 2) to test the risk of contamination when collecting wolf spiders using common sampling methods (pitfall trap and hand capture), and whether bleaching effectively decontaminated the spiders prior to DNA metabarcoding. With the results of this study, we found that wolf spiders collected from riparian forests had consumed only low amounts of aquatic prey. This has previously been observed in riparian forests within the study area (Krell et al. 2015). Thus, we decided to focus on the web-building species *Tetragnatha montana,* which are highly dependent on aquatic prey, in the field study (Chapter 3). For this, we collected *T. montana* individuals at ten forested stream sites across a pollution gradient, and used DNA metabarcoding to determine whether their diet differed at sites across a gradient of chemical pollution, which we quantified in Chapter 4, and which specific prey taxa were responsible for changes. We also compared the community of flying insects across different sites to see if the changes in spider diet could be linked to changes in the availability of flying aquatic and terrestrial insects.

2. *Does chemical stream pollution indirectly affect bat foraging behaviour and hunting success through a change in prey availability?* (Chapter 4)

Given the ecological importance and vulnerability of bats, as well as the lack of available information on effects of chemical pollution, we assessed the relationship between chemical stream pollution and the foraging behaviour of riparian bats at 14 forested streams. We evaluated changes in bat activity and bat hunting rates (success rates) across a pollution gradient using bioacoustic methods (Chapter 4). We included three European species (*Myotis daubentonii*, *M.* cf. *brandtii*, and *Pipistrellus pipistrellus*) with different degrees of specialization to riparian areas to see how the effect differed based on their dependence on aquatic prey. We also measured the availability of aquatic and terrestrial flying insects, as well as the emergence at each stream and the in-stream chemical pollution.

3. *Are riparian consumers exposed to chemical pollutants through their diet of emergent insects?* (Chapter 5)

Several studies have found evidence for the accumulation and transport of contaminants from the stream by emergent insects, which may cause them to enter the terrestrial food web in otherwise unexposed terrestrial areas. However, whether emergent insects accumulate and retain chemicals past metamorphosis depends on the properties of individual compounds and has not been well-studied for current-use pesticides. The results of the previous chapters also indicated the potential significance of dietary exposure for bats and spiders. To evaluate this risk, we conducted a field study in the same region as Chapters 3 and 4 to quantify the bioaccumulation of pesticides from emergent insects to riparian spiders (Chapter 5). We measured 82 pesticides in stream water, sediment, leaf litter, emergent insects, and spiders from ten stream sites in the same study region.

General Approach

To answer the questions above, we conducted field studies at forested streams in southern Rhineland-Palatinate, Germany (Figure 5). The sites were located in riparian forests at structurally natural streams (Figure 6) to reduce and homogenize the influence of anthropogenic alterations to the stream and riparian structure, which can affect emergence (Raitif et al. 2018), spider diet (Ramberg et al. 2020), and bat activity (Scott et al. 2010). The field study ran from April 21st to July 1st, 2020, which is during the main pesticide application period of the region (Vormeier et al. 2023). These sites were used in the field studies of Chapters 3 and 4 and were located along a pollution gradient present in the study region. In addition, Chapters 2 and 5 included select sites within this area.

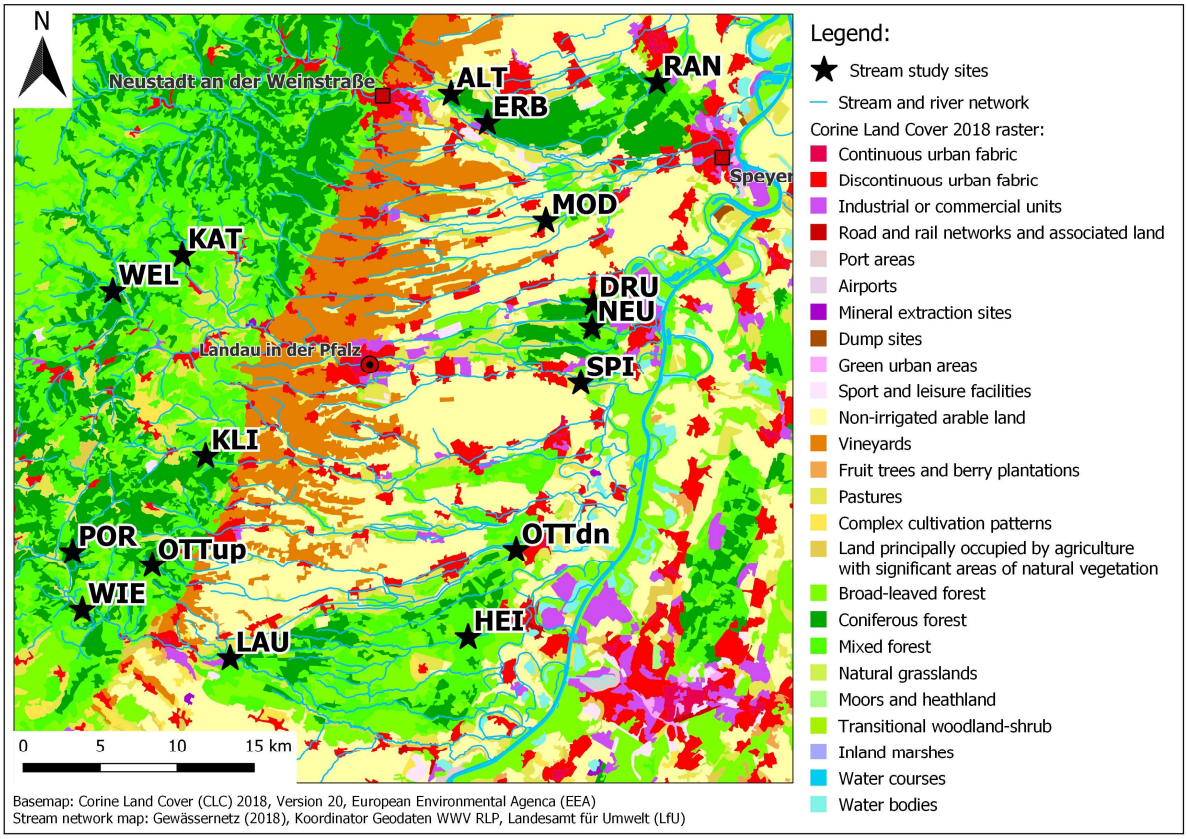

Figure 5: Land use cover and location of streams and field sites in the study region (used in Chapters 3 and 4), situated near Landau in der Pfalz, in southwestern Germany. Each site is labelled by its three-letter ID and is located at a forested section of stream. Land use cover classes are denoted by colour categories obtained from the CORINE Land Cover 2018 raster dataset. Cities (> 25,000 inhabitants) in Rhineland-Palatinate are denoted by red icons. The Rhine river flows from south to north in the east. Note, the two sites OTTup and HEI were excluded from the field study analyses due to early drying up of the stream (HEI) and different physical site conditions compared to other streams (OTTup).

Streams in the study region typically flow from the Palatinate Forest, a UNESCO Biosphere Reserve, in the west to the Rhine River in the east. Before reaching the Rhine, streams flow through the Upper Rhine Plain, which is characterized by agricultural, forested, and urban areas within the study region (Figure 5). The agricultural areas are dominated by viticulture, but also include orchards, vegetable crops and other agricultural land closer to the Rhine. The urban settlements in the study area are generally small (< 25,000 inhabitants), and water is treated by municipal wastewater treatment plants (Ministerium für Klimaschutz 2023).

Figure 6: Examples of forested stream sites included in the field study. Both bottom pictures include white emergence traps on the water surface. Photos by Maike Huszarik

A general pollution gradient exists across the landscape, with areas closer to the stream headwaters surrounded by forest and few pesticide or wastewater sources. As streams flow through the Rhine Plain, they are exposed to agricultural, urban, and industrial land. Many streams pass through riparian forests again before mouthing into the Rhine river. Thus, many of the downstream forest sites are subject to more point- and diffuse sources of agricultural and urban pollution. With this in mind, we chose the field study sites for Chapters 3 and 4 in riparian forests at various areas across this landscape to cover a gradient of chemical pollution. We then characterized the pesticide and wastewater pollution profiles of each stream with weekly measurements throughout the field study, as well as measuring the physicochemical and riparian habitat characteristics (Figure 7).

Figure 7: Fieldwork at the forested streams used in the field studies of chapters 3 and 4. This included measuring dissolved nutrients (**top left**), temperature, pH and conductivity (**top right** and **bottom left**), and the width and depth of the streams (**bottom right**). Photos by Maike Huszarik and Teagan Wernicke (bottom left).

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Chapter 2: External DNA contamination and efficiency of bleach decontamination for arthropod diet analysis

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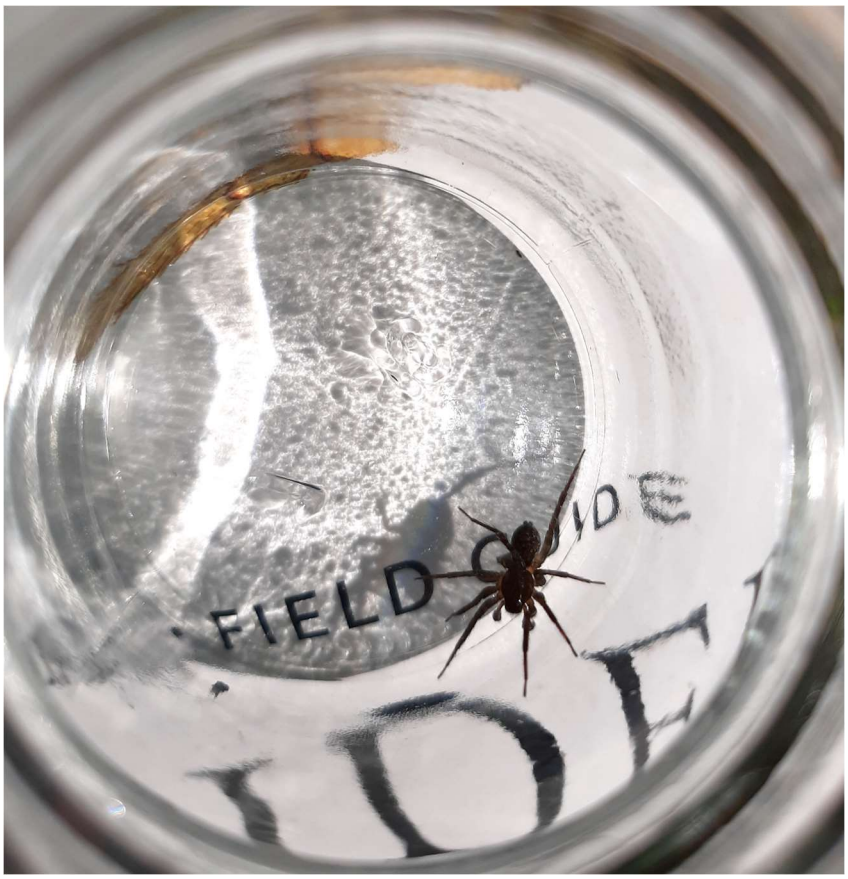

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ORIGINAL ARTICLE

External DNA contamination and efficiency of bleach decontamination for arthropod diet analysis

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Abstract

DNA metabarcoding is increasingly used to analyze the diet of arthropods, including spiders. However, high sensitivity to DNA contamination makes it difficult to apply to organisms obtained from mass-sampling methods such as pitfall traps. An alternative is to hand-sample spiders, but it is unclear how effectively this prevents external contamination, especially with new knowledge showing the wide spread of eDNA in the environment. Protocols using bleach to remove external DNA have been tested on several invertebrates, though testing with both mass-sampling methods and spiders is lacking. Here, we used wolf spiders (Lycosidae) to assess the risk of external DNA contamination from pitfall trapping and hand sampling, and the efficacy of bleach decontamination. We first conducted a contamination experiment where we placed spiders in pitfall traps containing trapping medium and a nonprey insect species to simulate external DNA contamination. We also compared sampling methods by collecting spiders using pitfall traps and hand sampling. Spiders from the contamination experiment and sampling method comparison were either bleached or untreated, then metabarcoded using multiple primer pairs. The contamination experiment resulted in the contamination of almost all spiders from pitfall traps, which was successfully eliminated with bleaching. Interestingly, there was no difference in the number of amplicon sequence variants (ASVs) detected per spider between pitfall trapping and hand sampling but bleaching resulted in significantly fewer ASV detections for both methods. Additionally, bleaching, but not sampling method, affected the taxonomic diet composition for both hand-sampled and pitfall-trapped spiders, indicating similar levels of external contamination. Our results are the first to confirm that DNA metabarcoding can be used together with bleaching for spiders sampled from pitfall traps, and that hand sampling does not necessarily exclude external DNA contamination. Thus, diet studies using metabarcoding should address the risk of external contamination with field-sampled arthropods, regardless of sampling method.

KEYWORDS
Araneae, DNA metabarcoding, gut content, hand sampling, Lycosidae, pitfall trap

1 | INTRODUCTION

DNA metabarcoding of gut content has become an important tool for studying the diet of arthropod predators (Birkhofer et al., 2017; Valentini et al., 2009), and to describe trophic links within food webs (Nielsen et al., 2018; Pringle & Hutchinson, 2020; Roslin & Majaneva, 2016). It is especially useful for cryptic feeders such as spiders (Greenstone & Shufran, 2003), which externally digest their prey and leave no morphologically identifiable remains in their gut or excrement. Furthermore, spiders are good candidates for food web studies using DNA metabarcoding, as they play important predatory roles in a wide range of ecosystems (Nyffeler & Birkhofer, 2017) and retain a relatively long snapshot of their diet, with prey DNA remaining detectable in their guts for days to weeks (Harwood, 2008; Uiterwaal & DeLong, 2020).

DNA metabarcoding is a highly sensitive method that can detect small amounts of DNA, ideal for analyzing degraded prey DNA in the gut. However, this makes DNA contamination from sample collection an important consideration for studies using DNA metabarcoding (Liu et al., 2020). To avoid contamination of arthropods with external DNA, careful hand sampling has been recommended (King et al., 2008) and is often used to individually collect spiders for metabarcoding (Hambäck et al., 2016; Macías-Hernández et al., 2018). Hand sampling also limits the possible sampling effort for a study, however, as it is time- and labor-intensive (Chapman et al., 2010). Furthermore, recent surveys of insect environmental DNA (eDNA) have shown that insect eDNA is detectable within the terrestrial environment on surfaces such as leaves (Valentin et al., 2020), flowers (Thomsen & Sigsgaard, 2019) and even in air (Roger et al., 2022), so it is possible for spiders and other arthropods to be covered with nonprey DNA just from moving through their environment. Thus, if spiders are already externally covered with eDNA, hand sampling would not exclude external DNA contamination (Greenstone et al., 2011). This new knowledge calls for the testing and updating of sampling recommendations to assess and account for this risk.

Ideally, diet analysis and food web studies should use effective sampling methods, as they benefit from large sample sizes (Pringle & Hutchinson, 2020). Pitfall trapping is one common approach to passively collect high numbers of arthropods, especially ground-dwelling spiders such as wolf spiders (Lycosidae) (McCravy, 2018). These traps may be filled with preservative trapping medium to prevent in-trap predation, allow traps to be emptied less frequently (wet pitfall trapping; Weeks Jr & McIntyre, 1997) and preserve DNA (Nakamura et al., 2020). However, pitfall traps are not a recommended sampling method for molecular analysis, as there may be external DNA contamination of sampled individuals with nonprey organisms in the traps if DNA is transferred via trapping medium (Shokralla et al., 2010). Nevertheless, the risk for external DNA contamination of samples from wet pitfall traps has not yet been directly evaluated, although the benefits obtained by the use of diet analysis for pitfall trap samples are apparent.

One possibility for dealing with possible external DNA contamination of samples for metabarcoding studies is through the removal of external DNA. Dissecting the gut from the exoskeleton (Athey et al., 2017) is one approach which only includes internal DNA. However, this method is time-consuming and difficult to apply to spiders, which have complex gut structures (Macías-Hernández et al., 2018). Another strategy is the decontamination of the spider cuticle by removing or destroying any external DNA. Greenstone et al. (2012) developed a protocol for bleaching the exterior of beetles prior to molecular gut content analysis, which successfully removed external DNA contamination. Bleach is well-known for decontaminating lab surfaces (Champlot et al., 2010), and has also successfully been used to decontaminate other organisms including lepidopteran larvae (Hausmann et al., 2021), ticks (Binetruy et al., 2019), and rotifers (Oh et al., 2020) used for molecular analyses. Bleaching has also been tested for spiders from a field experiment (Miller-ter Kuile et al., 2021), but without explicit contamination to directly assess the removal of external DNA. Experimentally testing bleach and directly comparing its effects on spiders sampled with different methods would help define the need for and effectiveness of decontamination prior to molecular diet analysis of whole spiders. Indeed, a decontamination step using bleach would offer a way to ensure that external DNA contamination does not affect diet results.

Thus, we aimed to explicitly test surface decontamination of spiders using bleach, and to compare the external DNA contamination of spiders sampled by wet pitfall trapping and hand sampling. We first conducted a contamination experiment by adding wolf spiders to wet pitfall traps containing an exotic nonprey insect to assess whether wet pitfall trapping results in external DNA contamination of the spiders with the exotic insect DNA, and the ability of bleaching to remove contamination. We also compared the detected amplicon sequence variants (ASVs) and diet composition obtained from wolf spiders sampled by hand and by pitfall trap, as well as the effect of bleaching with both sampling methods. We used a multiplex DNA metabarcoding approach, which included three primer pairs specifically designed for spider diet analysis (Krehenwinkel et al., 2019), rather than one pair, to cover a broad range of possible arthropod prey taxa. Together, our results can contribute to informing sample collection and handling to avoid external DNA contamination of arthropods, while allowing DNA metabarcoding to be used for samples with assumed contamination (i.e., due to mass-sampling methods) and increasing the types of samples suitable for molecular diet analysis.

2 | MATERIALS AND METHODS

2.1 | Contamination experiment

Adult female wolf spiders, *Pardosa amentata* (Clerk, 1757) and *Pardosa agrestris* (Westring, 1861), were individually hand-sampled in a vineyard in Rhineland-Palatinate, Germany (Figure 1, Table S1). They were kept at room temperature in individual containers for 3 days and fed *Sinella curviseta* (Brook, 1882; Collembola) to obtain a known gut content. Simultaneously, four pitfall traps were prepared

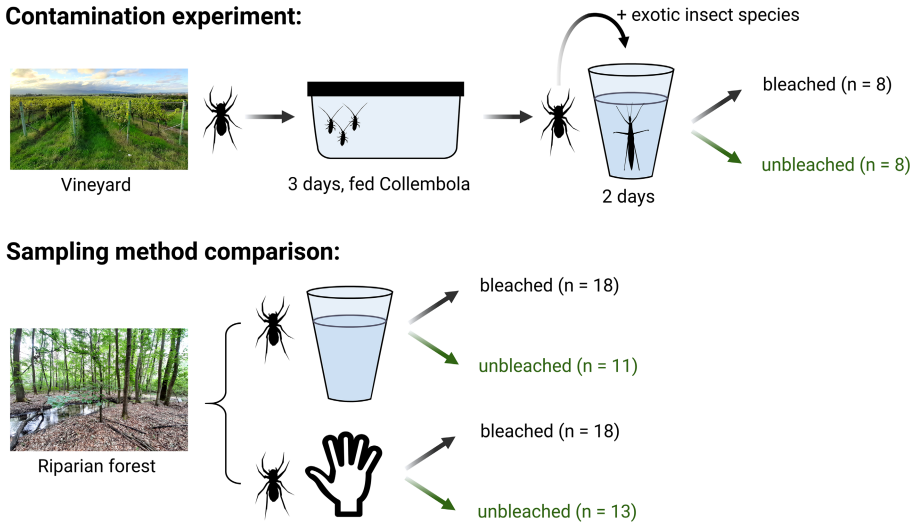

FIGURE 1 Overview of the collection and treatment of wolf spiders (Lycosidae) used for a contamination experiment and a comparison of two sampling methods for molecular diet analysis. Spiders for the contamination experiment (above) were collected by hand in a vineyard, then kept separated in the lab and fed *Sinella curviseta*, before being added to pitfall traps containing an exotic walking stick species, *Sungaya inexpectata*. For the sampling method comparison (below), spiders were either collected by hand or pitfall trap from riparian forests. Spiders from both parts of the study were then either bleached or left untreated, before being sequenced for DNA metabarcoding.

to simulate contamination occurring during trapping. We added multiple individuals of a non-native walking stick species, *Sungaya inexpectata* (Zompro 1996; Phasmatodea: Heteropterygidae), to unused plastic cups containing propylene glycol trapping medium (30% propylene glycol, 70% water, 1 mL/L dish soap and 10 mg/L denatonium benzoate – a deterrent for larger animals). As the spiders could not have previously fed on *S. inexpectata*, this species served as an indicator for DNA contamination caused by contact with nonprey organisms in the pitfall traps. After 3 days, the live spiders were added to the traps and immediately drowned, as would happen in the field. The traps were then set in forest ground (Table S1) to simulate field conditions, and covered to prevent additional material from entering. After 2 days, the spiders were removed, stored in 70% ethanol and frozen at −20°C. Finally, the ethanol was removed and the spiders were frozen individually in dry tubes at −20°C. Although the use of 70% ethanol and dry storage, common methods of storing arthropod samples to reduce brittleness, is not optimal for preservation of long-stranded DNA (Marquina et al., 2021), these methods are unlikely to have caused significant DNA degradation of our samples due to the short (<6 months) storage period (Stein et al., 2013), low temperatures (Vink et al., 2005), and short target fragment lengths.

2.2 | Sampling method comparison

We collected spiders by pitfall trap and hand sampling in the riparian zones of four forested streams in Rhineland-Palatinate, Germany (Figure 1, Table S1). Eight uncontaminated pitfall traps were set in the ground within 15 m of each stream bank. The pitfall traps were filled with 30% propylene glycol trapping liquid, as described for the contamination experiment, and emptied weekly over 5 weeks. Upon collection, the trap contents were immediately transferred to 70% ethanol and frozen at −20°C. The hand sampling of wolf spiders occurred on four occasions (Table S1), taking care to avoid external contamination. Each spider was frozen separately in a dry tube at −20°C. All female individuals of *Pardosa saltans* (Töpfer-Hofmann, 2000) and *Piratula hygrophila* (Thorell, 1872) from both hand and pitfall trap sampling were separated and individually stored in 96% ethanol at −20°C. Species identification of all spiders occurred on ice using Nentwig et al. (2019) and Roberts (1995).

2.3 | Bleaching

Approximately half of the spiders in each species and capture method group (pitfall trap, hand-sampled, experiment; Table 1, Figure 1) were bleached following Greenstone et al. (2012), to remove external DNA contamination. Bleaching was performed in the laboratory prior to DNA metabarcoding. Slightly more bleached spiders were included to ensure large enough sample sizes in case some individuals were damaged by the bleach treatment. Briefly, each spider was removed from its storage tube and added to a sterile tube containing 500 µL of 2.8% (w/w) commercial NaClO bleach (DANKlorix; Colgate-Palmolive GABA GmbH) for 40 min at 7.5°C, and gently shaken every 5 min. The bleach was then removed and the spiders were washed three times with distilled water and stored in 70% ethanol at −20°C.

2.4 | DNA extraction and PCR amplification

Ethanol was removed and spiders were dried in their tubes at 60°C for 1 h, then ground to a fine powder on a TissueLyser II (QIAGEN)

TABLE 1 The number of wolf spiders (Lycosidae) obtained with different sampling methods and treated with bleach for diet analysis with DNA metabarcoding.

	Pitfall trap		Hand-sampled		Experiment	
Spider species	Bleached	Unbleached	Bleached	Unbleached	Bleached	Unbleached
Pardosa saltans	8	5	8	5	–	–
Piratula hygrophila	10	6	10	8	–	–
Pardosa agrestris	–	–	–	–	2	2
Pardosa amentata	–	–	–	–	6	6
Total spiders (*n*)	18	11	18	13	8	8

using sterilized steel beads. DNA was extracted using a "high-salt" extraction method (Table S2; Aljanabi & Martinez, 1997), alongside seven negative control extractions. Next, prey DNA was selectively amplified in a multiplex PCR step using three primer pairs targeting variable regions of 18S (18S short and 18S long) and 28S rDNA, which are specifically designed to amplify a broad range of arthropods while suppressing spider amplification and providing taxonomic resolution comparable to COI at the order level (Krehenwinkel et al., 2019: 18SS, 18SL, and 28S; Table S3). For the multiplex PCR, 1.5 μL of the isolated DNA of each sample was mixed with 13.5 μL of the multiplex master mix (Table S4), then amplified (Table S5). Ten negative controls for DNA contamination containing only master mix were included. The success of the multiplex PCR was verified using gel electrophoresis. Then, all samples and negative controls were indexed and sequenced (Table 1).

The samples were indexed in a second PCR step using 96 unique combinations of 8 forward and 12 reverse indexing primers (Table S3). 0.5 μL of the multiplex PCR product was added to 8.5 μL master mix and 1 μL of the indexing primer pair (Table S6). This was then run with a shortened PCR program (Table S5), along with three negative controls containing only master mix. The success of the indexing PCR was verified with gel electrophoresis. The band strength on the gel was used to determine the amount of each sample to add to the final library for sequencing. The band strength was categorized as "weak," "middle," and "strong," and 4 μL, 2 μL, or 1 μL of sample was added, respectively, to include an approximately similar amount of DNA per sample in the library. The completed library was then shipped for the clean-up and sequencing steps. The library clean-up was performed at Trier University using 1X AMPure beads XP (Beckman Coulter). Finally, the library was sequenced with an Illumina MiSeq high-throughput sequencer using the MiSeq Reagent Nano Kit v2 (500-cycles; MS-103-1003; Illumina Inc.). Sequencing was performed at the Max Planck Institute for Evolutionary Biology in Plön, Germany.

2.5 | Sequence data processing and taxonomic assignment

The sequences were automatically demultiplexed by index barcode combination (bcl2fastq Conversion Software v1.8.4; Illumina Inc.). The adapter and primer sequences were removed using Cutadapt (Martin, 2011) at usegalaxy.eu (Afgan et al., 2018). The workflow was run once for each primer pair, and set to discard untrimmed reads (i.e. those belonging to the other primer pairs), with pair filtering set to both directions. The trimmed sequences were then processed with the DADA2 pipeline (v1.16; Callahan et al., 2016) in RStudio (R version 4.0.3; R Core Team, 2020). They were filtered (maxN = 0, maxEE = (2,2), truncQ = 2), and trimmed, with values for the "truncLen" variable based on the expected target sequence lengths of each primer pair and the quality plots produced with "plotQualityProfile" (18SL = 200, 160; 18SS = 150, 100; 28S = 200, 200). The sequences were then dereplicated, denoised, had chimeras removed and were merged to build the final amplicon sequence variant (ASV) table. In total, we identified 346,967 sequence reads and approximately 4565 reads per sample. The total number of reads and ASVs for each primer pair are presented in Table S7.

Next, taxonomic identities were assigned by matching the ASV sequences to entries in the GenBank nucleotide database (download date: 17 January 2022; Clark et al., 2016) using blastn MegaBLAST (BLAST+ version 2.12.0; Camacho et al., 2009), to obtain the first 100 matches, sorted by *e*-value. If the first 100 matches had the same *e*-value (i.e., no clear best sequences), more matches were obtained. Only matches with more than 85% identity, which has been tested as a threshold for assigning taxonomy at the order level for these primer pairs (Krehenwinkel et al., 2019), and at least 100 bp match length were included. However, the average match had over 97% identity. The NCBI BLAST name of the first 100 matches was used to assign each ASV to order level or higher, as this was sufficient to differentiate arthropods from nonprey groups. If the first 100 matches had the same BLAST name, then this taxonomic group was assigned to the ASV. If there were multiple matches, the name of the match with the best *e*-value and percent identity, query coverage and alignment length was selected. Specific ASVs were assessed and reblasted if conflicting orders were matched, and orders were assigned based on plausibility (i.e., considering unassigned uncultured environmental DNA matches, GenBank entries identified as contamination, mislabeled entries, and likely occurrence in study area). A second NCBI BLAST search (Johnson et al., 2008) was run only for spiders used in the contamination experiment, to identify Collembola ASV sequences with 100% identity matches to *S. curviseta*.

Amplicon sequence variants were then filtered to select only nonspider, noncrustacean arthropod orders, to avoid predator ASVs

from the spiders while keeping potential prey. In addition, ASVs only found in the negative DNA extraction and PCR controls were removed. After filtering and removing nontarget taxa, the diet analysis resulted in 77,117 target reads across all primer pairs (Table S7). To create an ASV presence-absence table, an ASV presence was defined as a read number greater than the minimum sequence copy of 0.001% of the maximum read number for that ASV (Drake et al., 2022). Singletons were excluded. As negative controls for DNA extraction and PCR did not show contamination of nonspider, noncrustacean arthropods, no adjustments for contamination were required.

2.6 | Statistical analysis

Statistical analyses were performed using R (R version 4.0.3; R Core Team, 2020). Data from each primer pair (18SS, 18SL, 28S) were analyzed separately, as it could not be confirmed that identical ASVs with identical taxonomic assignment from different genetic markers (i.e. different primer pairs) originated from the same organisms. First, to evaluate the effect of bleaching on the spiders from the contamination experiment, ASV counts for each spider were combined to form the three taxon groups "walking sticks" (the number of *S. inexpectata* ASVs), "known prey" (the number of ASVs with a 100% match to *S. curviseta*), and "other" (all other ASVs). The effect of bleaching on the average detections of each ASV group was evaluated using generalized linear models (GLMs) with a Poisson distribution, using the glm function in R.

Next, automated model selection was used to find the best model containing the variables affecting the number of ASVs detected per spider. First, a global GLM with a Poisson distribution was created, including all relevant predictor variables (spider species, sampling method and bleaching treatment) and their interactions, with the number of ASV detections as the response variable. All possible model subsets were computed and ranked by AICc (Burnham & Anderson, 2002; Hurvich & Tsai, 1989) using dredge (MuMIn; Barton, 2020). The best model (i.e. with lowest AICc) was chosen, except for 28S, where the second-best model was chosen as it included all three main predictor variables and had an AICc which was indistinguishable from the best model (delta AICc = 0.08; Burnham & Anderson, 2002). In addition, an initial GLM model had been conducted which included spider damage from bleach as a variable, to ensure that the exterior damage from bleaching observed in some spiders did not influence the diet analysis results. Damage was not significant for any primer pair 18SL: $Z(56,59) = 0.395$, $p = 0.495$; 18SS: $Z(55,59) = 1.135$, $p = 0.480$; 28S: $Z(57,59) = -2.265$, $p = 0.376$, so it was excluded in the final model selection. Finally, the effect of bleaching and sampling method on the spiders' recovered taxonomic diet composition was evaluated using a permuted MANOVA (adonis, vegan; Oksanen et al., 2019). The total number of ASV detections of each taxonomic group was calculated for each spider as the response variable. The predictor variables were spider species, bleaching treatment and capture method, as well as their interactions.

Generalized linear model assumptions and fit were verified for all models with check_model (performance, Lüdecke, Ben-Shachar, et al., 2021; see, Lüdecke, Patil, et al., 2021). To avoid problems with quasi-separation and over- and underdispersion, we derived the significance values for all GLMs from permutation tests (PermTest, pgirmess; Giraudoux, 2018). A significant result was considered $p < 0.05$. Figures were created using ggplot2 (Wickham, 2016) and ggpubr (Kassambara, 2020).

3 | RESULTS

3.1 | Contamination experiment

We detected walking stick ASVs in 7 of 8 unbleached spiders and none of the bleached spiders that had been placed in pitfall traps with walking sticks (*S. inexpectata*; Figure 2). Bleaching eliminated walking stick ASVs for all three primer pairs (18SL: $Z_{(14,15)} = 0.002$, $p < 0.001$; 18SS: $Z_{(14,15)} = 0.002$, $p < 0.001$; 28S: $Z_{(14,15)} = 0.002$, $p = 0.001$), but had no significant effect on the detections of the supplied prey *S. curviseta* or other taxa in any primer pair (all others $p > 0.41$; Figure 2).

3.2 | Sampling method comparison

The spider sampling method had no significant effect on the number of ASVs detected per spider in any primer pair (18SL: $Z_{(55,59)} = 2.450$, $p = 0.531$; 18SS: $Z_{(56,59)} = 0.000$, $p = 1.000$; 28S: $Z_{(56,59)} = 1.481$, $p = 0.417$; Figure 3). Furthermore, an interaction between the sampling method and bleaching was not included in the final models of 18SS nor 28S following model selection, and only showed a slight trend for 18SL ($Z_{(55,59)} = -2.390$, $p = 0.087$). Thus, sampling method also did not have an effect on the proportion of ASVs lost due to bleaching. Bleaching reduced ASV detections by 63.2% on average, namely 68.2% for hand-sampled and 58.3% for pitfall-trapped spiders. The negative effect of bleaching on insect DNA recovery was significant for all primer pairs (18SL: $Z_{(55,59)} = 4.221$, $p = 0.002$; 18SS: $Z_{(56,59)} = 2.027$, $p < 0.001$; 28S: $Z_{(57,59)} = 5.411$, $p = 0.002$; Figure 3). Finally, for both 18S primers, there was no effect of the spider species on the number of ASVs. However, 28S showed a significant difference in the number of ASVs found between *P. hygrophila* and *P. saltans* ($Z_{(57,59)} = 5.571$, $p < 0.001$), with *P. saltans* having approximately four times more ASVs detected per spider.

3.3 | Effect of bleaching and sampling method on detected diet composition of spiders

Springtails (Collembola), flies (Diptera), crickets (Orthoptera), and beetles (Coleoptera) composed most of the spider diet, with only minor differences in detected taxonomic diet composition between the three primer pairs (Figure 4). 18SS yielded the most

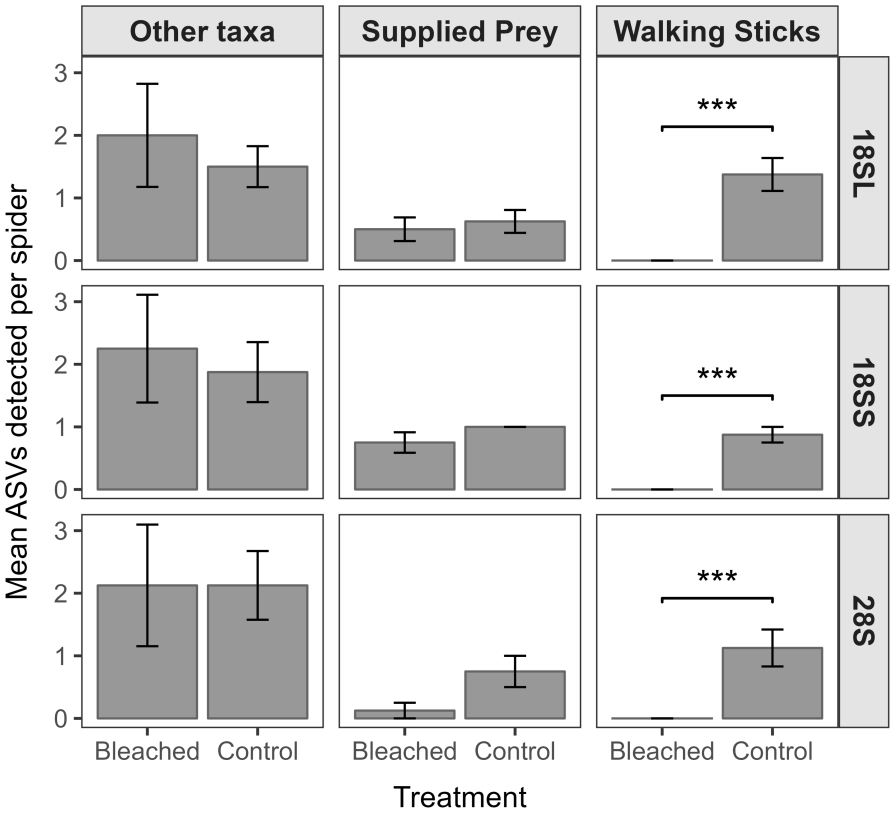

FIGURE 2 Effect of treatment with bleach on the average count of amplicon sequence variants (ASVs) obtained from wolf spiders (Lycosidae; $n = 8$ bleached, 8 control). "Walking sticks", *Sungaya inexpectata*, had been used to simulate external DNA contamination on the spiders from pitfall trap medium. The spiders had been fed the springtail species *Sinella curviseta* ("Supplied Prey") before being added to traps containing *S. inexpectata*. Spiders had either been treated with bleach to remove external DNA contamination (bleached) or not treated (control). The three primer pairs used for DNA metabarcoding are in separate panels on the vertical axis, and the taxonomic groupings of the ASVs on the horizontal axis. Significant differences are labeled with "***", indicating *p*-values of 0.001 or less, and standard errors are represented by black bars.

taxonomic groups, and 28S the fewest. Bleaching resulted in the recovery of a significantly different taxonomic diet composition in all three primer pairs, namely in fewer detections, rather than different taxa detected in the diet (18SL: $F_{(1,56)} = 4.31$, $p = 0.009$; 18SS: $F_{(1,56)} = 4.95$, $p = 0.003$; 28S $F_{(1,55)} = 6.38$, $p = 0.003$). Additionally, moths and millipedes were only detected in unbleached spiders. 18SL and 28S revealed a significant difference in the diet composition of *P. hygrophila* and *P. saltans* (18SL: $F_{(1,56)} = 3.17$, $p = 0.024$; 28S: $F_{(1,55)} = 10.64$, $p = 0.001$; Figure 4). 28S also showed an interaction between spider species and the bleaching treatment on diet composition ($F_{(1,55)} = 4.33$, $p = 0.012$). Furthermore, the sampling method was not associated with an effect on the diet composition of the spiders (18SL: $F_{(1,56)} = 0.203$, $p = 0.947$; 18SS: $F_{(1,56)} = 0.010$, $p = 0.652$; 28S $F_{(1,55)} = 0.009$, $p = 0.477$).

4 | DISCUSSION

4.1 | Contamination experiment

The results from our contamination experiment clearly confirm that wet pitfall trapping results in external DNA contamination of organisms used for molecular diet analysis. All unbleached spiders but one were contaminated with walking stick (*S. inexpectata*) DNA (Figure 2). Moreover, the fact that no bleached spiders had traces of *S. inexpectata* DNA indicates that the contamination in the pitfall traps was only external. Although this is the first study explicitly testing external DNA contamination from wet pitfall traps, our result is not unexpected. Propylene glycol can be used to store samples for molecular analysis (Nakamura et al., 2020), and can preserve DNA in aqueous solutions with concentrations as low as 20% (Ferro & Park, 2013). Our trapping medium contained 30% propylene glycol, which likely preserved the DNA of *S. inexpectata*, allowing it to come into contact with the spiders. Thus, such DNA contamination is also likely to occur in any other mass-sampling trap containing a DNA-preserving liquid.

There are several additional factors of trapping contamination that we did not explicitly evaluate with the experiment. For example, we did not consider the contribution of regurgitation to contamination within wet traps (King et al., 2008), nor the effect of time spent in trapping medium. However, when considering that insect eDNA can be widespread in the terrestrial habitat (Roger et al., 2022; Thomsen & Sigsgaard, 2019; Valentin et al., 2020), it is not surprising that mass-sampling traps are a source of contamination and are likely not the only concern when collecting arthropods. Our result calls for careful consideration when extracting DNA from whole organisms caught by mass-sampling traps, as any DNA on the target organism's cuticle can also be amplified by the primers targeting prey DNA from the gut.

4.2 | Bleaching decontamination

As expected, bleaching successfully removed the nontarget walking stick DNA from treated spiders in our contamination experiment, while not reducing the detection frequency of potential prey

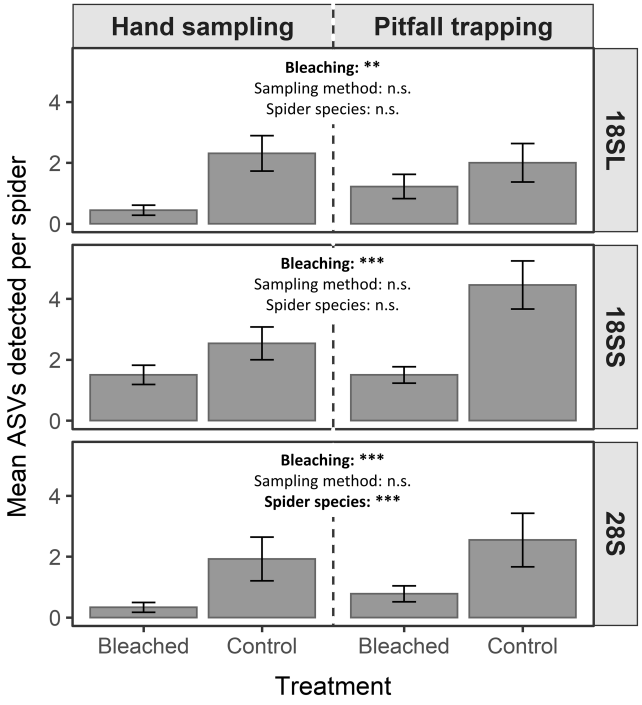

FIGURE 3 Effects of treatment with bleach on the average count of amplicon sequence variants (ASVs) found in wolf spiders (Lycosidae) sampled either by hand (n = 18 bleached, 13 control) or by pitfall trapping (n = 18 bleached, 11 control). The spider species included were *Pardosa saltans* and *Piratula hygrophila*. Spiders had either been treated with bleach to remove external DNA contamination (bleached) or not treated (control). The three primer pairs used for DNA metabarcoding are in separate panels on the vertical axis, and the spider sampling method on the horizontal axis. Significance is reported on the plots, with "n.s.", "**"and "***" representing p-values of >0.05, <0.01 and <0.001, respectively. Standard errors are represented by black bars.

(Figure 2). Bleached field-captured spiders also showed a strong reduction of arthropod ASV detections, with similar effects in both hand-sampled and pitfall-trapped spiders (Figure 3). However, a concern with bleaching is that it can destroy internal DNA if it enters the gut. The experiment did not show any effect of bleaching on the presence of ASVs matching with *S. curviseta*, which had been fed to the spiders to provide a known gut content. This indicates that the gut content was not significantly affected by the bleach. In fact, we detected other taxa which the spiders had probably consumed prior to being captured (Macías-Hernández et al., 2018), which were also not affected by bleaching. Additionally, a previous study testing the effectiveness of bleaching for surface decontamination of spiders also found that it did not significantly alter the measures of spider diet (Miller-ter Kuile et al., 2021). Thus, the results from our contamination experiment strongly suggest that bleach is a good treatment for removing external DNA, while keeping internal DNA intact.

Although there were no significant effects on gut content, our decontamination protocol could be further optimized. We exposed spiders to 2.8% NaClO for 40 min, based on Greenstone et al. (2012). However, Greenstone et al. (2012) used beetles in their study, which have a thicker cuticle than spiders and could likely withstand a stronger bleaching. While our results did not suggest that our protocol disrupted the DNA inside spiders, we could not directly confirm this. We did observe that the cuticle of a few spiders appeared slightly degraded following the bleaching treatment, but the initial statistical models confirmed that this visual damage did not significantly affect the number of ASVs detected. Other studies using bleach for surface decontamination have not found an effect on DNA in gut content (Binetruy et al., 2019; Hausmann et al., 2021; Miller-ter Kuile et al., 2021). However, their exposure times were shorter or at a lower concentration. Thus, we recommend exposing spiders and similar organisms with a thin cuticle to a shorter or less-concentrated bleach treatment to ensure external decontamination, while being certain to preserve internal DNA. Testing several concentrations and exposure times should be conducted to determine the ideal bleaching procedure for spiders and other arthropods.

In addition, bleaching may not be the only approach used for decontamination. Previous studies have already demonstrated that bleaching outperforms washing with ethanol for decontaminating invertebrate samples (Binetruy et al., 2019; Greenstone et al., 2012), as well as washing with distilled water and other decontaminants on surfaces (Champlot et al., 2010). However, bleaching may not be applicable to all samples, especially if they are particularly sensitive or if there are potential safety or environmental concerns. In this case UV exposure or washing with another nontoxic DNA-degrading liquid (as in Nilsson et al., 2022 or Champlot et al., 2010) could be tested. Nevertheless, the specific requirements for each study and organism should be considered and evaluated to decide on an appropriate protocol.

4.3 | Sampling method comparison

As carefully collecting spiders by hand has been suggested to avoid external contamination from mass-sampling techniques (King et al., 2008), we expected hand-sampled spiders to have fewer ASVs than those caught in pitfall traps in the sample method comparison. Surprisingly, our results did not reveal a difference in the number of ASVs detected between sampling methods (Figure 3). In fact, bleaching reduced the number of ASVs in both hand-sampled and pitfall-trapped spiders to a similar degree, which indicates that external DNA contamination was similar between hand-captured and pitfall-trapped spiders. The contamination of pitfall-trapped spiders can be explained by their contact with other arthropods in the traps via the trapping medium, as proven by the contamination experiment. However, the lack of difference between sampling methods indicates that most external DNA contamination is likely unrelated to pitfall traps, and cannot be avoided by hand sampling.

By combining the results of the method comparison and the contamination experiment, we can deduce the amount and source of external contamination of pitfall and hand-sampled spiders. Reductions through bleaching were similar across all arthropod orders in the sampling method comparison. Thus, there is no indication

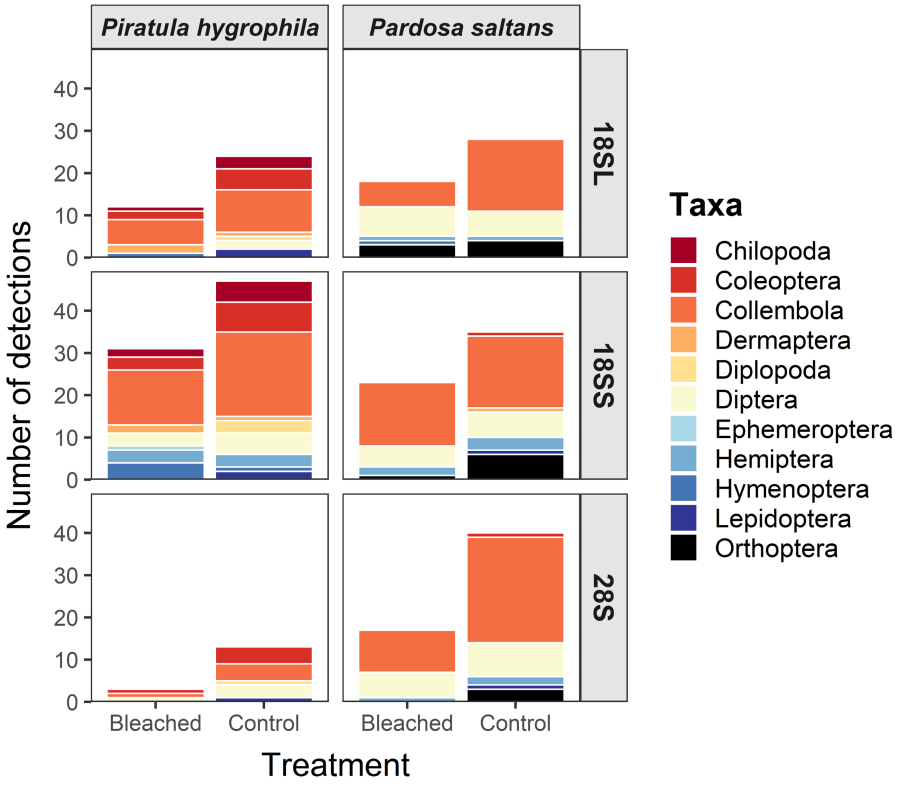

FIGURE 4 Taxonomic composition of gut content sequenced from *Piratula hygrophila* and *Pardosa saltans* using DNA metabarcoding. The spiders were collected by hand ($n = 18$ bleached, 13 control) and pitfall trap ($n = 18$ bleached, 11 control) from riparian forests in southwestern Germany. Spiders had either been treated with bleach to remove external DNA contamination (bleached) or not treated (control). The three primer pairs used for DNA metabarcoding are in separate panels on the vertical axis. Taxonomic composition was measured as the number of amplicon sequence variants (ASVs) detected per taxon per spider species. Note that there was no effect of sampling method on the diet composition and it is not shown in the figure.

that assumed prey, such as Diptera and Collembola, were less affected by bleaching than taxa that are less known to be consumed by wolf spiders, such as Chilopoda and Lepidoptera. Nonetheless, the contamination experiment suggests that bleach only acted externally, as bleaching only reduced *S. inexpectata* detections (Figure 2). Therefore, the strong, nonspecific effect of bleaching on DNA recovery from field-captured spiders combined with the poor effect on non *S. inexpectata* DNA in the contamination experiment suggest that both hand-captured and pitfall-trapped spiders had high levels of external contamination, albeit mainly with arthropods that are also part of their diet. This is not unexpected, because external contact of free-hunting spiders with arthropods will be most intense during prey attack. In addition, the generalist diet of the spiders in this study may mean that most of the organisms from the pitfall traps or local insect eDNA could also be potential prey. This contamination may not be considered severe if the DNA originates from prey or potential prey, but there still cannot be complete confidence in actual gut content versus external DNA if decontamination steps are not taken.

If wolf spiders are already externally contaminated in the field, we would expect that the bleached spiders from the contamination experiment would have shown a reduction of "other taxa" in addition to *S. inexpectata* detections. Although the spiders from our contamination experiment were hand sampled from the field prior to spending several days in the lab, they only showed *S. inexpectata* as external DNA contamination, and no difference in other taxa groups between bleached and control spiders. There is little information available about the fate of nonspider tissue and DNA on the spider cuticle. However, Valentin et al. (2021) found that insect eDNA rarely remained on surfaces for longer than a few days. Thus, we speculate that external DNA may have been lost from the spiders over time in the "clean" laboratory environment following hand capture. It could be interesting to investigate this further as another, specific, method of removing external DNA contamination in live samples.

Interestingly, our results indicate that hand sampling does not exclude external contamination of wolf spiders, and a decontamination step may be advisable regardless of sampling method. Greenstone et al. (2011) also tested whether hand sampling could eliminate contamination by releasing insects on plants where a different species had been incubated, and immediately recollecting them. They found that hand sampling did not necessarily eliminate contamination of their insect samples, indicating that there may still be a risk of external contamination due to the organisms coming into contact with eDNA in their environment, similar to our spiders. As discussed, much of the external DNA could originate from the handling of prey during attack and consumption. Furthermore, insect eDNA is found on plant surfaces and in air (Roger et al., 2022; Valentin et al., 2020), so spiders could have insect DNA on their cuticle simply from interacting with their environment. This is even more likely for free-hunting spiders which are much more mobile in their habitat than web-building species. If this is true, then spiders would also bring external DNA into pitfall traps, adding another source of contamination. Clearly, it is imperative to continue quantifying the risk of contamination of field-sampled organisms and whether it applies to different species and study designs.

4.4 | Effects of bleaching and sampling method on spider diet

In addition to the effect of bleaching and sampling method on the number of ASVs detected per spider, we also expected an effect of sampling method and bleaching on the recovered taxonomic diet composition of the spiders. However, we only observed that bleaching and spider species altered the taxonomic composition of recovered DNA, with no effect of sampling method (Figure 4). The difference in diet between the spider species is not surprising, given that *P. hygrophila* is more specialized on riparian areas whereas *P. saltans* is a forest specialist (Nentwig et al., 2019; Roberts, 1995). In addition, it makes sense that bleached spiders had a different DNA profile than control spiders if bleaching had removed external DNA contamination, which was likely slightly different than prey DNA in the gut. On the other hand, the fact that there was no difference between hand-sampled and pitfall-trapped spiders appears to indicate that both groups were exposed to similar taxa. This reinforces the idea that spiders from both sampling methods had external DNA contamination which was removed by bleaching, but also that hand sampling and pitfall trapping are equally suited to study the diet of wolf spiders.

4.5 | Future directions

Our results not only add to existing literature investigating bleaching as a decontamination method for invertebrates, but demonstrate that a decontamination step would enable food web studies to use mass-sampling techniques for collecting spiders, and other arthropods, for molecular diet analysis. This has the potential to greatly increase the sample size and reduce sampling effort required for such studies. For example, it would be easier to sample nocturnal ground-dwelling spiders or those in remote areas using pitfall traps, as the traps can be left unattended. In addition, different sampling methods are more efficient at collecting different species (McCravy, 2018), so increased sampling flexibility for molecular diet analysis would be a clear advantage. This means that DNA metabarcoding could more easily be used to fill knowledge gaps in the diets of a wider variety of species.

This study also highlights the need for updated sample-handling protocols which consider the risk of external contamination of field organisms with eDNA. We show that contaminating eDNA may originate from potential prey taxa of spiders, which could be mistaken for gut content. Thus, it is important for future studies to quantify the risk of external contamination of arthropod samples and to consider whether a decontamination step is necessary prior to performing molecular diet analysis. One strategy for this may be taking samples of storage ethanol (Shokralla et al., 2010) or the cuticle (Binetruy et al., 2019) to sequence as a control. However, this would not aid in distinguishing between gut and external DNA if both are potential prey taxa, as found in our study. In this case, external decontamination would ensure that sequenced DNA is only internal.

5 | CONCLUSION

In summary, our results confirm that there is a risk of external DNA contamination when using wet pitfall traps, but also that wolf spiders sampled by hand can be as contaminated as those from mass-sampling traps. Thus, external DNA contamination is a concern regardless of the sampling method. We also show that bleaching is effective as an additional decontamination step, enabling the use of DNA metabarcoding to analyze the diet of externally contaminated spiders. With additional testing and adjustment of the protocol, bleaching can also be applied to other mass-sampling techniques where external contamination is a concern, as well as for other arthropods. This would open the possibility of using metabarcoding for larger studies where samples cannot be individually collected, and help to provide important diet information to food web ecologists. Our results question the assumption that hand-sampled spiders are not contaminated with external DNA and call for a more careful approach to sample preparation in the context of DNA metabarcoding whole organisms.

AUTHOR CONTRIBUTIONS

ME, NR and LE conceptualized the study. ME, KS, SK, and HK supervised all study stages. NR and LE carried-out field sampling. MH, NR, SK and LE performed laboratory work. MH and NR conducted bioinformatics and MH performed data analysis. MH, ME, HK, SK, NR, and KS contributed to the interpretation of the data. MH wrote the manuscript with the assistance of all coauthors.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The raw data for this study are available from Figshare (doi: 10.6084/m9.figshare.21197788).

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SUPPORTING INFORMATION

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Chapter 3: Shift in diet composition of *Tetragnatha montana* along a chemical stream pollution gradient

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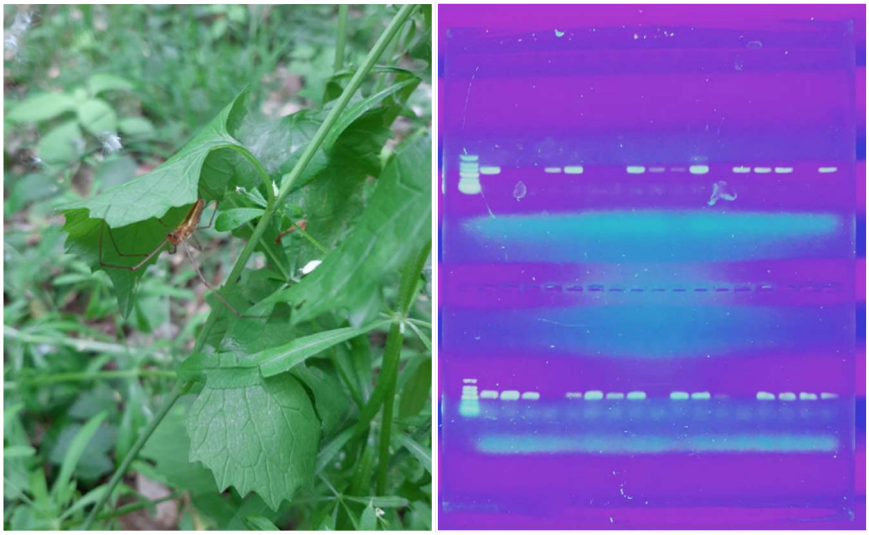

Shift in diet composition of *Tetragnata montana* along a chemical stream pollution gradient

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Abstract

Terrestrial insectivores in riparian areas, such as spiders, depend on emergent aquatic insects as high-quality prey. However, chemical pollution entering streams from agricultural and urban sources can alter the temporal dynamics and composition of the aquatic insect emergence, which may also affect the riparian food web. Few studies have examined the effects of stressor-induced alterations in aquatic insect emergence on spiders, especially in terms of chemical pollution and diet composition. We used DNA metabarcoding to describe the diet of *Tetragnatha montana* spiders collected from ten forested streams with differing levels of pesticide and wastewater pollution. We found that spiders consumed more Chironomidae and fewer other aquatic Diptera at more polluted streams. Pollution-related effects were only observed in the spider diet, and not in the number nor composition of flying insects trapped at each site. Our results indicate that riparian spider diets are highly sensitive to stream pollution, even without a change in the overall proportion of aquatic prey consumed. A high reliance on aquatic prey at polluted streams may lead to an increased risk for spiders of dietary exposure to chemical pollutants transferred by emergent insects.

Keywords: Molecular gut content analysis, food web interactions, pharmaceuticals, pesticide toxicity, riparian forest, Araneae

Introduction

Terrestrial and aquatic ecosystems are closely linked by a transfer of nutrients across the land-water interface. These nutrient transfers can act as subsidies to the recipient ecosystem (Polis et al. 1997) and have been well-documented, both from land to water and, more recently, from water to land (Muehlbauer et al. 2014; Schulz et al. 2015; Soininen et al. 2015). Aquatic subsidies, namely emergent insects with an aquatic larval phase, represent high-quality prey for terrestrial predators (e.g., spiders, Kowarik et al. 2021, birds, Schilke et al. 2020, and bats, Fukui et al. 2006). They are rich in types of essential long-chain polyunsaturated fatty acids which are not found in terrestrial insect prey (Hixson et al. 2015; Guo et al. 2017). In addition, the timing and mass of emergent aquatic insect fluxes can occur when less terrestrial prey is available (Nakano and Murakami 2001; Kato et al. 2003), and are important for supporting energy-intensive life stages of predators, such as during developmental (Marczak and Richardson 2008) or reproductive periods (Twining et al. 2018). Thus, a change in emergent aquatic insect prey abundance, timing, or quality could have negative consequences for riparian insectivores (Uno 2016; Kopp and Allen 2021).

Spiders living in riparian areas are major consumers of emergent aquatic insects (hereafter emergent insects; Paetzold and Tockner 2005). Riparian orb weavers such as *Tetragnatha* spp. are particularly reliant on emergent insect prey (Gergs et al. 2014; Krell et al. 2015; Wieczorek et al. 2015), and their distribution along streams is influenced by the presence of emergent insects (Kato et al. 2003; Tagwireyi and Sullivan 2015). Spiders link terrestrial and aquatic ecosystems, as they not only consume aquatic subsidies, but also serve as prey for other predators such as birds and bats (Vallejo et al. 2019). In addition, emergent insect subsidies can alter top-down effects in the terrestrial food web by causing a shift in spider diet (Graf et al. 2017).

Streams are among the ecosystems most strongly influenced by humans (Albert et al. 2021). Not only do stressors affect streams locally, but streams can also transport and transfer stressors downstream into otherwise unexposed areas, such as nature reserves (Wolfram et al. 2023). Chemical pollutants from agricultural and wastewater sources are one of the main stressors affecting the functionality of stream ecosystems (Brauns et al. 2022). In particular, pesticides and pharmaceuticals (Barber et al. 2013; Burdon et al. 2019), as well as excess nutrient concentrations can cause severe alterations in the stream, including effects on the emergence of aquatic insects (Kalcounis-Rueppell et al. 2007; Bunzel et al. 2013). For example, chemical

pollutants can cause direct mortality of aquatic insect larvae, resulting in a reduction of total emergence (Kraus et al. 2021a) or a shift to more tolerant taxa in the stream community (Cuffney et al. 1984). There may also be pollution-induced changes in emergence timing or quality (Ohler et al. 2023), leading to a de-coupling of the aquatic subsidy transfer. In addition, emergent insects may accumulate and transfer pollutants to terrestrial predators when consumed (Kraus et al. 2021b; Previšić et al. 2021; Roodt et al. 2023).

Several field studies have observed effects of stream pollution on riparian insectivores, although many aspects, such as the role of specific trophic links, remain unresolved. For example, stream pollution was associated with a reduction in the diversity of riparian spider assemblages (Graf et al. 2019), as well as poorer development and shifts in the sex ratio of bird nestlings fed with aquatic subsidies (Morrissey et al. 2014). Moreover, the contribution of aquatic prey to the diet of riparian spiders changed along a pollution gradient, though this change was specific to different spider species (Graf et al. 2020). Many of the previous studies evaluating effects of aquatic insect emergence on consumer diets are based on stable isotope analysis (SIA). SIA is effective at determining the main sources and types of prey consumed, but cannot confirm which taxa are responsible for changes, or lack thereof, in aquatic diet contribution (Birkhofer et al. 2017). Molecular gut content analysis using DNA metabarcoding of prey DNA is a relatively novel and highly sensitive method which is able to detect species-level changes in spider diets (Pompanon et al. 2012; Huszarik et al. 2023a). Using a high-resolution molecular approach would add to the knowledge obtained by SIA and help to better understand how stream pollution affects riparian food webs.

The aim of our study was to describe changes in the diet of riparian spiders along a stream pollution gradient using DNA metabarcoding. We quantified the chemical pollution from pesticides and wastewater in streams flowing through riparian forests. We chose to collect *Tetragnatha montana* as they are common along streams and are known to have a high proportion of aquatic prey in their diet (Graf et al. 2020). We also captured potential flying insect prey with malaise traps at each stream to characterise their relationship with spider diet. We predicted that the proportion of aquatic prey in the spider diet and the abundance of flying insects would behave similarly in response to stream pollution: both being either higher or lower at more polluted sites. We also predicted that Diptera tolerant to stream pollution, such as Chironomidae (Chang et al. 2014), would be more common in the diet of spiders collected from more polluted streams.

Methods

Field study sites

The field study was conducted at ten stream sites located in riparian forest areas in Rhineland-Palatinate, Germany (Table S1, Figure S1). Streams in this area flow from the Palatinate Forest, a UNESCO Biosphere Reserve, to the Rhine River, passing through vineyard, other agriculture, urban, and forest land uses. Each site consisted of a 40-m stretch of stream surrounded by predominantly deciduous forest, away from direct exposure to agricultural or urban areas. All stream sites had a relatively natural stream structure, as per the Rhineland-Palatinate structural quality classification (no more than "moderately altered"; Koordinator Geodaten WWV RLP 2018). The field study was conducted during the main pesticide application period of the region, when the highest load and effects of pesticide pollution are expected to occur (Vormeier et al. 2023), from April 21st to July 1st, 2020. The sites were also part of a larger field study; for more details see Huszarik et al. (2023b).

Sites were visited once per week to measure physicochemical stream characteristics and collect water samples for pollutant analysis. The stream width and depth were measured. The concentration of nitrite (NO_2^-), nitrate (NO_3^-), ammonium (NH_4^+), phosphate (PO_4^{3-}) and sulphate (SO_4^{2-}) were measured with a nutrient analysis kit (VISOCOLOR® ECO reagents with PF-12 Spectrophotometer; Macherey-Nagel GmbH, Germany), and the water temperature, pH, dissolved oxygen, and conductivity with a multi-parameter meter (Multi 3620 IDS or Multi 340i, WTW Xylem Analytics GmbH, Germany). Weekly measurements for each physicochemical variable were averaged per stream site over the study period. The vegetation of the stream sites was characterised on June 23rd, 2020 to assess tree canopy cover over the streams, vegetation surface clutter (i.e. any vegetation on the stream surface), and shrub separation (Coulloudon et al. 1999; Tables S2, S3). Vegetation, such as tree canopy cover or vegetation emerging from the water, influences *Tetragnatha* diet (Tagwireyi and Sullivan 2015) and the production of emergent insects in streams (Laeser et al. 2005; Marshall et al. 2022). All stream variables are presented in Table S4.

Analysis of chemical pollutants in stream water

Weekly water grab samples were collected mid-stream using 1L amber glass bottles for the measurement of pesticide and wastewater pollutants. To capture peak contamination levels which may be missed by grab sampling (Rabiet et al. 2010), high water level event samples

were collected at all sites during one rain event using 1L bottle samplers (Figure S2). Analytes of chemical pollutants were extracted from the stream water by solid phase extraction (SPE), following Machado et al. (2016), using Oasis® HLB 6cc 500 mg SPE cartridges (Waters Corporation, Milford, USA). The extracts were eluted from the cartridges, dried and re-suspended in 500 µL of a 70:30% (v/v) mixture of water with 0.1% (v/v) formic acid and MeOH (MS grade; Sigma-Aldrich Chemie GmbH, Germany). Further details of the extraction, chemical analysis and quality assurance procedures are described in Huszarik et al. (2023b).

High performance liquid chromatography tandem to triple quadrupole mass spectrometry by electrospray ionization (HPLC-ESI-MS/MS) was used to analyse the samples for 77 pesticides and 4 established wastewater indicators (Table S5) with an Agilent 1260 Infinity II HPLC system tandem to an Agilent 6495 triple quadrupole mass spectrometer (MS/MS; Agilent Technologies, Inc., Santa Clara, CA, USA). Agilent MassHunter Workstation (Quantitative analysis for QQQ v10, Agilent Technologies, Inc., Santa Clara CA, USA) was used to process the HPLC-ESI-MS/MS data.

The sum pesticide toxicity of each stream sample was calculated as the logarithmic sum toxic unit (Schäfer et al. 2013) for freshwater invertebrates:

$$sumTU = \log 10 \left(\frac{C_i}{EC_{50_i}} \right) \quad (1)$$

where C_i is the normalised concentration of pesticide i, and EC_{50_i} is the concentration affecting 50% (EC_{50}) of organisms in an acute test with pesticide i. Acute exposure (24-96 hr) EC_{50} values for the most sensitive freshwater invertebrate were mainly obtained from the ECOTOX database (U.S. Environmental Protection Agency 2021) using the R package Standartox (Scharmüller et al. 2020), or the PPDB in case of missing values (Lewis et al. 2016). The sumTU was averaged for each site over the study period. Wastewater pollutants indicating treated (carbamazepine, diclofenac, and sulfamethoxazole) and untreated (caffeine) wastewater (Čelić et al. 2019; Li et al. 2020) were only evaluated qualitatively, as concentrations of wastewater effluent in streams can change hourly (Paíga et al. 2019) and sites were not visited at the same time of day. Thus, we tallied all detections of wastewater indicators for each stream site over the study period.

Collection of potential flying insect prey

We sampled flying insects at the stream shore on four occasions (May 12th/13th, May 19th, June 2nd, June 9th/10th) using "Sea, Land, Air" style (SLAM) malaise traps (McCravy 2018;

MegaView Science Co., Ltd., Taichung, Taiwan). The SLAM traps were set 1 m above the water surface, directly above the stream shore with openings parallel to the stream (Figure S3). The collection bottles were filled with propylene glycol trapping medium (33% propane-1,2-diol, 66% water, 1 mL/L dish soap and 10 mg/L denatonium benzoate for deterring larger animals) and emptied after one week. Flying insects were identified to order, or to family level for orders (i.e. Diptera and Coleoptera) including families with both aquatic and terrestrial larval development (Brohmer et al. 2009; Köhler 2015). A list of the taxa and their aquatic or terrestrial designation is provided in Table S6.

Spider collection

Adult *Tetragnatha montana* spiders were collected from all field sites between 29.5.2020 and 4.7.2020, during the afternoon (13:00 to 19:00h; Table 1). Spiders were sampled in areas immediately up- or downstream from the field sites, to avoid disturbing the streambeds where other sampling was taking place. Spiders were collected individually from their webs above the water surface or along the stream shore (maximum 5 m from the stream) using sterile tweezers. They were stored individually in PVC vials and then frozen at -18°C for one night. On the following day, they were transferred to 98% EtOH and stored at -20 °C before further processing.

Table 1: Number (N) of female (F) and male (M) *Tetragnatha montana* spiders collected at stream sites for diet analysis.

Site	F	M	Total N
ERB	9	2	11
KAT	11	8	19
KLI	10	4	14
LAU	21	8	29
MOD	9	6	15
NEU	8	10	18
POR	14	6	20
SPI	21	4	25
WEL	17	4	21
WIE	22	2	24
Total	154	54	196

DNA Extraction

DNA extraction was performed at Stockholm University in Sweden. Spiders were first dissected, under sterile conditions, to reduce the amount of spider material included in DNA extraction. Complete dissection of the stomach is difficult to perform on spiders due to their

complex digestive system (Macías-Hernández et al. 2018). Instead, spiders were dissected on sterile filter paper: Once excess ethanol had evaporated and the spider was dry, legs and pedipalps or epigynes were removed, and the spider was carefully cut in half lengthwise. One half of the abdomen and cephalothorax was used for DNA extraction. If the abdomen was too small to dissect (abdomen < 4 mm), the entire abdomen and cephalothorax were used, without legs. The dissected spider parts were stored dry at -20 °C until DNA extraction.

DNA extraction was performed semi-automatically using the Mag-Bind® Blood & Tissue DNA HDQ 96 kit (Omega Bio-Tek, Inc., Norcross, GA, USA) containing magnetic beads, following a modified protocol (Guide for tissue, OBT_M6399_Kduo_100µL_v1.1, SI Section S1). Briefly, each spider sample was homogenized, then incubated overnight in a tissue-lysis buffer and proteinase K solution. The next day, RNA was removed with 5 µL of 10 mg/mL RNAse, and samples were incubated at room temperature for 15 minutes before being spun down for 10 minutes (40000g; CT15RE, VWR Hitachi, Lutterworth, UK). Samples were prepared for extraction by adding buffers to the extraction plate wells as described in the protocol, then adding 250 µL of the sample supernatant, binding buffers and 20 µL of magnetic beads. Automated extraction was performed with the KingFischer™ Duo Prime Purification System (Thermo Fischer Scientific, Inc., Waltham, MA, USA). Following extraction, the concentrations of DNA were measured per sample with the NanoDrop™ One (Thermo Fischer Scientific, Inc., Waltham, MA, USA). Samples were then stored at -20 °C. A blank extraction sample was included with each extraction day to control for DNA contamination (n = 3).

PCR Amplification

Prey DNA was amplified using unique combinations of tagged NoAranR (reverse primer, 5'-3' TGTTCATCCDGTNCCWG; Hambäck et al. 2021) and LCO1490 (forward primer, 5'-3' GGTCAACAAATCATAAAGATATTGG; Folmer et al. 1994). This primer combination is designed to preferentially amplify insect DNA while reducing amplification of spider DNA (Hambäck et al. 2021) in a single indexing polymerase chain reaction (PCR) step ("tagging PCR protocol"; Bohmann et al. 2022). The tagging system used was an 8-base-pair combination of both forward and reverse primers (Table S7; Binladen et al. 2007). It should be noted that whereas these primers reduce amplification for most spider DNA, the reduction is poorer for *Tetragnatha* spp. than for other spiders. Ten microlitres of each primer, 25 µL of Multiplex PCR Master Mix, 2x (QIAGEN GmbH, Germany) and 5 µL of extracted DNA (concentration 20-30 µg/µL) were combined under sterile conditions, and each sample within

a PCR run (up to 64 samples) received a unique tagged primer combination. Any samples with DNA concentrations over 40 ng/µL were diluted with sterile ultrapure water before being added to the PCR reagents. For PCR blanks, 5 µL of sterile ultrapure water was added instead of DNA. Approximately 25% of the primer combinations per plate were blanks to monitor the frequency of sequencing errors (Bohmann et al. 2022). Details of the PCR protocol are provided in the SI (Table S8). The amplified samples were stored at 4 °C and successful amplification verified using gel electrophoresis. The concentration of amplified DNA was measured using a Qubit 2.0 Fluorometer with the Qubit dsDNA HS Assay Kit (Thermo Fischer Scientific, Inc., Waltham, MA, USA).

Following amplification, samples were pooled into seven PCR libraries (one library per PCR run), adjusting the volume based on the DNA concentrations measured after the PCR to ensure that each sample contributed the same amount of DNA. Pools were cleaned using AMPure XP beads following the manufacturer's protocol (Beckman Coulter, Brea, CA, USA) to remove excess nucleotides and primers. The concentration of DNA was re-measured with the Qubit HS kit. Pools were cleaned again using the MinElute PCR Purification (QIAGEN GmbH, Germany). A unique combination of forward and reverse Illumina TruSeq® DNA Single Index (Set A: i2, i4, i5, i6, i7, i13, i19; Illumina, Inc., San Diego, CA, USA) tagged adaptors were then added to each pool using a phosphorylation and adapter ligation step (SI Section S2). Finally, the pools were cleaned using the MinElute Gel extraction Kit (QIAGEN GmbH, Germany), where fragments within the target length (~300 bp) were extracted from a gel. Samples were concentrated, pooled into one library, and then sequenced by the SNP&SEQ Technology Platform at the Science for Life Laboratory in Uppsala Sweden using a MiSeq™ system (Illumina, Inc., San Diego, CA, USA).

Bioinformatics

Output sequences were processed using ObiTools (Boyer et al. 2016) in the galaxy web interface (use.galaxy.eu, 2023, Jalili et al. 2020). Paired-end sequences of high quality (score > 40) were assembled using 'Illuminapairedend' and demultiplexed using 'NGSfilter' after filtering for size. We then used 'obiuniq' to identify and count unique sequences before clustering operational taxonomic units (OTUs) using a 97% similarity threshold, tabulated for each spider individual.

Taxonomic assignments were matched to OTU sequences using BOLD (Accessed 16.05.2023; Ratnasingham and Hebert 2007) within the Boldigger interface (version 2.1.2; Buchner and

Leese 2020). Sequences were aligned using the BOLDigger pipeline to find the top 20 matches, including the "Correction of top hits via BOLD API" option. A threshold of 97% similarity was used for taxonomic assignment. Assignments were made to the lowest taxonomic level of the best match. Any sequences which had multiple species with the same similarity, private or early release sequence matches, or suspicious matches, were BLASTed in GenBank using Megablast (blastn version 2.13; Camacho et al. 2009) to obtain the top 30 matches. Any better match, based on identity score, match length, and E-value, was selected. The geographic range of taxonomic matches was evaluated using the Global Biodiversity Information Facility (GBIF.org, accessed May 2023), and all species with occurrence records in Germany or neighbouring countries were included. Any OTUs with only one read in a single sample or blank were removed. All non-arthropod OTUs were removed, as well as *Tetragnatha* spp. OTUs, to retain only prey OTUs.

Several approaches are available to account for errors during PCR or sequencing (Drake et al. 2022). We did not find evidence for DNA contamination in blank samples, and therefore set a minimum read number threshold of 0.1% of the total OTU read number for an OTU to be detected in a sample. If the maximum number of reads in a blank was higher than this threshold, which only occurred for five OTUs, the maximum read count in a blank was used as the threshold for that OTU to exclude any erroneous detects (Cirtwill and Hambäck 2021). Following filtering, the detections of OTUs with identical species names were combined to give the presence of each prey taxon per spider. Next, prey taxa were grouped in higher taxonomic groups (family or order) based on their terrestrial or aquatic larval origin, referred to hereafter in the text as "terrestrial" and "aquatic", respectively (Table S6). The number of detections of each prey taxon and taxa group were then totalled for each spider before calculating the per-site proportions of prey detections belonging to each taxa group or taxon.

Data analysis

A correlation matrix was calculated using Spearman's rank correlation for non-parametric data to identify highly correlated ($\rho > 0.8$) variables, which were excluded unless highly biologically relevant. Next, a principal component analysis (PCA; VEGAN; Oksanen et al. 2022) of variables describing physical stream characteristics and in-stream chemical pollution was calculated to identify environmental gradients existing across the stream sites. We then selected three variables based on their importance for insect emergence and riparian spiders and representing these gradients to use in further analysis steps. The average sumTU is biologically relevant as

it related to negative effects of pesticides on insect emergence and indirect effects on spider diet, as well as being highly correlated with most chemical pollution variables and aligned with a water quality gradient present across stream sites. Stream width and tree canopy cover were also selected as they represented stream size and vegetation coverage, both of which are relevant for insect emergence and spiders (Laeser et al. 2005; Tagwireyi and Sullivan 2015; Raitif et al. 2018; Marshall et al. 2022), as well as for their alignment with a habitat characteristics gradient.

The effect of sumTU, stream width, and canopy cover on the composition of both flying insects and *T. montana* diet at different streams was tested using a PERMANOVA ("adonis", VEGAN; Oksanen et al. 2022). In addition to the coarse (order to family level) classification of the prey taxonomic groups, the spider diet composition at the species level was also tested by including those 13 prey species which had been detected in spiders at three or more streams (*Polypedilum aegyptium* [Chironomidae], *Hilara beckeri* [Empididae], *Lasius brunneus* [Formicidae], Cecidomyiidae *sp.*, *Rheotanytarsus curtistylus* [Chironomidae], *Phyllaphis fagi* [Aphididae], *Hilara sp.* [Empididae], *Hilara lurida* [Empididae], *Micropsectra notescens* [Chironomidae], *Austrolimnophila ochracea* [Limoniidae], *Agapetus ochripes* [Glossosomatidae], *Micropsectra pallidula* [Chironomidae], *Microtendipes pedellus* [Chironomidae]). Permuted linear models were used as *post-hoc* tests to determine significant effects on individual taxa groups. We also used generalized linear models (GLMs) to assess the effects of average sumTU, stream width, and canopy cover on the total number of flying insects caught in SLAM traps, the proportion of aquatic flying insects, the average number of prey detections per spider at each site, and the proportion of aquatic insects in the spider diet. The relationship of sumTU, stream width, and canopy cover was first tested with spider sex to ensure that the diets of males and females were not significantly different and could be tested together. The negative binomial family ("glm.nb", MASS; Venables et al., 2002) was used for count data, and the gaussian family with permutation was used for interval data. Model assumptions were checked using "check_model" (PERFORMANCE; Lüdecke et al. 2021). Statistical analyses were performed using R (version 4.2.2; R Core Team 2022), and figures were created using GGPLOT2 and GGPUBR (Wickham 2016; Kassambara 2022). A significant effect was considered when $p < 0.05$.

Results

Environmental gradients at stream sites

The studied streams formed a gradient of chemical pollution, which consisted of pesticide detection and toxicity, wastewater pollution, dissolved nutrients and other variables related to water quality across the stream sites, but also included a weak association with shrub separation. The average sumTU was highly correlated with almost all other stream pollution variables. The pollution gradient aligned with the first PCA axis (Figure 1). The second gradient, consisting of stream width and depth, as well as canopy cover and vegetation, other than shrub separation, aligned with the second PCA axis. The pollution and stream size gradients were orthogonal and, thus, largely independent of each other.

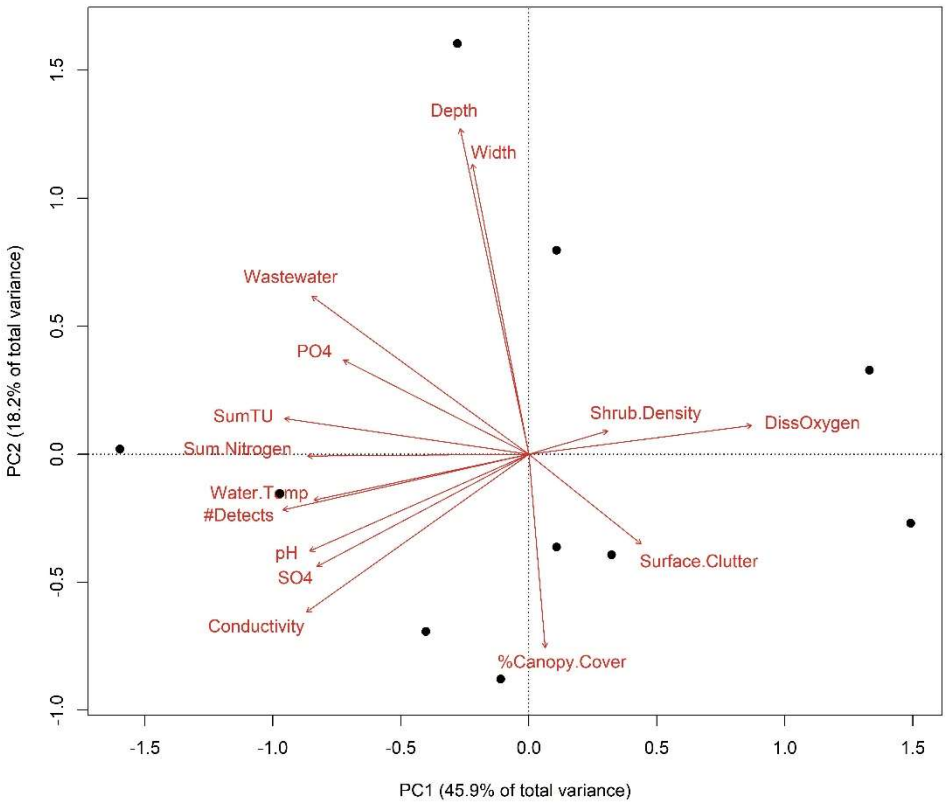

Figure 1: A principal component analysis (PCA) of environmental variables (red arrows) measured at stream sites (black points). Abbreviations: *Depth* water depth, *Width* stream width, *Shrub.Density* the separation score of shrubs along the stream shore, *DissOxygen* the average concentration of dissolved oxygen, *Surface.Clutter* the average score of water surface coverage by vegetation clutter, *%Canopy.cover* the percentage of tree canopy cover, *Conductivity* the average water conductivity, *SO4* the average concentration of dissolved sulphate, *pH* the average pH of the stream water, *#Detects* the average number of pesticides detected, *Water.Temp* the average water temperature, *Sum.Nitrogen* the average concentration of dissolved nitrate, nitrite and ammonium combined, *SumTU* the average sum toxicity of pesticides for freshwater invertebrates, *PO4* the average concentration of dissolved phosphate, *Wastewater* the total number of wastewater indicators detected.

Change in flying insect prey and Tetragnatha montana *diet along pollution gradient*

The flying insect community at the streams was a mixture of terrestrial and aquatic taxa, with an average of 35±13% of taxa identified as aquatic, or 45±9% of taxa identified as either aquatic or unknown origin (Figure 2A). There tended to be fewer flying insects at sites with higher sumTU and canopy cover, while there were no significant relationships with the proportion of aquatic insects trapped at each site (Table S9, Figure S4, Figure 2A). Diptera was the most numerous order of flying insects at all streams, with Terrestrial Diptera (19.1±1.7%) and Chironomidae (18.5±4.0%) dominating the catch of the Malaise traps both proportionally and numerically (Figure 2A, Figure S4). There was no overall significant relationship between the taxonomic composition of flying insects and in-stream pesticide toxicity, canopy cover nor stream size (Table S9). However, the number of Plecoptera was negatively correlated with sumTU (ρ = -0.84, p = 0.018). Far fewer Plecoptera were present at streams with a sumTU higher than -1.1, with none found at the two most polluted streams.

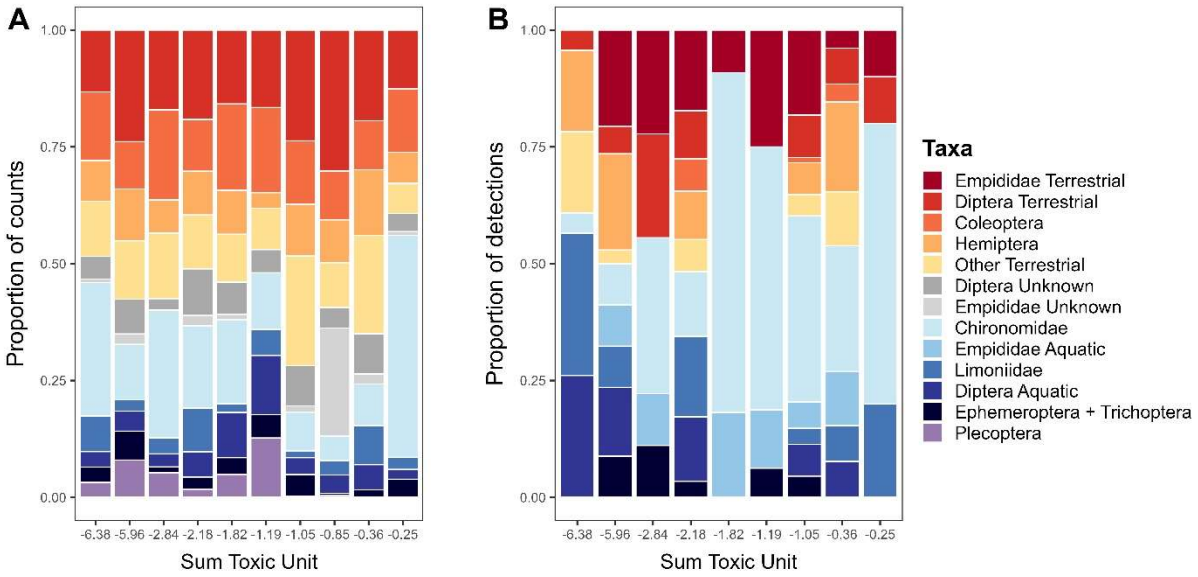

Figure 2: Composition of flying insects sampled with Malaise traps (**A:** proportion of total insect counts) and the diet composition of *Tetragnatha montana* spiders (**B:** proportion of prey detections from DNA metabarcoding) sampled at different stream sites in riparian forests. Stream sites are arranged from low to high chemical pollution and are labelled by their average in-stream pesticide toxicity (sum toxic unit) on the x-axis. A more positive sum toxic unit (log scale) represents a higher toxicity, whereas a more negative value represents a lower toxicity. Taxa coloured from red to yellow are terrestrial, blue and purple are aquatic, and grey are of mixed origin. It should be noted that in (**A**), a few individuals of aquatic families are included in Coleoptera and that Empididae were not separated into aquatic and terrestrial. Site "-0.85" (ERB) is only included in (**A**) as no prey taxa were detected in spiders from that site.

DNA metabarcoding of the collected spiders (Table 1) produced 150,258 total reads of prey taxa and 200 prey OTUs after filtering. Spiders consumed 105 taxa in total, of which 62 were aquatic. On average, 1.3±2.0 taxa (maximum 14) were detected per spider, except for one site

where we detected no prey DNA in any spiders. There was no difference between male and female spider diet composition ($F_{(1,14)} = 1.03$, $p = 0.397$), detections ($t_{(1,14)} = -1.09$, $p = 0.289$), nor proportion of aquatic prey ($t_{(1,14)} = -1.64$, $p = 0.134$), although females generally had slightly more prey detections (female average: 1.3±0.3, male average: 0.8±0.2) and slightly more aquatic prey (female average: 68±5%, male average: 52±8%) in their diet.

The diet of *T. montana* was comprised of 64 ±5% aquatic prey, on average (Figure 2B). Diptera were the most common prey taxa, with Chironomidae (35±8%), Empididae (22±4%), and Limoniidae (10±3%) representing the most frequently consumed families. While the overall diet composition of *T. montana* did not differ with stream size, stream pollution, nor canopy cover, spiders consumed significantly more chironomids ($t_{(1,7)} = 2.52$, $p = 0.042$) and fewer other aquatic dipterans (i.e., Culicidae, Dixidae, Dolichopodidae, Pediciidae, Psychodidae, Ptychopteridae, Simuliidae, and Tipulidae) at more polluted sites ($t_{(1,7)} = -3.05$, $p = 0.022$; Figure 2B). There were no relationships between stream pollution, canopy cover nor stream size on the number of prey detections per spider, on the proportion of aquatic prey in the spider diet, nor on the diet composition at the species level (Table S9).

Discussion

Effect of stream pollution on Tetragnatha diet

As we predicted, chemical pollution in streams was associated with a shift in the diet composition of *T. montana*, which consumed more chironomids and fewer other aquatic Diptera at more polluted sites (Figure 2B). This finding was the strongest evidence for an indirect effect of stream pollution on *T. montana*, as we found no significant changes in the number of prey detections, nor in the proportion of aquatic prey in their gut content. The results of our study expand upon a similar study by Graf et al. (2020), who found that the proportion of aquatic prey in the diet of *T. montana* along agricultural streams was only affected by intensive agriculture land use and not by in-stream pesticide toxicity. With our study, we show that even when the overall proportion of aquatic prey does not change, a shift in the taxa consumed by spiders can occur in response to toxicity and stream pollution gradients.

Although the percentage of aquatic prey consumed by *T. montana* was high at all sites, chironomids clearly dominated the diet composition at sites with a sumTU > -2 (Figure 2B). This dietary shift may be explained by increased availability of chironomids due to a change from sensitive to tolerant species emerging from the streams, and spider prey preference. In another study on German streams, Liess and Von Der Ohe (2005) observed a significant reduction in the proportion of sensitive aquatic invertebrates (species at risk; SPEAR) in stream communities, as well as an increase in the abundance of tolerant taxa, at sites with a TU higher than -3. Certain chironomids are well-known to be tolerant to pollution (Chang et al. 2014; Rico and Van den Brink 2015), and could have had an advantage at more polluted streams in our study, most likely due to higher nutrient levels. In comparison, the aquatic Diptera that were consumed more frequently at less polluted sites included taxa associated with standing water (Culicidae) and others more likely to be sensitive to pollution (Liess and Von Der Ohe 2005; Lock et al. 2014).

If increased availability were the sole explanation for the change in spider diet, chironomids should also have been more common in the flying insect community sampled by the malaise traps. This was not the case, although we had expected to find similar responses in the spider diet and flying insects. The fact that chironomids were consumed more often by spiders at polluted sites, and frequently consumed overall, may be attributed to a preference of *T. montana* for chironomid prey. There are few studies describing the exact diet of *T. montana*, but they likely mainly consume small aquatic dipterans (Nyffeler 1999; Henschel et al. 2001).

In this case, *T. montana* could have switched to other prey at less polluted sites where chironomids were less available and more diverse taxa occurred. Another reason for the difference between spider diet and flying insects may be due to the sampling methods. *Tetragnatha* spiders are attracted to areas rich in aquatic prey (Kato et al. 2003), and build their webs directly over the water surface. The "SLAM"-style malaise traps were located on the stream shore and one meter above the water level. It may be that weak-flying chironomids were better "sampled" by the spiders than the malaise traps. Using additional methods such as benthos sampling and different trap positions will help to further explain the differences we see between the flying insect community and spider diet compositions in our results.

Effect of stream pollution on available prey

There were no clear effects of chemical stream pollution on the overall flying insect community composition. However, there were fewer flying Plecoptera at the most polluted streams. Plecoptera are highly sensitive to stream water quality and pollutants (Chang et al. 2014; Rico and Van den Brink 2015) and were likely not present in polluted streams due to a combined effect of poorer water quality, with less oxygen and higher water temperature, together with more dissolved nutrients and pollutants. Chemical pollution from pesticides (Muenze et al. 2017; Liess et al. 2021) and wastewater (Stalter et al. 2013; Pallottini et al. 2017) can cause a shift in the macroinvertebrate stream community from diverse assemblages in terms of taxa, sensitivity, and ecological traits to more uniform communities. For example, the application of the insecticide methoxychlor in a stream caused the stream community to shift to small, tolerant, and quickly-reproducing taxa (Cuffney et al. 1984). However, this shift may not always be detectable when measuring the abundances of insects (Stenroth et al. 2015), and the effects of stream pollution at the levels observed in this study may not have been strong enough to be detected in the flying insect community, other than the reduction of Plecoptera. It would be interesting to include sites with a stronger gradient of pollution in the future to determine how the coupling between aquatic and terrestrial systems are affected in more extreme scenarios.

Advantages of DNA metabarcoding for analysis of dietary effects

Many studies evaluating effects of contaminants on consumers of aquatic insects have used stable isotope analysis (SIA) to reveal changes in their diets. While SIA is effective for determining the trophic level and the aquatic signature of the diet and can reflect a longer temporal snapshot, it can also be misinterpreted and leave unanswered questions, particularly

when the signatures of specific taxa are not easily distinguished (Nielsen et al. 2018). We propose that DNA metabarcoding is an excellent method to complement stable isotope analysis and examine dietary changes in more detail, as shown by Hambäck et al. (2016). The taxonomic specificity that can be achieved with DNA metabarcoding can clarify which taxa are responsible for changes in the diet, especially if SIA results are unclear, as in Hambäck et al. (2016) and Graf et al. (2020). DNA metabarcoding enabled us to detect a dietary shift in *T. montana*. We would likely not have seen an effect of stream pollution in our study using only SIA, as there were no large differences in the proportion of aquatic prey detected between streams. Furthermore, knowing the species occurring in spider diet can reveal additional information about the consumed prey community, such as general size composition, sensitivity to stressors, or feeding traits. Including methods such as DNA metabarcoding has great potential for adding new information to our knowledge of changes in aquatic-terrestrial food webs in response to stressors.

Consequences of dietary shift

The shift of *T. montana* diet towards consuming more chironomids at more polluted sites and maintaining the same proportion of aquatic prey in their diet suggests an increased risk of pollutant uptake for spiders, rather than a higher reliance on terrestrial prey due a decrease in aquatic prey availability. Increasing evidence shows that emergent insects can accumulate and export certain pesticides (Roodt et al. 2022), metals (Naslund et al. 2020) and pharmaceutical compounds (Richmond et al. 2018; Previšić et al. 2021). This has also been associated with effects in consumers, such as changes in microbiome of spiders (Millar et al. 2022) and bats (Mehl et al. 2021). Kraus et al. (2021b) summarizes possible consequences of in-stream pollutants on consumers of emergent insects: pollutants may act to reduce availability of insect emergence ("exposure driving subsidies"), and insect emergence may bioaccumulate certain pollutants, resulting in pollutant transfer to consumers ("subsidies driving exposure"). Although both dynamics may co-occur, i.e. in the case of highly bioaccumulating compounds which remain in adult insects but also reduce insect emergence (Kraus 2019), the levels of stream pollution observed at our sites were not high enough to decrease the overall abundance of insect prey. In the same study area, Roodt et al. (2023) found that certain pesticides accumulated in *Tetragnatha* spiders via emergent insects from the stream. Given this, the spiders in our study were likely increasingly exposed to contaminants at more polluted streams via their emergent insect prey. Pollutant exposure can result in consequences for spiders, such

as possible poorer body condition due to sublethal effects (Pietz et al. 2023), as well as pollutant transfer to the greater riparian food web.

Conclusion

DNA metabarcoding proved highly suitable to detect shifts in the diet of a terrestrial insectivore along a gradient of stream pollution. Spiders consumed more chironomids at more polluted streams in the absence of a significant change in the proportion of aquatic prey in their diet. Their continued reliance on aquatic prey at polluted streams likely resulted in an increased dietary exposure of spiders to chemical pollutants, which could affect spiders themselves and propagate further into the riparian ecosystem. Future studies should investigate which direct implications this dietary shift and chemical pollution exposure may have on riparian spiders and their food web.

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Author contribution statement: The study was conceptualized by **MH**, **APR**, **VS**, **RS**, **PH**, and **MHE**. The data were collected, prepared, and processed by **MH**, **APR**, **TW**, **ML**, **ELF**, **DA** and **PH**. Statistical analyses were performed by **MH** with the assistance of **VS** and **MHE**. The study was supervised by **RS**, **PH**, and **MHE**. The manuscript was written by **MH** with assistance from all authors.

The authors do not declare any competing interests.

The data for this study is available at figshare (doi: 10.6084/m9.figshare.24161229).

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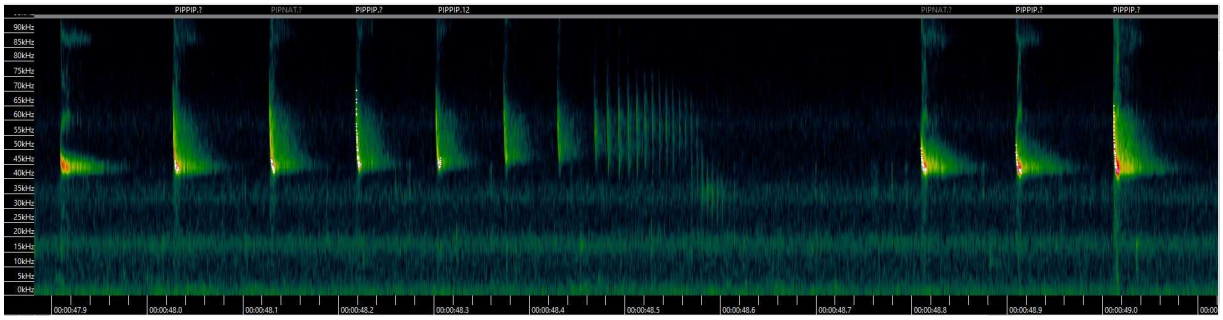
Chapter 4: Increased bat hunting at polluted streams suggests chemical exposure rather than prey shortage

Maike Huszarik, Alexis P. Roodt, Teagan Wernicke, Fernanda Chávez, Annika Metz, Moritz Link, Eva Lima-Fernandes, Ralf Schulz, Martin H. Entling



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Increased bat hunting at polluted streams suggests chemical exposure rather than prey shortage

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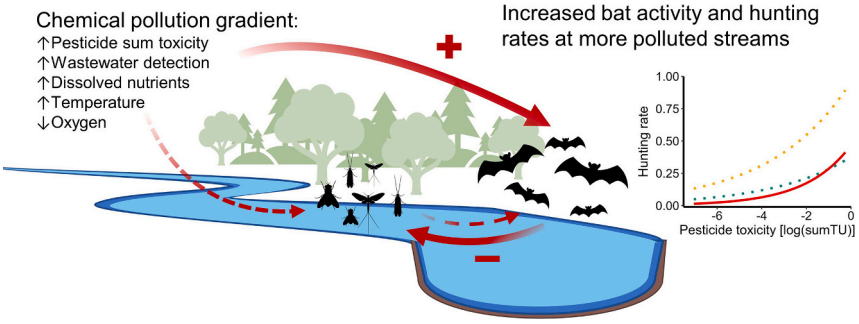
HIGHLIGHTS

- Water quality may negatively impact insectivorous bats hunting in riparian areas.
- We measured 77 pesticides and 4 wastewater indicators in 14 forested streams.
- The abundance of emergent insect prey was not reduced by stream pollution.
- Hunting rate and activity of *Myotis* bats were highest at more polluted streams.
- Bats may be exposed to stream pollutants through consumption of contaminated prey.

GRAPHICAL ABSTRACT



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ABSTRACT

Streams and their riparian areas are important habitats and foraging sites for bats feeding on emergent aquatic insects. Chemical pollutants entering freshwater streams from agricultural and wastewater sources have been shown to alter aquatic insect emergence, yet little is known about how this impacts insectivorous bats in riparian areas. In this study, we investigate the relationships between the presence of wastewater effluent, in-stream pesticide toxicity, the number of emergent and flying aquatic insects, and the activity and hunting behaviour of bats at 14 streams in southwestern Germany. Stream sites were located in riparian forests, sheltered from direct exposure to pollutants from agricultural and urban areas. We focused on three bat species associated with riparian areas: *Myotis daubentonii*, *M.* cf. *brandtii*, and *Pipistrellus pipistrellus*. We found that streams with higher pesticide toxicity and more frequent detection of wastewater also tended to be warmer and have higher nutrient and lower oxygen concentrations. We did not observe a reduction of insect emergence, bat activity or hunting rates in association with pesticide toxicity and wastewater detections. Instead, the activity and hunting rates of *Myotis* spp. were higher at more polluted sites. The observed increase in bat hunting at more polluted streams suggests that instead of reduced prey availability, chemical pollution at the levels measured in the present study could expose bats to pollutants transported from the stream by emergent aquatic insects.

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1. Introduction

Bats are known to be vulnerable to disturbances and stressors in their ecosystems, and require high-quality food sources to maintain their energy-intensive lifestyle (Jones et al., 2009). Many European populations have suffered declines in the past as a result of habitat degradation, human disturbance and chemical pollution, among other stressors (Browning et al., 2021; Jones et al., 2009). In response, all European bat species are protected under the 1991 EUROBATS agreement and the Habitats Directive (Council Directive 92/43/EEC). Although some populations appear to be recovering, bats remain at risk from a plethora of threats, many of which are poorly understood (Browning et al., 2021; Frick et al., 2020). In particular, chemical pollutants and water pollution have been identified as significant, yet understudied, threats to European bats (Browning et al., 2021; EFSA et al., 2019).

Streams and surrounding riparian areas represent important habitats for many bat species, either as flight paths, sources of water or foraging areas (Grindal et al., 1999; Scott et al., 2010). Emergent aquatic insects, which spend their larval stages in streams before emerging as flying adults, represent an important, high-quality prey source for bats (Guo et al., 2017; Hixson et al., 2015). Some bat species specialise in hunting over water surfaces and mainly consume aquatic insects, such as Daubenton's bat (*Myotis daubentonii,* Kuhl 1817; Nissen et al., 2013; Vesterinen et al., 2018). However, even less-specialised species take advantage of riparian areas (Bellamy et al., 2013; Stahlschmidt et al., 2012), and streams have been found to be "hotspots" of bat activity in forests, especially in areas of high aquatic insect emergence (Fukui et al., 2006; Power et al., 2004). Ensuring good habitat quality of streams, and the aquatic prey they provide, benefits many bats (Bellamy et al., 2013).

Chemical pollution is a major challenge for stream quality and freshwater ecosystems (Malaj et al., 2014), as well as for bat conservation (Frick et al., 2020). This stressor has the potential to affect large stream stretches, as pollutants can be transported to otherwise unexposed areas downstream (Barber et al., 2013; Wolfram et al., 2023). Chemical pollutants enter streams from point and non-point sources, such as effluent from a wastewater treatment plant (WWTP), and runoff from agricultural fields, respectively (Neumann et al., 2002). Once in the stream, they can negatively affect organisms, altering the stream community with potential food web effects in riparian areas (Burdon et al., 2019; Graf et al., 2017; Manning and Sullivan, 2021). Both pesticides and wastewater effluent found in streams have been associated with decreases in insect emergence (Kalcounis-Rueppell et al., 2007; Kraus et al., 2021; Marshall et al., 2022; Roodt et al., 2023a). Thus, chemical pollution in streams could reduce the availability of high-quality aquatic prey for riparian bats.

Few studies have evaluated how effects of chemical stream pollution propagate into the riparian food web, especially in the context of bats. Some have observed changes in bat activity and hunting behaviour in connection with altered insect emergence around WWTPs, with varying responses (Abbott et al., 2009; Kalcounis-Rueppell et al., 2007; Vaughan et al., 1996). On the other hand, we are not aware of any studies evaluating indirect food web effects of current-use pesticides in streams on bats, though several reviews have stressed the importance of this knowledge gap (Browning et al., 2021; Torquetti et al., 2020; Voigt and Kingston, 2016). Pesticide toxicity in streams has been associated with a reduction in the number of riparian spiders preying on emergent insects and changes in the riparian spider community (Graf et al., 2019), and similar effects can be expected for bats hunting in riparian areas. An additional consideration is that emergent insects can take up pollutants and transport them from the stream into the terrestrial ecosystem (Kraus et al., 2021; Previšić et al., 2021; Richmond et al., 2018), potentially leading to negative impacts on bats through dietary exposure. Although studies have looked at effects of historical pollutants on bats, little is known about effects of chemicals used today (Torquetti et al., 2020).

Our aim was to investigate potential indirect effects of chemical pollution in streams on the activity and hunting rate of riparian bats. To do this, we conducted an 11-week field study at 14 streams along a pollution gradient in southwestern Germany. We analysed 77 pesticides and 4 wastewater indicators in addition to measuring nutrient concentrations and other physicochemical stream parameters, collecting emerging and flying insects, and recording bat activity and hunting behaviour at the sites. We focused on three bat species known to forage at streams with different degrees of specialisation: the common pipistrelle (*Pipistrellus pipistrellus*, Schreber 1774), Brandt's bat (*Myotis* cf. *brandtii*, Eversmann 1845), and Daubenton's bat (*M. daubentonii*). We hypothesised that stream pollution would negatively affect bat foraging behaviour by reducing the available emergent insect prey. Specifically, we predicted that sites with a higher pesticide sum toxicity and wastewater detection would have fewer emergent aquatic insects. We also predicted that bat activity and hunting rates would be lower at sites with more stream pollution, due to a reduction of the available emergent insect prey. We predicted that effects would be strongest for Daubenton's bat, the species most specialised in hunting at streams.

2. Materials and methods

2.1. Study area and stream sites

The field study was conducted at 14 streams located in southern Rhineland-Palatinate, Germany. The study area is bordered by the Palatinate Forest, a UNESCO Biosphere reserve, to the west, and the Rhine river to the east (Fig. S1). It is characterised by second and third order streams running west to east through forest then vineyards and agricultural land mixed with urban settlements and forested areas.

Forty-metre-long stream sections were selected to represent a gradient of chemical pollution while maintaining a homogeneous and natural habitat structure. We chose sections classified as no more than "moderately altered" according to the stream structural quality classification from https://wasserportal.rlp-umwelt.de/servlet/is/2025/ (accessed March 2020), to avoid the influence of anthropogenic alterations of stream structure in the study (Table S1). Stream sections were generally calmly-flowing with a dominance of smooth surfaces, to match the preferred foraging habitat of *Myotis daubentonii* (Warren et al., 2000). We attempted to keep stream size and pollution as independent as possible by including streams with low and high potential pollution levels across the range of sizes selected for the study. All sites were located in deciduous or mixed forest to standardise the riparian habitat, and were sheltered from direct exposure to agricultural and urban areas. The percentage of land cover types within a 100 m buffer around the sites, derived from aerial photographs (40 cm ground resolution, Map: WMS RP DOP40 v.2023-02-25 ©GeoBasis-DE/LVermGeoRP, 2023, dl-de/by-2-0, http://www.lvermgeo.rlp.de; QGIS version 3.12; QGIS Development Team, 2023) are provided in Table S1.

Sites were visited weekly over 11 weeks (April 21st 2020 to July 1st 2020) to sample water, stream physicochemical characteristics, emergent and flying insects (i.e. available prey for bats), and ultrasonic bat calls. In addition, the riparian vegetation near the streams was characterised and high-water event samples were collected on one occasion each.

2.2. Physicochemical stream characteristics

Stream width and depth were recorded several times throughout the study period. Dissolved nutrients nitrite (NO_2^-), nitrate (NO_3^-), ammonium (NH_4^+), phosphate (PO_4^{3-}) and sulphate (SO_4^{2-}) were measured in-stream using a nutrient analysis kit (VISOCOLOR® ECO reagents with PF-12 Spectrophotometer; Macherey-Nagel GmbH, Germany). Nutrient concentrations below the level of detection (LOD) were reported as half of the LOD. Water temperature, dissolved oxygen (mg/L), conductivity (μS/cm) and pH were measured with a multi-parameter meter (Multi 3620 IDS or Multi 340i, WTW Xylem Analytics GmbH, Germany).

Additionally, we placed two temperature loggers (HOBO Pendant® Temperature/Light 8K Data Logger #UA-002–08, Onset) 10 m apart on the stream shore to measure hourly air temperature on nights when bat detectors were recording. All physicochemical parameter measurements were averaged for each site over the study period (Table S2).

The vegetation of the riparian areas was characterised at each site on one occasion (June 23rd 2020). Canopy cover, shrubs, and vegetation obstructing the stream surface have been shown to affect the activity and behaviour of bats along streams (Biscardi et al., 2007; Boonman et al., 1998; Ober and Hayes, 2008) and the insect emergence due to changes in stream productivity (Marshall et al., 2022). The percent canopy cover was calculated as the average of three pictures taken of the tree canopy from the upper, middle, and downstream sections of each site. The pictures were taken mid-stream, 1 m above the water surface facing directly upwards. They were converted to blue-channel greyscale and then analysed in black and white pixels using ImageJ 1.53e (Ecological forester, 2011; Schneider et al., 2012). The distance between shrubs (shrub separation; Coulloudon et al., 1999) in the riparian area was classified on a scale for both stream banks, then averaged (Table S3). The percentage of the stream water surface interrupted or covered by clutter (i.e., vegetation disrupting the water surface or blocking a bat's flightpath) was also classified on a scale (Table S4) for the upper, middle and downstream sections of the site, then averaged. The height of clutter above the stream surface was measured along the sampling site and averaged. The vegetation surveys were conducted by the same observer at all sites.

2.3. Quantification of chemical stream pollution

Each week, 1 L water grab samples were taken for the analysis of chemical pollution by filling clean amber glass bottles mid-stream, below the water surface. In addition to grab samples, high-water event samples were collected during rain events. Run-off triggered by rain washes chemicals from agricultural fields and other surfaces into streams and can lead to peak concentrations of chemical pollutants, which may be missed by regular grab sampling (Rabiet et al., 2010). Event samplers consisting of two upright 1 L amber glass bottles with a small opening between the bottle and lid (Fig. S2) were attached to a stake and placed in the streams. The lowest bottle was 2–3 cm and the highest approximately 10 cm above the normal water line. We checked samplers during rain events and collected any full event bottles. If the bottles were not filled, a grab sample was taken. Event samples from one occasion were included for each site, taken during a rain event which occurred at all streams during the study (June 5th – 7th, 2020). All water samples were kept on ice during transport to the laboratory and then stored at 4 °C for 24–48 h to allow for settling of sediment prior to extraction and analysis of chemical pollutants.

2.3.1. Extraction of analytes

Chemical pollution analytes were extracted from 10 weekly grab samples and one event sample per site using solid phase extraction (SPE), following the method of Machado et al. (2016). At least one blank sample of 1 L ultra-pure water (18.2 MΩ-cm, D3750 2 μm endfilter, Barnstead™/Werner Reinstwassersystem, Thermo Fischer Scientific, Waltham, MA, USA) was included with each weekly extraction ($n = 14$), as well as solvent blanks run during the analyte elution ($n = 5$). Further details are presented in Section S1.1 of the Supplementary Information.

2.3.2. Concentration measurements

High-performance liquid chromatography tandem to triple-quadrupole mass spectrometry by electrospray ionization (HPLC-ESI-MS/MS) was used to analyse the samples for 77 currently used pesticides and 4 established wastewater indicators (Table S5). Measurements were performed with an Agilent 1260 Infinity II HPLC system tandem to an Agilent 6495 triple quadrupole mass spectrometer (MS/MS; Agilent Technologies, Inc., Santa Clara, CA, USA). A ZORBAX Eclipse Plus C18 HPLC column (3 × 150 mm, particle size 2.7 μm; Agilent Technologies, Inc., Santa Clara, CA, USA) kept at 45 °C was used to achieve chromatographic separation. The sample injection volume was 10 μL with a flow rate of 0.45 mL/min. At least two multiple reaction monitoring (MRM) transitions were used per compound to confirm the identity of and quantify the selected analytes, except for proquinazid, which only had one transition (Table S6). Processing of the HPLC-ESI-MS/MS data was performed with the Agilent MassHunter Workstation (Quantitative analysis for QQQ v10, Agilent Technologies, Inc., Santa Clara CA, USA).

2.3.3. Analytical quality assurance and data analysis

Analytical standards were prepared for the calculation of the limits of detection (LODs) and limits of quantification (LOQs) based on calibration curves (Table S5). In addition, the accuracy and reproducibility of the extraction method were evaluated with five ultrapure water samples containing a mixture of all analytes at a known concentration. Analytes with recoveries between 70 and 120 % and relative standard deviations between replicates (RSD) of 15 % or less were quantified in the samples (Table S5). Any analytes ($n = 23$) which did not meet these standards were only considered qualitatively and not included in the toxicity calculations. However, fipronil, which had a recovery of 50 %, was quantified as an exception due to its high ecotoxicological relevance and frequent occurrence in analysed samples.

Measured concentrations of each chemical pollutant analyte were normalised to the actual volume of water used for the SPE of each sample and for HPLC-ESI-MS/MS analysis (Section S1.2). Next, any concentration below the LOD was set to zero and concentrations between the LOD and LOQ were set as half of the LOQ (George et al., 2021). The LOQs of boscalid and caffeine were adjusted to account for a quantifiable background signal in blank samples (Table S12). Further details are provided in Section S1.2 of the supplementary information.

2.4. Presence of wastewater

Four of the measured analytes had been selected to indicate the presence of wastewater effluent in the streams. Caffeine is highly abundant in global freshwaters but is effectively removed with wastewater treatment (Li et al., 2020), allowing it to be used as an indicator of untreated wastewater. Carbamazepine, diclofenac, and sulfamethoxazole are three common pharmaceuticals present in surface waters but which are not effectively removed by treatment in WWTPs (Čelić et al., 2019). Concentrations of wastewater effluent and some pharmaceuticals have been shown to vary throughout the day (Nelson et al., 2011; Paíga et al., 2019). As we could not visit all stream sites at similar times of day, we avoided potential bias by only considering whether each indicator was detected (i.e. >LOD) in a sample, and calculated the total number of detections during the study period for each stream site.

2.5. Pesticide sum toxicity calculation

We used the logarithmic sum of toxic units (sumTU; Schäfer et al., 2013) to quantify the potential sum toxicity of the pesticide mixture measured in the stream samples:

$$sumTU = log10\left(\frac{C_i}{EC_{50_i}}\right) \quad (1)$$

where C_i is the normalised concentration of pesticide i, and EC_{50_i} is the concentration affecting 50 % (EC_{50}) of organisms in an acute test with pesticide i. Because we were interested in the direct effects of pesticides on emergent aquatic insect larvae in the streams, we calculated the sumTU for freshwater invertebrates. We used the EC_{50} for the most sensitive freshwater invertebrate from acute toxicity tests (24–96 h) for each analyte, based on available data (Table S5) mainly obtained from the USEPA ECOTOX database (U.S. EPA, 2021) using the Standartox package for R (Scharmüller et al., 2020), or the Pesticide Properties

Database (Lewis et al., 2016). The sumTU was then calculated for each sample (10 grab and 1 event), and averaged for each stream to obtain the average pesticide sum toxicity. A larger sumTU indicates a higher sum toxicity, whereas a more negative sumTU indicates a lower sum toxicity. We assigned a sumTU of −9.4, a factor of ten smaller than the lowest calculated sumTU, to three individual samples without detections of pesticides used in the sumTU calculation.

2.6. Measuring available emergent and flying insect prey

We used a combination of traps to approximate 1) the production of emergent aquatic insects and 2) the abundance of flying terrestrial and aquatic insect prey available for bats at each stream. Each site had two pyramid-shaped emergence traps with 0.25 m^2 surface area, based on Cadmus et al. (2016), in place on the water surface throughout the entire study period to continuously sample adult insects emerging from the stream. Traps were placed at least 10 m apart, when possible, in different parts of the stream channel, and had 125 mL of propylene glycol trapping medium (33 % propane-1,2-diol, 66 % water, 1 mL/L dish soap and 10 mg/L denatonium benzoate for deterring larger animals) in 500 mL collection bottles. Captured emergent insects were collected from the bottles weekly throughout the study. Flying terrestrial and aquatic insects were sampled at all sites on four occasions (May 12/13, May 19/20, June 2/3, June 9/10) using SLAM-style Malaise traps (McCravy, 2018; MegaView Science Co., Ltd., Taichung, Taiwan; Table S7). Each site had one SLAM trap suspended 1 m directly above the stream shoreline, secured so that the open sides were parallel to the stream. The trap bottles contained 125 mL of propylene glycol trapping medium (as for emergence traps) and were collected after one week.

Both flying (i.e. from SLAM traps) and emergent insect samples were kept on ice for transport to the lab, where they were removed from the trapping medium and stored in 80 % ethanol at 4 °C. Emergent insects were identified to order level (Brohmer et al., 2009; Chinery, 2012), and flying insects were identified to family level for orders with aquatic and terrestrial families (Brohmer et al., 2009; Köhler, 2015). The total number of individuals was used to estimate the abundance of emergence and flying insect prey at each site over the study period. The total number of emergent insects was corrected to account for differing trap numbers (Table S7) due to losses of some samples during storms.

2.7. Recording bat activity and hunting success

Bats emit echolocation calls during flight, which can be used to assess their overall activity and specific behaviours with bioacoustic methods. We deployed full-spectrum ultrasonic bat detectors (Audiomoth v1.1.0 with Firmware v1.2.2, Open Acoustic Devices; Hill et al., 2019) at each site for one night per week to automatically record bat calls. Detectors were wrapped in one layer of household cling film to protect them from moisture and dirt, and were taped to the trunks of trees approximately 40 m apart, at a height of 1.5 m approximately 1 m away from the shoreline. The microphones faced the stream and were unobstructed by vegetation. Recording was programmed to begin one hour before sunset and end one hour after sunrise, with a sample rate of 192 kHz, medium gain and continuous 1-h recording periods. Bats were only recorded on nights without precipitation and high wind speeds.

Audio recordings were processed with Kaleidoscope Pro (version 5.6, Bats of Europe 5.4, Wildlife Acoustics, Inc.), which split the recordings into 60-s-long files and filtered out noise files (i.e., without recognized ultrasonic signal detections). We used the default signal parameters in "Bat analysis mode" and the Auto-ID function with sensitivity set to "Balanced" to produce an initial species classification for each recorded minute, grouping minutes with similar calls for later manual identification. Only the 22 species known to occur in Rhineland-Palatinate (Lindermann, 2017) were included in the Auto-ID list.

Because automatic identification software is not yet fully reliable (Rydell et al., 2017), each minute was manually identified (sonogram settings FFT size 128, WIN size 64 in Kaleidoscope) after the initial classification by Auto-ID. As bats at or in close proximity to the stream would be within a few metres of the microphone, we assumed that all species using the streams would be detectable by the bat detectors (Barataud, 2020). We only considered those sequences containing at least one call recognized by Kaleidoscope (i.e., surpassing Kaleidoscope's noise threshold, with visual zero-crossing points) and excluded noise files, where any calls were likely too quiet or of too poor quality. There may have been some loss of calls as noise due to interference from the water surface, though we ensured that the detector placement was similar to keep this likelihood equal for all sites.

Five nights per site (May 5/6, May 18/19, June 2, June 12, June 23/24) were included. Specific procedures and details for manual identification are provided in Section S2 of the Supplementary Information. We counted the number of minutes containing bat calls of each species ("active minutes") as a proxy for bat activity. Bats emit special call types directly before prey capture, known as feeding buzzes. We counted the number of feeding buzzes in each minute, which we differentiated from drinking buzzes (Griffiths, 2013; Russo et al., 2016), as described in Section S2. The hunting rate, or the number of feeding buzzes per active minute, could then be calculated following (2):

$$Hunting\ rate_i = \frac{n_{feeding\ buzz_i}}{n_{active\ minutes_i} + 1} \quad (2)$$

where $n_{feeding\ buzz_i}$ is the number of feeding buzzes recorded and $n_{active\ minutes_i}$ the number of active minutes of bat species *i*.

We focused on three bat species for this study: the common pipistrelle, *Pipistrellus pipistrellus*, Daubenton's bat, *Myotis daubentonii*, and Brandt's bat, *M.* cf. *brandtii*, as they were the most common across the study area, present at all stream sites, and are all known to forage at forested streams (Roswag et al., 2019; Todd and Williamson, 2019; Warren et al., 2000). The calls of Brandt's bat are almost indistinguishable from the whiskered bat, *M. mystacinus* (Kuhl, 1817) (Russ, 2021). However, both species share overlapping ecological niches (Roswag et al., 2019), with Brandt's bat more restricted to woodlands. Thus, we assumed that most calls were likely to be Brandt's bat, though whiskered bats may have been included. The soprano pipistrelle, *P. pygmaeus* (Leach, 1825) was also common at some sites, but was excluded since it does not normally occur in the Palatinate forest (Lindermann, 2017).

2.8. Statistical analysis

Statistical analyses were conducted with R (version 4.2.2; R Core Team, 2022). All variables were summarised for each site over the entire study period, either as a total or an average value. To avoid correlation between variables, a correlation matrix was constructed with Spearman's rank correlation. Out of highly correlated variables ($\rho > 0.8$), only those with the highest expected relevance for bats were retained in the analysis.

A principal component analysis (PCA) was conducted based on the environmental variables measured (VEGAN; Oksanen et al., 2022). We then added the number of both emerged and flying aquatic Diptera and Ephemeroptera, Plecoptera, and Trichoptera (EPT), the number of all flying insects, and the activity and hunting rate of the three bat species to the biplot as passive variables, using "predict" in R to calculate their positions on the first two principal component axes.

Next, three series of generalized linear models (GLMs) were fitted. We used automated model selection and model averaging for each GLM to test the relationships between: 1) the number of emergent and flying aquatic insects explained by the environmental variables, 2) bat activity and hunting rates explained by the environmental variables and 3) bat activity and hunting rates explained by the number of emergent and flying insects (prey availability). Average pesticide sum toxicity (sumTU), stream width, and tree canopy cover were used as proxies of the various groups of correlated environmental variables and PCA axes:

water pollution/water quality, stream size, and vegetation, respectively.

For each GLM, a global model containing all variables was fitted (Table S10). The error distribution family and link functions were selected to match the distribution of the dependent variable (linear and gamma distributions fitted with "glm" in R, negative binomial with "glm.nb"; LME4; Bates et al., 2015; tweedie with "glmmTMB";GLMMTMB; Brooks et al., 2017). The hunting rate of the common pipistrelle required log transformation for one GLM. Model assumptions were checked ("check_model", PERFORMANCE; Lüdecke et al., 2021) and a VIF <3 was deemed acceptable. Each model was tested for spatial autocorrelation using Moran's I test (SPDEP; Bivand et al., 2013) and inspected with variograms (GSTAT; Pebesma, 2004). In the case of significant spatial patterns, the AICc values of the original model was compared to models containing spatial correlation structures to choose the best-fitting model (Zuur et al., 2009).

The "dredge" function (MUMIN; Bartoń, 2022) was used to compute all possible models from the global model and rank them by AICc, with a maximum of two explanatory variables allowed per model due to the small number of sites. An average model (MUMIN; Bartoń, 2022) was then calculated from all models within 4 points of difference in AICc from the best model (Burnham and Anderson, 2002). We considered the output of the conditional average model. Results of all GLMs are presented in Table S10. We also conducted generalized linear mixed effect models (GLMMs) with time as a fixed and site as a random effect to evaluate the temporal dynamics in the relationship between bats and insects. As these results are not directly related to our main hypotheses, they are presented in Table S14. A significant result was defined as $p < 0.05$. Plots were created with GGPLOT2 (Wickham, 2016) and GGPUBR (Kassambara, 2022).

3. Results

3.1. Chemical stream pollution

We found differing profiles in pesticide sum toxicity (average sumTU) and wastewater pollution (total number of detections) across stream sites (Fig. 1). In addition, average pesticide toxicity, wastewater indicator detections, and count of pesticide detections were highly correlated, and also highly correlated with nitrogen and sulphate concentrations measured in the streams (Table 1).

Of the 81 measured analytes, we detected 69 pesticides and all 4 wastewater indicators in at least one water sample (Table S8). An average of 17.9 (standard deviation ±13.5) pesticides and 2.1 (standard deviation ±1.4) wastewater indicators were detected per sample, with a maximum of 50 pesticides detected in a single sample. At least one chemical pollutant was detected in every water sample but two. The insecticide fipronil was detected in 83 % of samples, followed by mecoprop and metholachlor-S (herbicides,75 %), and 2,4-D (herbicide, 73 %; Table S8). In addition, diclofenac was the most commonly detected wastewater indicator, present in 77 % of samples (Table S8). There were no strong temporal changes in pesticide toxicity nor wastewater detections throughout the study period (Fig. S3).

There was a wide range in pesticide toxicity across streams, driven by few, toxic compounds. The average sumTU per site had a large range which was skewed towards higher toxicity (Table 1) and the maximum measured sumTU in an individual water sample was −0.061 (SPI, week 6). Fipronil, a non-agricultural insecticide, drove the sum toxicity for most sites due to its ubiquitous presence and high toxicity, whereas herbicides generally had the highest concentrations in the samples (Table S8). There was no strong peak in pesticide toxicity detected by high-water event samples, so they were considered together with weekly grab samples.

3.2. Relationships between water quality, stream size, vegetation, emergent and flying aquatic insects and bats

The streams were characterised by two independent environmental gradients (Fig. 2). Variables reflecting water quality and chemical pollution were grouped along the first axis, and explained most of the variation between sites. Streams with higher pesticide toxicity and more pesticide detections were warmer, had more wastewater detections, and higher nitrogen, sulphate and phosphate concentrations, but less dissolved oxygen. Oxygen levels were never measured below 7.07 mg/L during the study period. The second axis mainly represented stream size and vegetation characteristics. Wide streams tended to be deeper and to have less canopy cover and a lower surface vegetation clutter score than narrow streams. In terms of variation within streams over the study period, water temperature tended to increase and dissolved oxygen to decrease over time, while most other variables were either consistent or varied with no clear temporal trend (Table S13). Ranges within sites are reported in Table S2.

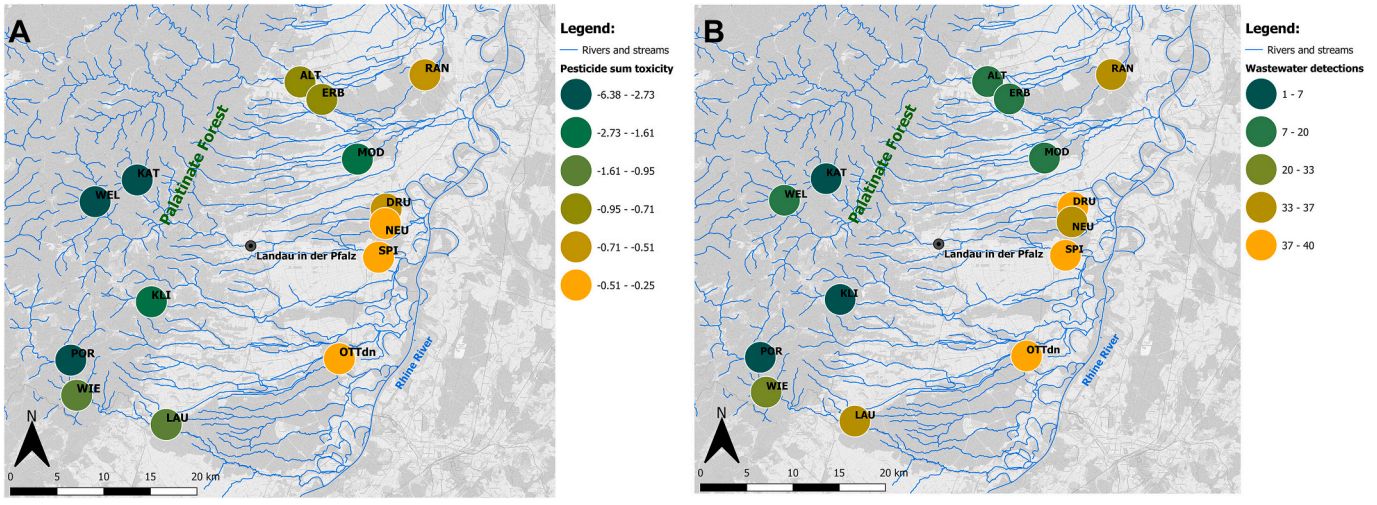

Fig. 1. Chemical pollution measured at 14 stream sites in southwestern Germany over 11 weeks. Dark grey fields are forested areas and vineyards, while light grey represents other agricultural and urban land. The Rhine river and Palatinate forest are labelled, as well as the city of Landau in der Pfalz. A) Average pesticide sum toxicity of the streams, measured as the logarithmic sum toxic unit (sumTU), is represented by the colours of the circles (range of average sumTU: −6.4 to −0.2). B) Total number of wastewater indicator detections in streams are represented by the colours of the circles (range 10 to 44 total detections). Note that the sumTU is on a logarithmic scale. The basemap is OpenStreetMap, available under the Open Database Licence (CC BY-SA 2.0). The stream layer "Gewässernetz 2017", available from WWV RLP (CC BY 4.0). The maps were created in QGIS 3.12.1-București.

Table 1
Chemical pollution, physicochemical and vegetation measurements per stream from 14 stream sites. Physicochemical variables are averages of measurements conducted over an 11-week field study, whereas vegetation characteristics were recorded on one occasion. The ranges and median values of each variable from the 14 streams are stated. In addition, the correlation of each variable to the average pesticide mixture toxicity (sumTU) given by Spearman's ρ.

Variable	Unit	Type	Range	Median	Correlation (ρ) with average sumTU
Pesticide sum toxicity	sumTU	Average over study period	−7.05 to −0.25	−0.95	–
Pesticide detections	Count	Average over study period	1.6–38.0	11.3	0.86
Wastewater detections	Count	Total over study period	1–40	25.5	0.87
Caffeine detections	Count	Total over study period	0–10	3.5	0.52
Pharmaceutical detections	Count	Total over study period	0–33	20	0.89
Width	m	Average over study period	2.1–8.8	3.9	0.11
Depth	cm	Average over study period	9–94	16	0.18
pH	–	Average over study period	6.7–8.1	7.6	0.44
Night air temperature	°C	Average over study period	10.1–13.9	12.7	0.65
Water temperature	°C	Average over study period	10.9–15.7	14.1	0.50
Conductivity	$\mu S/cm^2$	Average over study period	64.6–603.9	201.6	0.64
Dissolved O_2	mg/L	Average over study period	7.9–10.9	9.5	−0.55
Dissolved Nitrogen combined	mg/L	Average over study period, summed NO_2^-, NO_3^-, NH_4^+	0.6–2.7	1.5	0.86
Dissolved PO_4^{3-}	mg/L	Average over study period	0.1–0.2	0.1	0.57
Dissolved SO_4^{2-}	mg/L	Average over study period	10.0–32.8	13.9	0.72
Shrub separation score	–	Average of both banks	0–2	0.25	−0.23
Surface clutter score	–	Average of three locations on stream	0–2.5	1	−0.20
Surface clutter height	cm	Average over 40 m of stream	30–150	75	−0.082
Canopy cover	%	Average of three locations over stream	44.9–86.9	80.5	0.23

3.3. Insect emergence and flying insects

The PCA showed that more polluted streams tended to have higher numbers of emerging Diptera and EPT (Fig. 2). However, none of the relationships between the number of emerging insects and the stream toxicity, canopy cover, or stream width were significant (Fig. 3, Table S10). Contrary to the emergence pattern, the number of flying aquatic Diptera, EPT, and of all flying terrestrial and aquatic insects tended to either be higher at less polluted sites or have no clear relationship with stream pollution as shown in the PCA, again with no significant relationships (Fig. 2, Fig. 3, Table S10).

3.4. Bat activity and hunting rate

Streams with more pollution were associated with higher hunting rates of all three bat species and higher activity of both *Myotis* species, similar to emergent Diptera and EPT in the PCA (Fig. 2). The activity of Daubenton's bat ($z = 2.0$, $p = 0.04$, Fig. 4A) and hunting rate of Brandt's bat ($z = 2.2$, $p = 0.03$, Fig. 4B) were significantly higher at streams with higher pesticide toxicity (Table S10). Though the hunting rate of Daubenton's bat tended to be higher at more polluted sites, this was not significant (Fig. 2, Fig. 4B, Table S10). The activity of the common pipistrelle showed no clear increase with stream toxicity (Fig. 4A). The common pipistrelle was the only bat whose activity was related to stream vegetation and structure: they were significantly more active at sites with less canopy coverage ($z = 2.2$, $p = 0.03$, Table S10) and tended to prefer larger, more open streams (Fig. 2).

Overall relationships between bats and insects were highly variable. The activity of both *Myotis* species and hunting rate of all three bat species were similarly positioned to the number of emergent insects in the PCA (Fig. 2). The overall comparison of bats and insect abundance between streams revealed only few clear relationships (Table S10). While the activity of Brandt's bat was significantly higher at streams with a higher number of emerging Diptera ($z = 2.1$, $p = 0.004$; Fig. 5B), there was no relationship between their hunting rate and the number emerging or flying insects. The activity ($z = 2.9$, $p = 0.003$) and hunting rate ($z = 2.6$, $p = 0.01$) of Daubenton's bat were both negatively related to the abundance of flying EPT (Fig. 2, Fig. 5AC). There were no significant relationships between the hunting rate of Daubenton's bat and emerging and flying Diptera, though they showed a positive trend (Fig. 5D, Fig. 2). There were no strong relationships between the number of insects and the activity nor hunting rate of the common pipistrelle. When including temporal variation by analysing the data of each sampling period, the foraging behaviour of all three bat species increased with the number of insects (Table S14). In particular, the hunting rates of all bats increased significantly with both the number of emerging and flying Diptera at the stream sites.

4. Discussion

4.1. Chemical stream pollution

We found a clear gradient of stream pollution across our sites in terms of pesticide, wastewater and nutrient load. Measurements of pesticides and wastewater were highly correlated with other water-quality parameters, such as decreased oxygen, increased pH, water temperature, and nutrient concentrations (Fig. 2). Therefore, polluted streams in our study tended to be more polluted overall, and not due to specific sources of pollution. In addition, pollution was not related to stream width or depth, as shown in the PCA (Fig. 2), confirming that our selection of streams across a pollution gradient was not strongly biased by stream size.

The pesticide pollution measured in our study is comparable to levels measured in similar German streams. The average and maximum number of pesticides detected per sample as well as the toxicity range in our streams are similar to those measured in the "Kleingewässermonitoring" (KGM), a Germany-wide stream monitoring programme conducted in 2018–2019 (Liess et al., 2021; Weisner et al., 2021). Moreover, the pesticide sum toxicity and number of detected pesticides we measured is similar to that measured in streams in the same area in 2019 (Schneeweiss et al., 2022). Schneeweiss et al. (2022) compared pesticide toxicity and its effects between unpolluted upstream sections in the Palatinate forest and polluted stream sections adjacent to agricultural areas. Our stream sites were all in forested areas, with 12 sites located in protected areas and three sites in nature conservation areas (Table S1). Although none of our sites were adjacent to agricultural or urban areas, almost all had a pesticide profile similar to that measured in agricultural stream sections by Schneeweiss et al. (2022). The fact that we measured similar pollution levels at stream sites downstream from pollutant sources demonstrates the potential for streams to import pollutants into otherwise unexposed natural areas (Wolfram et al., 2023), which are hotspots for bat activity and foraging (Fukui et al., 2006; Stahlschmidt et al., 2012).

While our focus was on using the pesticide sum toxicity as a general

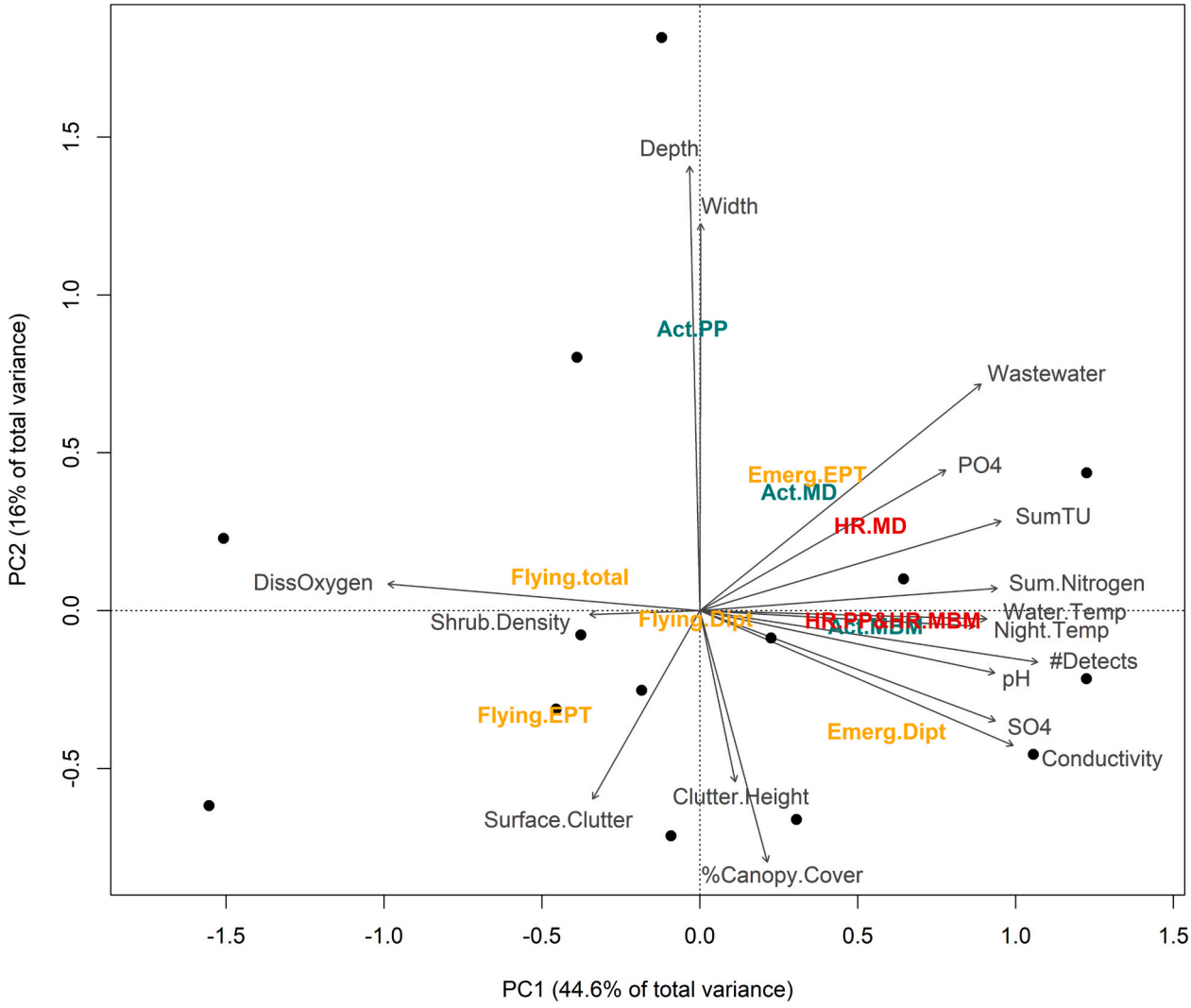

Fig. 2. Principal component analysis biplot showing the main environmental gradients among different stream sites (black points) explained by measured environmental variables (grey labelled arrows). Bat activity (blue) and hunting rate (red) of three bat species (MBM: Brandt's bat *Myotis* cf. *brandtii*, MD: Daubenton's bat *M. daubentonii*, PP: common pipistrelle *Pipistrellus pipistrellus*), as well as the number of flying and emergent aquatic insects (yellow; Dipt: Diptera, EPT: Ephemeroptera, Plecoptera and Trichoptera, Total: all flying insects) are included as passive variables in the biplot. Abbreviations: Clutter.Height is the average height of vegetation clutter on the stream surface, %Canopy.cover is the percentage of tree canopy cover, Conductivity is the average water conductivity, SO4 is the average concentration of dissolved sulphate, #Detects is the average number of pesticides detected, pH is the average pH of the stream water, Sum.Nitrogen is the average concentration of dissolved nitrate, nitrite and ammonium combined, Night.Temp is the average air temperature on nights when bat calls were recorded, Water.Temp is the average water temperature, SumTU is the average sum toxicity of pesticides for freshwater invertebrates, Wastewater is the total number of wastewater indicators detected in the streams, PO4 is the average concentration of dissolved phosphate, Width is the stream width, Depth is the water depth, Shrub.Density is the density score of shrubs along the stream shore, DissOxygen is the average concentration of dissolved oxygen, Surface.Clutter is the average score of water surface coverage by vegetation clutter. Note that the hunting rates of *M.* cf. *brandtii* and *P. pipistrellus*, "HR.PP&HR.MBM", overlaps the activity of *M.* cf. *brandtii*, "Act.MBM" in the centre of the right quadrants.

indication of the level of pesticide pollution in our streams, there was one substance of concern. Fipronil, an insecticide which is still used as a veterinary drug against ectoparasites (CVMP, 2023) and for indoor pest control (EC, 2011), was banned for agricultural use in the European Union in 2017 (EC, 2016). However, it was the most detected pesticide in our study. Fipronil also drove the sumTU in most streams due to its high toxicity for freshwater invertebrates (Miller et al., 2020; Weston and Lydy, 2014). The presence of fipronil in surface waters is a widespread issue and has been attributed to use on household pets and entry via wastewater effluent (CVMP, 2023; Bradley et al., 2017; Miller et al., 2020; Teerlink et al., 2017). This may also explain its frequent occurrence in our study. Due to its high potential ecological risk, the presence and implications of fipronil in streams merit further investigation.

4.2. *Response of emerging and flying insects to pollution*

While we predicted that fewer aquatic insects would emerge from streams with higher pesticide toxicity, we did not observe negative responses of insect emergence to stream pollution. We also did not find any drivers clearly explaining the differences in the numbers of emerging nor flying aquatic insects at different stream sites, other than a tendency for more insects emerging at more polluted sites (Fig. 2). Previous studies have documented a reduction in insect emergence due to pesticide toxicity and wastewater effluent in streams (Kalcounis-Rueppell et al., 2007; Kraus et al., 2021; Marshall et al., 2022; Miller et al., 2020). The average sum toxicity measured in most of our streams was relatively high and had the potential to negatively affect sensitive

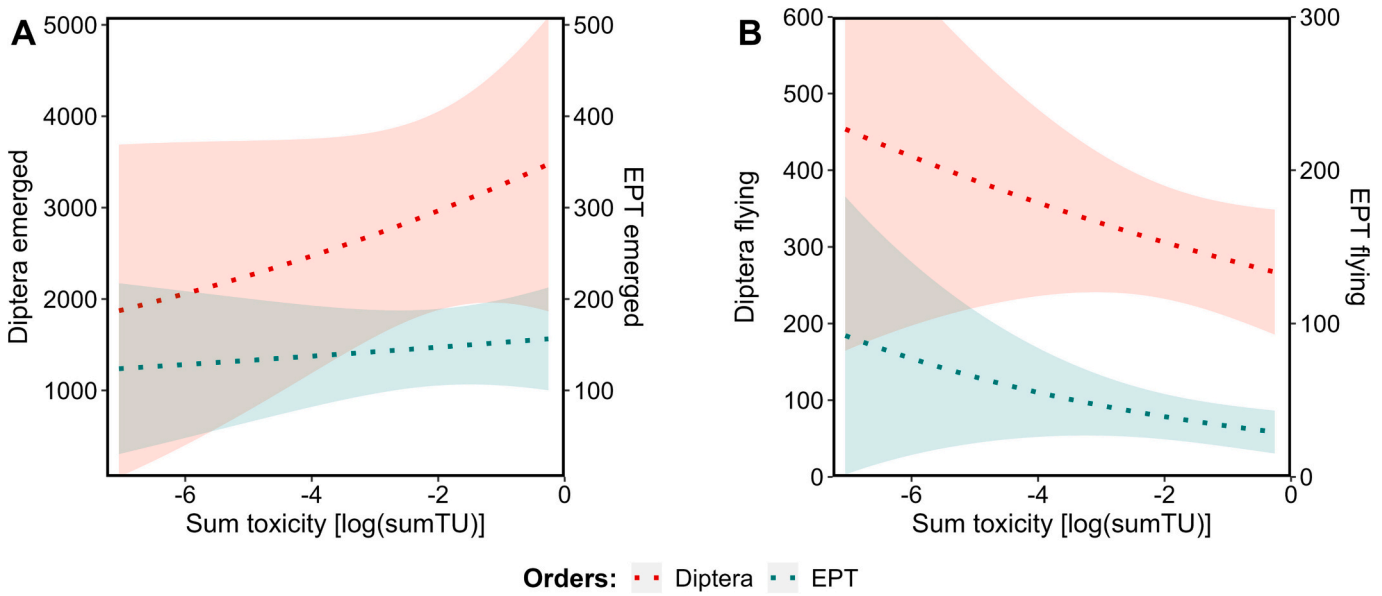

Fig. 3. Relationships between the number of A) emergent and B) flying insects captured at streams with varying degrees of pesticide toxicity, measured in logarithmic sum toxic units (sumTU). The order group EPT is the combination of Ephemeroptera, Plecoptera, and Trichoptera individuals, and is on a separate axis from Diptera. No relationship is statistically significant. The relationships were calculated using generalized linear models based on measurements from 14 stream sites, and the 95 % confidence intervals are shown by the shaded areas surrounding the model lines.

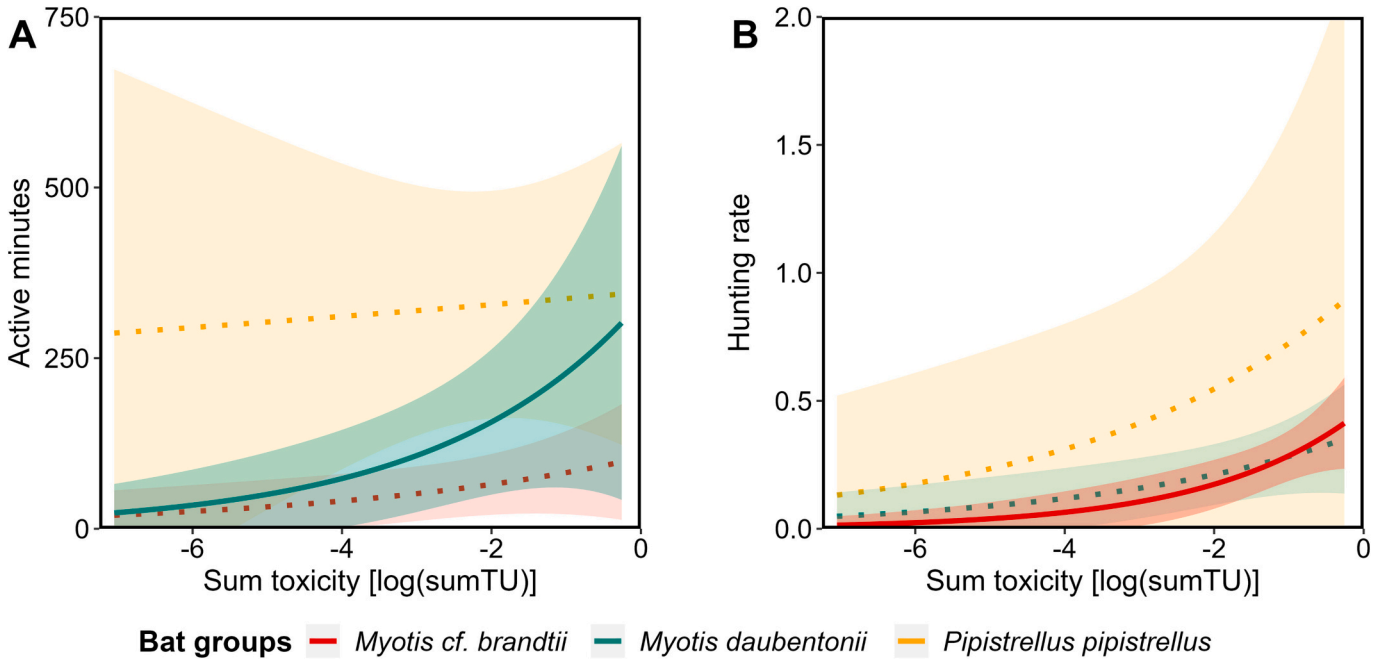

Fig. 4. Relationships between the (A) activity and (B) hunting rates of three bat species and the toxicity of pesticide mixtures measured in forested streams. Bat activity was measured as the number of active minutes, i.e., the number of minutes that a species was recorded calling. The hunting rate is the number of "feeding buzz" hunting calls per active minute. The stream toxicity was calculated as the log sum toxic unit (sumTU) obtained from the measurement of 77 pesticides in the stream water. The relationships were calculated using generalized linear models based on measurements from 14 stream sites. Significant relationships ($p < 0.05$) are shown by solid lines and the 95 % confidence intervals are shown in the shaded areas surrounding the model lines.

stream insects (Liess and Von der Ohe, 2005; Liess et al., 2021; Miller et al., 2020).

Rather than a reduction of all insect emergence, the communities at our stream sites may have shifted to more tolerant species at polluted sites and more sensitive species at less-polluted sites, as has been observed in other studies (Burdon et al., 2016; Ohler et al., 2023; Schneeweiss et al., 2022). Liess et al. (2021) calculated a maximum sumTU of −3.27 as a threshold for maintaining a good in-stream ecological quality for invertebrates at 95 % of streams based on their field study. The average sumTU for all but two of our sites and maximum sumTU for all sites were above −3 (Table S11) and, by this definition, not protective for sensitive species when considering that our streams are similar to those of Liess et al. (2021). Furthermore, the lack of competition from more sensitive species in situations of constant pesticide exposure could lead to higher success of tolerant species (Liess et al., 2013). For example, Ohler et al. (2023) recorded higher biomass

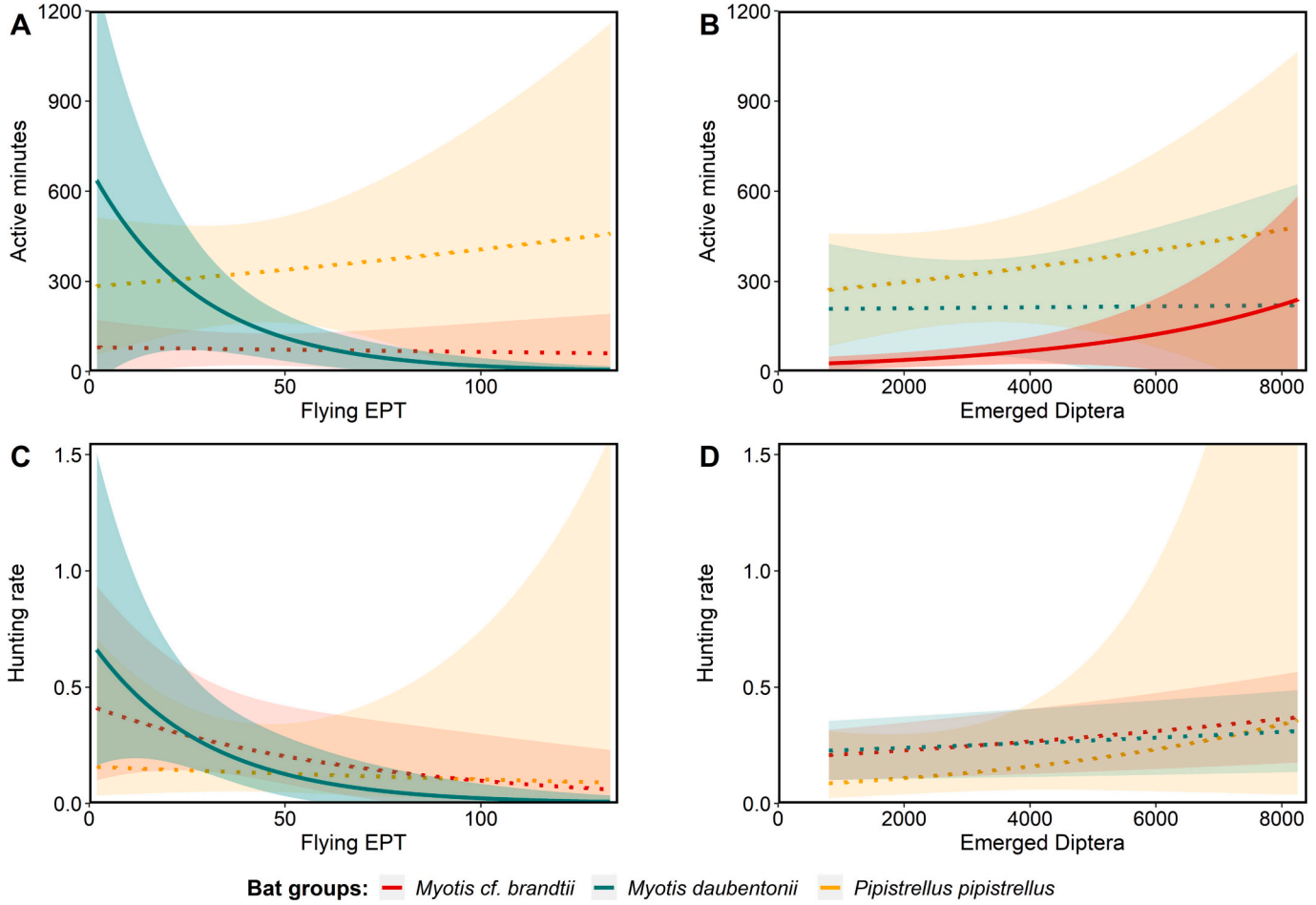

Fig. 5. Relationships between the activity and hunting rates of three bat species and A/C) the number of Diptera emerging from forested streams, and B/D) the number of Ephemeroptera, Plecoptera, and Trichoptera (EPT) flying at the stream banks. A&B show bat activity, in terms of active minutes, while C&D show bat hunting rates. The hunting rate is the number of "feeding buzz" hunting calls recorded per active minute. Significant relationships ($p < 0.05$) are presented as solid lines. The relationships were calculated using generalized linear models based on measurements from 14 stream sites and the 95 % confidence intervals are shown in the shaded areas surrounding the model lines.

and abundance of tolerant emergent insects, as well as a temporal shift in emergence from agricultural streams compared to forested streams, which may also have occurred at more polluted sites in our study. We found that pesticide toxicity and wastewater presence were highly correlated with nutrient load and higher temperatures (Fig. 2, Table 1), and tolerant taxa could have taken advantage of higher productivity in polluted streams (Abbott et al., 2009; Raitif et al., 2018). Although we cannot confirm a shift to more tolerant taxa in the prey community at our streams, studies sampling the benthic invertebrate community or including finer taxonomic scales using methods such as DNA metabarcoding of insect samples or the bat diet would be capable of revealing such effects of stream pollution.

4.3. Bat activity and hunting rate

Bat activity and hunting rates were either unrelated to pollution or higher at more polluted sites (Fig. 4). The activity of Daubenton's bat, as well as the hunting rate of Brandt's bat were higher at sites with more pesticide pollution (Fig. 2). However, we did not observe strong relationships of bat foraging behaviour and the number of aquatic insects, although bats are known to track insect emergence at streams (Fukui et al., 2006). Only the activity of Brandt's bat showed a positive relationship with the abundance of emergent Diptera in the overall between-stream comparison (Table S10). However, all bats tracked the number of Diptera in the temporally-resolved dataset (Table S14). This is expected, as Diptera make up the majority of their diet (Galan et al., 2018; Vesterinen et al., 2018), though the small size of the numerically-dominant Chironomidae could mean that they are less valuable in terms of nutritional quality than the larger EPT.

While we did not see clear relationships between bats and the number of insects along the pollution gradient, results of previous studies may help to explain the higher *Myotis* spp. activity and hunting rates at polluted sites. For example, studies investigating bat activity upstream and downstream from wastewater treatment plants found that, in some cases, bats were more active downstream, which was explained by a higher insect emergence due to a suspected increase in dissolved nutrients (Abbott et al., 2009; Vaughan et al., 1996). Likewise, positive effects of increased nutrients and temperature on emergent insects in our study could have negated toxic effects of pollution. Higher prey abundances may have encouraged bats to spend more time foraging at these sites. We saw that the sites with more emergent insects and bat foraging behaviour also tended to be more polluted, warmer, and have higher concentrations of dissolved nutrients (Fig. 2). Additional factors such as proximity to roosts may also have contributed to the numbers of bats spending time at certain streams, but we were not able to control for this in our study. Furthermore, although there was no significant spatial autocorrelation in our study (Table S10), we cannot exclude some influence of spatial patterns inevitably present in our study area on the

stream habitats at the sites. Future experimental field studies are needed to clarify the effect of different drivers leading to increased bat activity at polluted sites.

While we expected riparian bats to respond positively to the number of aquatic insects present at the streams, we observed a negative relationship between the activity and hunting rate of Daubenton's bat and flying EPT abundance (Fig. 5). As bats are attracted by high prey densities, there must be other reasons for the negative correlations between Daubenton's bat and flying EPT. One explanation could be opposing habitat preferences. Daubenton's bats prefer to hunt over calm and open water surfaces (Boonman et al., 1998; Todd and Williamson, 2019; Warren et al., 2000), while most EPT prefer fast-flowing streams (Beermann et al., 2018). Some EPT such as Plecoptera, which were more frequently sampled by the malaise traps, are highly sensitive to stream pollution and poor water quality (Chang et al., 2014) and may have been less common at polluted sites preferred by bats, though we cannot confirm this with our results. Alternatively, negative correlations between prey and predator abundances could indicate top-down regulation (Polis et al., 1997). Top-down regulation of insect densities by bats has been suggested for agricultural systems (Tuneu-Corral et al., 2023) and documented in urban parks (Villarroya-Villalba et al., 2021), and a forest experiment (Beilke and O'Keefe, 2023). Thirdly, bats may need to exert less hunting effort in areas with more abundant prey due to more rapid satiation. However, the negative correlation between lower hunting rates at sites with high EPT abundance suggests that this was not the case. Exploring potential explanations for the negative relationship between Daubenton's bat and flying EPT abundance would require further study, possibly including dietary analysis, and is out of the scope of the current investigation.

In accordance with our predictions, the relationships that we observed between bats and stream-specific variables (insects and pollution) were strongest for the two *Myotis* species (Figs. 4 and 5), which are more associated with streams than the common pipistrelle. Daubenton's bat is a specialised riparian species, often hunting directly above the water surface (Kalko and Schnitzler, 1989). Brandt's bat is also associated with riparian areas, though to a lesser degree (Roswag et al., 2019). Thus, it is not surprising that they had the strongest relationships to insect emergence and pollution. On the other hand, the common pipistrelle is a generalist bat and is widely distributed in a variety of habitats, including riparian areas (Lundy and Montgomery, 2009). The common pipistrelle was the most common species in our study. It was also the only species that responded to structural characteristics around the stream sites, confirming its generalist habitat choice. The semi-open conditions for such an edge-space forager are best met by the streams with a relatively open canopy (Kusch et al., 2004), as seen in our results.

4.4. Implications of bat response to pollution

We observed higher activity and hunting rates of riparian bats at streams with more pollution and poorer water quality. Though the correlative nature of our study does not allow for the establishment of a mechanistic relationship, foraging at polluted sites may lead to detrimental effects for bats. Emergent insects are known to take up pesticides and pharmaceuticals from the water, and can transport them into the terrestrial ecosystem (Kraus et al., 2021; Previšić et al., 2021; Roodt et al., 2023b). Kraus (2019) describes the balance between pollutant toxicity and insect emergence, where fewer insects emerge due to negative effects at higher toxicity levels, but the higher emergence at lower levels can lead to a higher pollutant flux from the stream, depending on the accumulation potential of the pollutants. This may also apply to pesticides if they are retained into the adult stage of emergent insects. Although Kraus (2019) suggests that current-use pesticides are more likely to reduce emergence flux via mortality rather than to accumulate in and be transported by the insects, Roodt et al. (2022, 2023a) experimentally demonstrated that certain pesticides, including insecticides such as neonicotinoids, are retained by chironomids through metamorphosis. In addition, a study by Roodt et al. (2023b) conducted in the same area as our study confirmed that certain pesticides are transferred by emergent insects, especially dipterans, and bioaccumulate in spiders feeding on stream emergence. Combined with this knowledge, our results suggest that, at the observed concentrations of chemical pollutants in our streams, the unaffected numbers of emergent insects and higher bat hunting rates at polluted sites led to a dietary exposure of bats to chemical pollutants from streams.

Many pesticides and other contaminants have already been reported in bats, for example across Germany (Schanzer et al., 2022), but the contribution and significance of stream pollution to this is not yet known. In addition, although dietary exposure to contaminants in streams may negatively affect bats, a lack of research in this area makes specific consequences difficult to predict (Torquetti et al., 2020). Changes in the microbiome of bats after hunting near WWTPs have been reported, likely due to pharmaceuticals in the water and emergent insects (Mehl et al., 2021). This could also occur at some streams in this study, as sulfamethoxazole, an antibiotic, was measured in the water. In addition, Roodt et al. (2023b) reported the bioaccumulation and biomagnification of neonicotinoid pesticides in spiders at our streams via emergent insects, which can also apply to bats. A detailed review and risk assessment by Mineau and Callaghan (2018) suggest that exposure of bats to neonicotinoids may lead to immunological, behavioural, reproductive and mortality effects, though few studies have tested bats directly.

In terms of sublethal effects of contaminants such as pesticides, neurological effects leading to poorer hunting efficiency or migration performance, effects on metabolism reducing survivability of hibernation or reductions in reproductive success would have detrimental consequences for bat populations (Amaral et al., 2012, Eidels et al., 2016; Hsiao et al., 2016). Bats may be particularly vulnerable to effects of chemical exposure as they require high amounts of energy for flight and hibernation, have a long lifespan and produce few offspring (Jones et al., 2009). Thus, it remains imperative to evaluate the risks of chemical pollutant exposure to bats, including the role played by streams.

Bats face a plethora of threats globally (Browning et al., 2021). Both indirect effects of pollution through prey loss and direct effects from contaminant uptake put bats at risk. We have only included chemical stream pollution in this study, which is a globally relevant stressor (Stehle and Schulz, 2015), but it is also important to consider interactions with other stressors affecting bats such as habitat loss, climate change, and disease (Frick et al., 2020). For example, increased contaminant uptake with effects on immune functions may reduce bats' ability to cope with diseases such as white-nose syndrome (Cable et al., 2022; Korine et al., 2017), or parasites (Pilosof et al., 2014). Any effect of pollutants on bat survival or reproduction adds to that of other stressors causing high mortality in bats, and this pressure is expected to increase in the future with climate change (O'Shea et al., 2016). This can have serious implications for the recovery and conservation of vulnerable bat populations. Furthermore, the potential threat of consuming insects from polluted freshwater may be exacerbated by the insect decline recorded over the last decades (Hallmann et al., 2017). The stronger decline in terrestrial than aquatic insect species could further increase the reliance of bats on insects from freshwater ecosystems (Van Klink et al., 2020).

4.5. Conclusion

There was no net negative effect of wastewater or pesticide pollution on the abundance of emergent aquatic insects. Thus, the pollution levels measured at our stream sites did not appear to reduce prey availability for bats. The higher foraging rates of bats at polluted sites may instead have resulted in increased pesticide exposure. However, negative effects of pesticides on insect emergence and prey availability can be expected in systems with higher levels of pesticide and wastewater pollution. It is

also concerning that streams in our study transported micropollutants into protected areas. Given the sensitivity of bats to stressors in their habitats and the lack of knowledge associated with emerging contaminants and bats, chemical pollution in streams remains a topic of concern, especially in the context of multiple stressors that bats are facing globally. Thus, we encourage further ecotoxicological investigation for the conservation of these important and vulnerable mammals.

CRediT authorship contribution statement

Maike Huszarik: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Alexis P. Roodt:** Methodology, Validation, Investigation, Writing – review & editing. **Teagan Wernicke:** Methodology, Investigation, Writing – review & editing. **Fernanda Chávez:** Investigation, Writing – review & editing. **Annika Metz:** Methodology, Investigation, Writing – review & editing. **Moritz Link:** Methodology, Investigation, Writing – review & editing. **Eva Lima-Fernandes:** Methodology, Investigation, Writing – review & editing. **Ralf Schulz:** Supervision, Funding acquisition, Writing – review & editing. **Martin H. Entling:** Conceptualization, Formal analysis, Supervision, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The raw data for this study are available in the supplementary information and upon request.

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Appendix A. Supplementary data

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Chapter 5: Aquatic-terrestrial transfer of neonicotinoid insecticides in riparian food webs

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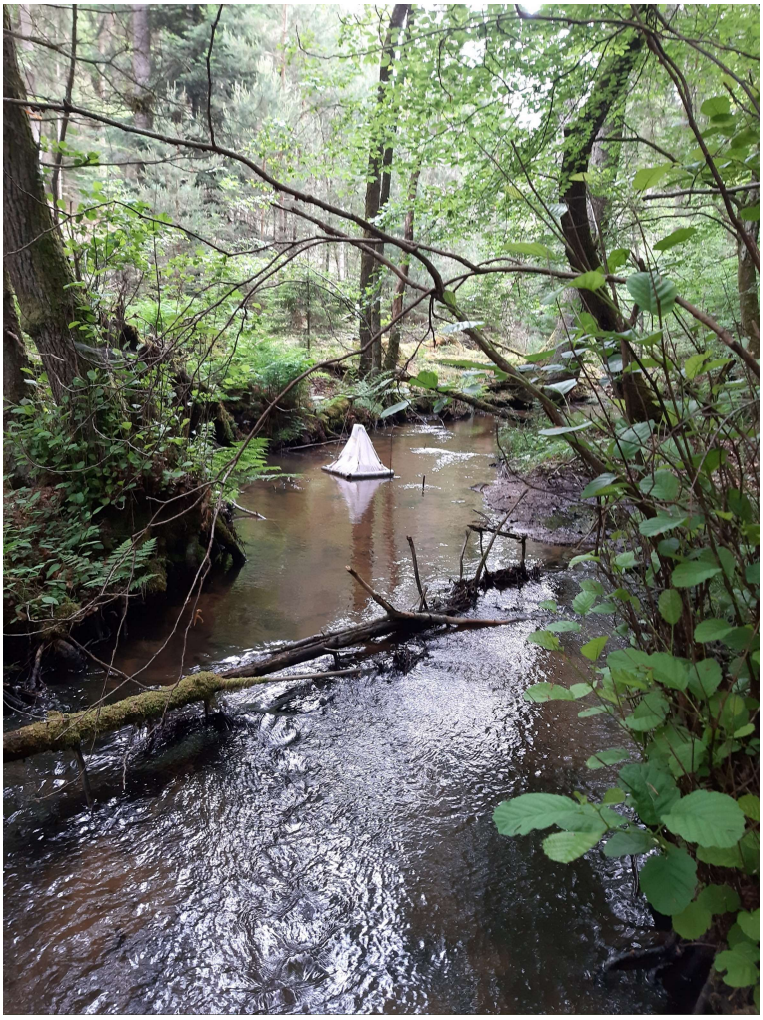

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Aquatic-terrestrial transfer of neonicotinoid insecticides in riparian food webs

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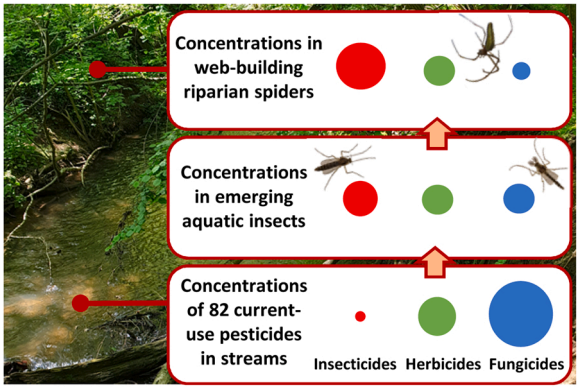
HIGHLIGHTS

- Neonicotinoids had the highest concentrations in the emerging insects and spiders.
- Concentrations of fungicides decreased between the aquatic environment and spiders.
- Riparian spiders could form a reservoir of neurotoxic insecticides in the food web.

GRAPHICAL ABSTRACT



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ABSTRACT

Current-use pesticides are ubiquitous in freshwaters globally, often at very low concentrations. Emerging aquatic insects can accumulate pesticides during their aquatic development, which can be retained through their metamorphosis into terrestrial adults. Emerging insects thus provide a potential, yet largely understudied linkage for exposure of terrestrial insectivores to waterborne pesticides. We measured 82 low to moderately lipophilic organic pesticides ($logK_{ow}$: −2.87 to 6.9) in the aquatic environment, emerging insects and web-building riparian spiders from stream sites impacted by agricultural land use. Insecticides, mainly neuro-active neonicotinoids were ubiquitous and had the highest concentrations in emerging insects and spiders (∑ insecticides: 0.1–33 and 1–240 ng/g, respectively), although their concentrations in water were low, even when compared to global levels. Furthermore, neonicotinoids, although not considered to be bioaccumulative, were biomagnified in riparian spiders. In contrast, concentrations of fungicides and most herbicides decreased from the aquatic environment to the spiders. Our results provide evidence for the transfer and accumulation of neonicotinoids across the aquatic-terrestrial ecosystem boundary. This could threaten food webs in ecologically sensitive riparian areas worldwide.

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1. Introduction

Emerging aquatic insects link aquatic to terrestrial ecosystems by transporting matter and energy, supporting diverse communities of terrestrial insectivores at the land-water interface [1]. These insects are an important source of essential fatty acids which are not readily substituted by terrestrial insect prey [2–4]. Degradation of aquatic ecosystems through the introduction of micropollutants results in declines of sensitive insect orders and may negatively affect populations of insectivores, such as insectivorous birds or riparian spiders [5–7]. Recently, there has been growing interest in investigating the transport of micropollutants from contaminated surface waters to the surrounding terrestrial habitats by emerging aquatic insects [8–10]. As a result, this route of micropollutant transfer has been shown for a wide range of chemical classes, including metals [11–13], metal-based nanoparticles [14], polychlorinated biphenyls (PCBs) [15], per- and polyfluorinated alkyl substances (PFAS) [16], halogenated organic pollutants [17], pharmaceuticals [18] and pesticides [19–21]. The retention and transport of micropollutants can thus result in the dietary exposure of terrestrial insectivores, such as spiders and birds [10,19,22–25]. Among them, web-building riparian spiders are potential sentinels of aquatic pollution due to the high proportion of emerging insects in their diets [26,27].

Despite the presence of hundreds of pesticides in global surface waters, their transport by emerging insects has only been studied for a small fraction. For example, Laboratory studies of nine fungicides and herbicides found compound-specific and sex-specific effects on fungicide and herbicide concentrations in midges over their full lifecycle [20,21]. Additionally, insecticide-specific elimination rates during development affected the concentrations of three insecticides in adult emerging insects [21]. In a field study, Kraus et al. [19] detected seven pesticides and metabolites (out of targeted analyses for 16) in two taxonomic orders of emerging insects from wetlands impacted by agriculture. This study reported insecticide concentrations up to 577 ng/g and suggested the exposure of terrestrial insectivores as a consequence. The dietary exposure of terrestrial predators to pesticides through consumption of emerging insects has, however, been limited to calculations based on published consumption rates [19,20]. Empirical knowledge combining the detection of pesticides in both emerging aquatic insects and riparian predators (e.g. web-building riparian spiders) is lacking.

Systemic pesticides, characterised by high water solubility, regularly occur in aquatic environments at low concentrations [28–32]. This includes neurotoxic insecticides, among them the highly debated neonicotinoid insecticides [7,33]. Neonicotinoids often occur as mixtures and exhibit a chronic exposure profile [30,34,35]. Their mode of action results in negative impacts on aquatic invertebrate communities [35] and terrestrial food webs [36]. Despite their bioaccumulative potential being considered as low [36], bioaccumulation of neonicotinoids has recently been reported in aquatic macroinvertebrates in field studies [37,38]. Furthermore, in a laboratory study, the neonicotinoid thiacloprid was retained by emerging midges, in contrast to two other non-neonicotinoid insecticides [21], yet it remains unclear whether this applies to other neonicotinoids.

Drivers of pesticide bioaccumulation by emerging aquatic insects are not clear. Bioaccumulation and trophic magnification potential of organic molecules are related to their chemical lipophilicity (octanol-water partition coefficient K_{ow}) and metabolisation rates for moderately to highly lipophilic chemicals ($logK_{ow} > 5$) [39]. Many currently used pesticides are, however, characterised by low to moderate lipophilicities ($logK_{ow} < 5$). Furthermore, once accumulated by emerging insects during their aquatic development, concentrations of contaminants can be modified during metamorphosis [8]. The retention of lipophilic organic molecules ($logK_{ow} > 5$) by emerging aquatic insects across metamorphosis potentially reflects biomagnification in food webs, correlating non-linearly with increasing lipophilicity [8]. On the other hand, small organic molecules with low to mid polarities ($logK_{ow} < 5$) show the reverse relationship and it is unclear whether this relationship reflects the potential for trophic transfer within food webs [8,20]. These observations have, however, been based on a limited number of chemicals and chemical classes. Evaluations for a larger number of pesticides, which fall into this lipophilicity range, and how they correlate with the prevalence and concentrations in adult emerging insects and terrestrial consumers, such as riparian spiders, are lacking.

We provide new insight into the prevalence and concentrations of a larger number of currently used pesticides in aquatic habitats, emerging insects and riparian spiders. For this, we validated an analytical methodology for the measurement of 82 currently used pesticides in small-volume insect samples (30 mg) by high-performance liquid chromatography tandem to triple quadrupole mass spectrometry by electrospray ionization (HPLC-ESI-MS/MS). We then investigated the prevalence and concentrations of these pesticides in the aquatic environmental compartments (water, sediment and aquatic leaf litter) and the terrestrial biological compartment (adult *Tetragnatha* spp. riparian spiders) collected from ten stream sites differing in the degree of agricultural impact. The sampling sites were sheltered from direct impacts by agricultural activities, such as spray drift during pesticide application. We furthermore aimed to establish the link between aquatic and terrestrial compartments by quantitative and qualitative evaluation of these same pesticides in stream water, rainwater and three orders of emerging aquatic insects (namely, Diptera, Ephemeroptera and Trichoptera), which were collected from a subset of the sampling sites. We categorised pesticides based on their detection frequencies in different compartments to elucidate the potential importance of a range of pesticide properties. Finally, we test the hypothesis that the transport of thiacloprid by emerging insects occurs at low concentrations commonly found in global surface waters, and also occurs for other neonicotinoid insecticides.

2. Materials and methods

2.1. Study sites and sampling overview

Sampling took place at stream sites in the upper Rhine valley of the Palatinate region of South-West Germany (Fig. S6), an area that includes a variety of crops typically grown in Europe. In 2020, water and emerging insect samples were collected from two streams, namely the Modenbach (MB, 49°16'50.4"N 8°16'53.0"E) and Spiegelbach (SPI, 49°11'13.6"N 8°18'44.6"E). In 2021, adult riparian spiders (*Tetragnatha* spp.), sediment samples and aquatic leaf litter were collected from these two sites along with a further eight sites, namely the Katzenbach (KB, 49°16'12.0"N 7°57'58.0"E), Eußerbach (EB, 49°14'20.1"N 7°58'34.4"E), Ranschbach (RB, 49°11'57.0"N 8°04'55.0"E), Queich Site 1 (QS1, 49°12'01.0"N 8°05'40.0"E), Queich Site 2 (QS2, 49°12'04.7"N 8°08'16.1"E), Queich Site 3 (QS3, 49°12'19.1"N 8°11'32.0"E), Queich Site 4 (QS4, 49°12'39.0"N 8°13'43.0"E) and Queich Site 5 (QS5, 49°13'19.0"N 8°16'12.2"E). Two of these sites, namely KB and EB are located within a forested region with very limited agricultural activities and were therefore considered the least impacted by pesticides. The remaining eight sampling sites lie on streams which flow from West to East through a region which is characterised by intensive agriculture (Fig. S6). The sampling sites were therefore carefully selected to be sheltered from agricultural activities by areas of dense natural vegetation to minimise the potential impacts of deposition as a result of spray drift. RB, which was separated by approximately 60 m of forest from the nearest agriculturally used land, was the site nearest to agricultural activities among all sampling sites. Potential atmospheric deposition resulting from rainfall was, however unavoidable. Therefore, rainwater samples were also collected from two sites during 2021, rainwater sampler 1 was located at a site within the forest (Eußerthal Ecosystem Research Station, 49°15'15.2"N 7°57'42.3"E) and rainwater sampler 2 was located within the agricultural landscape (QS2, 49°12'04.7"N 8°08'16.1"E) (Fig. S6).

2.2. Water and emerging aquatic insect sampling in 2020

Weekly water grab samples and emerging insect samples were collected from 28.04.20 to 14.07.20. Water grab samples were collected mid-stream in clean amber glass bottles (1 L) from the two sampling sites, MB and SB (Fig. S6). Bottles were completely submerged and capped under the water surface during sample collection. All water samples were transported directly to the laboratory where 10 mL was transferred to a clear glass storage vial capped with an aluminium foil lining. The samples were then frozen and stored at - 20 °C until pesticide analysis. Three emergence traps were installed at each site to collect emerging aquatic insects. Floating emergence traps were constructed based on previously published designs [40]. Briefly, each trap covered a surface area of 0.25 m^2. A pyramid-shaped mesh tent on top of the floating base had an opening where a polypropylene sampling bottle was attached to collect flying insects. Of the three traps at each sampling site, two had a bottle with 125 mL of trapping liquid (30% propylene glycol, 70% deionized water by volume, 1 mL/L dish soap and 10 mg/L denatonium benzoate) to capture and preserve adult emergent insects, while the third contained no fluid. Emerging insects caught in the trapping fluid were collected weekly, while those in the bottles without fluid were collected after 24–48 h. Live insect samples were frozen and stored at − 80 °C. Samples in trapping fluid were stored at 4 °C. Both live-caught and fluid-caught insects were identified to the order level [41,42]. The frozen samples of the live caught insects were kept on ice during identification and sorting to prevent degradation. Order-specific biomass was estimated by measuring the body length of each insect caught with catching fluid to the nearest millimetre. Dry biomass was then calculated based on order-specific reference values using the method of Sabo et al. [43]. The frozen insect samples were then freeze dried and weighed using an MT5 analytical microbalance (d = 0.001 mg, Mettler-Toledo GmbH, Gießen, Germany) before being stored at − 80 °C. In order to obtain the required biomass for the pesticide measurements, insects were pooled by order which resulted in 15 samples with final sample weights of 26.49 ± 7.19 mg. Six replicate dipteran samples were obtained from MB, but only one from SB. One ephemeropteran sample was obtained for MB and three for SB, while both sites had sufficient trichopteran biomass to yield two samples each.

2.3. Water, sediment, aquatic leaf litter and riparian spider sampling in 2021

All ten sampling sites were sampled twice during 2021 (Fig. S6). Once in June (21.06.21 to 25.06.21) and once in July (12.07.21 to 16.07.21), which covered the summer pesticide application period. Sediment samples were collected by scooping the surface layer of the sediment using a square shovel (10 ×10 ×2 cm). Five sediment samples were randomly collected at each site and pooled to create a composite sample. Aquatic leaf litter, which serves as a habitat for many macroinvertebrates, was collected from the streambed at each site. Between 10 and 20 sexually mature *Tetragnatha* spp. spiders were collected from vegetation or from their webs directly overhanging the water surface at each site. A daily grab water sample was collected from QS2 (Fig. S6) for 47 consecutive days covering the sampling period (07.06.21 to 23.07.21). Rainwater samples were collected using in-house constructed samplers consisting of a stainless-steel funnel (diameter 30 cm) fixed to a brown glass bottle (1 L) housed in a styrofoam insulated box, which was installed approximately 1.5 m above ground level away from overhanging vegetation. Rainwater in the forest (Rainwater sampler 1) was sampled for 13 days in June (09.06.21 to 22.06.21), which coincided with the first round of field site sampling. Rainwater at QS2 (Rainwater sampler 2) was sampled during both rounds of field sampling in June (21.06.21 to 24.06.21) and July (09.07.21 to 14.07.21). Additionally, the volume of precipitation at rainwater sampler 1 was recorded by an MWS10-Weather station (Reinhardt System- und Messelectronic GmbH, Dießen am Ammersee, Germany) and at rainwater sampler 2 by udometer.

Water, sediment and leaf litter samples were all frozen after collection and stored at − 20 °C before processing for pesticide measurements. The live spiders were kept individually in plastic containers covered with a 1 mm nylon mesh at 20 °C for 72 h before being frozen and stored at − 80 °C prior to further processing and pesticide measurements. The 72-hour waiting period after collection allowed the spiders to clear their gut content while ensuring a high survival rate. Spiders that died during the 72-hour depuration period were not included in pesticide measurements. Frozen samples of each sample type were freeze dried. Aquatic leaf litter samples were then checked for macroinvertebrates, which were removed before the samples were ground and homogenised using a mortar and pestle. Both sediment and ground leaf litter samples were sieved to 1 mm. Separate subsamples of each sediment and leaf litter (n = 20 each) were weighed on a Sartorius CP225D balance (d = 0.01 mg, Sartorius Lab Instruments GmbH & Co. KG, Göttingen, Germany) for pesticide analysis. Subsamples of sediment and leaf litter had average weights (± standard deviation) of 5.04 ± 0.03 and 1.02 ± 0.01 g, respectively. Spiders were weighed on an MT5 analytical microbalance. Overall, female spiders were more abundant and had greater dry weights than male spiders across all 10 sites. In order to obtain suitable samples for pesticide measurements, individual spiders were pooled by sex and sampling site. This resulted in a total of 45 samples of female spiders, from all ten sites, each containing three to nine individuals with an average weight of 29.80 ± 2.60 mg. Similarly, a total of 34 samples of male spiders containing one to four individuals with an average weight of 6.39 ± 1.61 mg were prepared. Overall, six to eleven spider samples were analysed for pesticides per sampling site.

2.4. Pesticide concentration measurements

Pesticides were measured in all samples by HPLC-ESI-MS/MS. Analytical standards were obtained from Restek (Bad Homburg, Germany). Solvents (LC-MS Grade) were purchased from Honeywell (Seelze, Germany). Instrument parameters used for the measurements are provided in the supplementary information (Table S5).

2.4.1. Pesticides measurements in sediment and aquatic leaf litter samples

The methods used for the extraction and analyses of sediment and aquatic leaf litter have been reported elsewhere [44]. Briefly, samples of sediment and leaf litter were spiked with 50 μL of deuterated internal standards (pirimicarb-D6, thiacloprid-D4 and thiamethoxam-D3 in acetone) to achieve a final concentration of 2 μg/kg in the measured extract. The samples were air dried for 30 min before either 5 or 1 g of ammonium formate was added to the extraction tube for sediment and leaf litter samples, respectively. Subsequently, 10 mL of acetonitrile containing 2.5% formic acid was added and the samples were shaken for 60 min in an overhead shaker. Samples were then centrifuged for 6 min at 3000 rpm. The supernatants from sediment samples were then filtered through a 0.2 μm PTFE filter prior to pesticide measurements. The supernatants from leaf litter samples were first transferred to a vial containing graphitised carbon black (GCB) powder 7.5 mg/mL and vortexed for 30 s before centrifugation and filtering prior to pesticide measurements.

2.4.2. Pesticides measurements in water samples

A direct-injection HPLC-ESI-MS/MS method was validated and used for measurements of pesticides in water samples. Details of the validation method and pesticide LOQs are provided in the supplementary materials. Frozen water samples were defrosted and centrifuged at 16000 rpm at 20 °C for 10 min, after which 350 μL was transferred to an amber-glass vial. Each sample was diluted with 150 μL of methanol containing a mixture of deuterated internal standards (pirimicarb-D6, thiacloprid-D4 and thiamethoxam-D3, 3 μg/L) and 0.3% formic acid. Water samples were always measured directly after being prepared. A calibration series with 11 concentrations covering the concentration

range from 0.3 to 2000 ng/L was prepared using MS-grade water, in addition to solvent blanks.

2.4.3. Pesticides measurements in insects and spider samples

The extraction method was previously optimised in our laboratory (results not shown here). The method was validated using criteria published by the International Council for Harmonisation, guideline Validation of Analytical Procedures: Text and MethodologyQ2(R1) [45]. Details of the validation method and pesticide LOQs are provided in the supplementary materials.

Samples of freeze-dried insects and spiders were pulverised using a Tissuelyzer (Retsch MM 301, Haan, Germany) and 2.5 mm diameter steel pellets. Samples of dry insect material were weighed into 2 mL polypropylene tubes using an MT5 analytical balance (d = 0.001 mg). Internal standards (pirimicarb-D6, indoxacarb-D3 and thiacloprid-D4) were added to each sample for a final extract concentration of 0.48 ng/mL. Extractions were performed with 1 mL of acetonitrile containing 0.1% formic acid. Samples were vortexed for 30 s, after which they were sonicated for 5 min and centrifuged for a further 5 min at 16000 rpm. Subsequently, a dispersive solid phase extraction clean-up was performed by pipetting 850 μL of the extract to a new sample tube containing 24 mg of Z-Sep+ and primary-secondary amine (PSA). The mixing, sonication and centrifugation steps were repeated as before. After centrifugation, 700 μL of the extract was pipetted into a glass vial which was placed under a gentle stream of nitrogen gas until all the solvent had evaporated. The residues were then dissolved in 500 μL of a mixture of water and methanol (70:30, v/v) containing 0.1% formic acid and 0.5 ng/mL thiamethoxam-D3 as an internal standard. Matrix and sample-weight matched calibration series were prepared with ten concentrations ranging between 0.01 and 16 ng/g dw, in addition to matrix blanks.

2.5. Data evaluation and statistics

Order-specific biomass of emerging insects was calculated by multiplying the number of individuals in each sample by the estimated dry biomass calculated from their length measurements (Table S1). The site-specific average weekly pesticide flux was then estimated by multiplying the average weekly emergence flux by the average total pesticide concentration (Table S2). Biota-water accumulation factors (BWAFs) were calculated for each measured pesticide concentration in individual insect samples by dividing the concentration by the respective site-specific median concentration in the weekly water samples. Differences in BWAFs between orders of emerging insects (Fig S4), or concentrations of pesticides in spiders and emerging insects were tested for significance using a Kruskal-Wallis rank sum test with post hoc Dunn's test using Bonferroni correction when differences were detected ($p < 0.05$). A principle component analysis was performed on pesticides that had been categorised according to their frequency of detection in abiotic (sediment, aquatic leaf-litter and water) and biotic compartments (Emerging insects and spiders). Pesticides were categorised as either "transferred" or "not-transferred". The categorisation was performed using frequency data for sediment, leaf litter and spider samples from the sites QS1 to QS5, MB and SB because these sites were similarly contaminated by agriculture (as opposed to the less impacted upstream sites KB and EB). Additionally, representative frequencies of pesticide detection in water were available for these sites. Pesticides that were frequently detected in the abiotic compartments (>70% detection frequency in at least one compartment), but had no detections in spider samples, or emerging insects in the case of MB and SB, were categorised as "not transferred". Similarly, pesticides that satisfied these criteria, but were consistently detected in spider samples (and emerging insects at MB and SB) were categorised as "transferred". This process yielded eleven pesticides which were categorised as "transferred" and seven as "not-transferred" (Data S7). A principle component analysis was performed on values representing physicochemical properties, toxicity and environmental persistence of these pesticides (Table S3). These parameters were chosen because they have the potential to impact the transport and bioaccumulation of pesticides across the aquatic-terrestrial food web. The parameters included the logarithmically transformed values for the Henry's law constant (HLC), aqueous solubility (S), topological polar surface area (TPSA), monoisotopic mass (MIM) octanol-water partition coefficient (K_{ow}), first dissociation constant (pK_{a1}), water-phase half-life (DT50) and the chronic 28-day no observed effects concentration for *Chironomus riparius* (MidgeNOEC28). For neutral pesticides, the pK_{a1} was assigned the value 14. Additionally, pesticides for which no appropriate NOEC was available, the proxy value of 100 mg/L was used. Statistical analyses were performed in R version 4.2.2. [46].

3. Results

3.1. Prevalence and relative concentrations of pesticides in riparian spiders and the adjacent aquatic environment

Twenty-nine pesticides were detected in spider samples across all ten sampling sites (Data S1). This included thirteen fungicides and five herbicides at relatively low concentrations, with sum average concentrations (SACs): < 6.2 and < 1.6 ng/g, respectively (Fig. 1 A and B). Eleven insecticides were measured at higher concentrations, with SACs: 2.1–94.2 ng/g (Fig. 1 C). In contrast, aquatic leaf litter and sediment samples frequently contained many more fungicides (20–23 at the eight most impacted sites, with median detection frequencies of 88% and 38% in leaf litter and sediment, respectively (Data S2). This included all the fungicides detected in the spiders. The fungicide concentrations were also the highest of the three pesticide classes (SACs: 60.8–340.4 ng/g in leaf litter and 2.1–85.5 ng/g in sediments, Fig. 1. A). Up to six herbicides were detected in leaf litter and sediment with median detection frequencies of 70% and 25%, respectively (Data S2). The herbicides were present at lower concentrations (SACs: 1.9 – 18.3 ng/g in leaf litter and < 0.1 ng/g in sediments, Fig. 1. B), two of which were also present in spider samples (Data S1). Eight insecticides were detected in sediment and leaf litter with the lowest frequency of all classes (median detection frequencies of 25% and 13% in leaf litter and sediment, respectively, Data S2). They also had the lowest concentrations of any class (Fig. 1. C, SACs: < 12.7 and < 0.6 ng/g in leaf litter and sediments, respectively). Five of the insecticides detected in the leaf litter and sediment were also detected in spider samples (Data S1).

Insecticides were the largest and most frequently detected group of pesticides in spider samples from individual sites (Fig. 2, Data S1). Spider samples collected from the two least impacted sites, KB and EB, contained up to five insecticides, while those collected from the remaining eight most impacted sites contained up to ten (Data S1). The insecticides included four neonicotinoids, namely acetamiprid, clothianidin, imidacloprid and thiacloprid; fipronil and its sulfone metabolite; two ryanoid insecticides, namely, chlorantraniliprole and cyantraniliprole; the butanolide insecticide, flupyradifurone; tebufenozide and spinosad. The four neonicotinoids were among the most frequently detected insecticides and were present in 16 – 100% of spider samples across all ten sites (Data S1). These four neonicotinoids also had the highest site-specific average concentrations (up to 46 ng/g) compared to other insecticide classes (up to 14.9 ng/g, Fig. S1). Moreover, the overall median concentrations of acetamiprid, clothianidin, imidacloprid and thiacloprid (3.2, 0.9, 14.4 and 12.6 ng/g, respectively) were all higher than the median concentration of other (non-neonicotinoid) insecticides (0.5 ng/g, Fig. 2 A). Concentrations of individual fungicides and herbicides were also comparatively low compared to the neonicotinoids (site-specific average concentrations < 7 ng/g dw, Fig. S2) and overall median concentrations of 0.4 and 0.5 ng/g, respectively (Fig. 2 A).

Water samples taken at three out of the ten sites accounted for pesticides that were present in spiders but not present in leaf litter or sediment (Data S1–3). Complex mixtures of between 15 and 32, 9 – 35

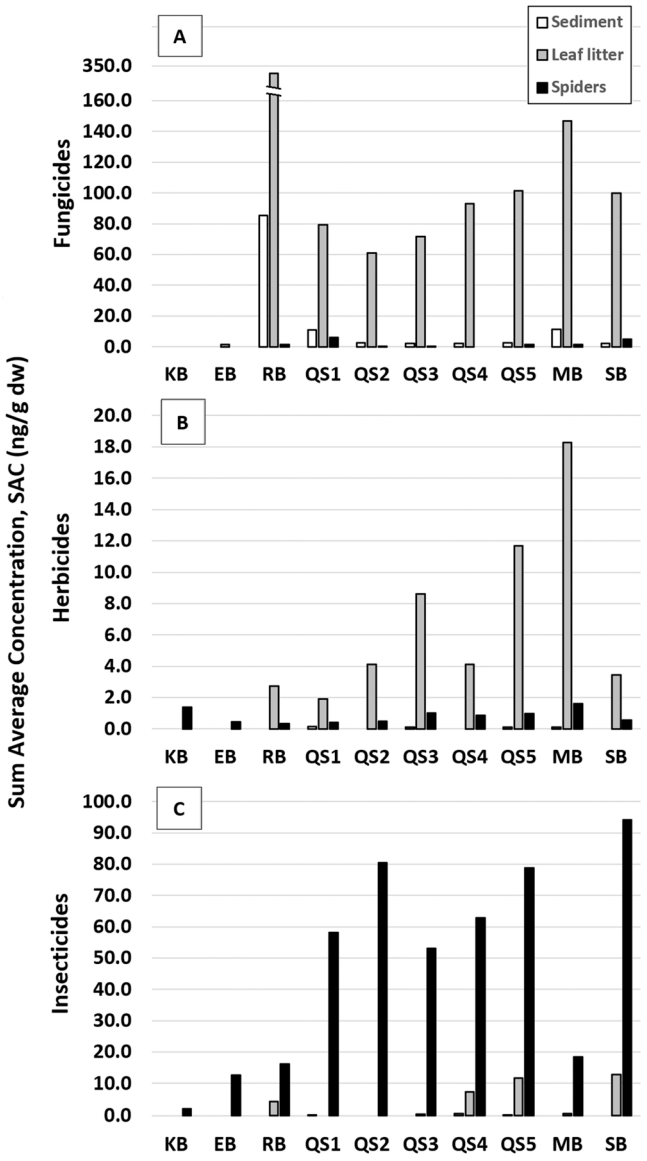

Fig. 1. Sum average concentrations (SACs) of fungicides (A), herbicides (B) and insecticides (C) in sediment (white bars), leaf litter (grey bars) and spiders (black bars) from ten sampling sites with differing degree of agricultural impact.

and 26 – 36 pesticides were detected in individual water samples collected from QS2, MB and SB, respectively (Data S3). Up to twenty fungicides were chronically present at all three sites (median detection frequencies: 73%, 77% and 65% at QS2, MB and SB, respectively) with the highest median concentration (0.01 ng/mL, Fig. 2B.) of the three pesticides classes. Individual sampling sites had moderate to high concentrations (∑median concentrations: 0.9, 0.1 and 0.2 ng/mL at QS2, MB and SB, respectively, Data S3). Up to eleven herbicides were frequently detected with low to high concentrations (median detection frequencies: 70%, 27% and 92% and ∑median concentrations: 0.04, 0.8 and 0.1 ng/L at QS2, MB and SB, respectively, Data S3) and an overall median concentration of 0.004 ng/mL (Fig. 2 B). Up to nine insecticides had the lowest overall median concentration of 0.003 ng/mL and were measured with moderate to high frequency and low to moderate concentrations at individual sites (median detection frequencies: 40%, 45% and 85% and ∑median concentrations: 0.05, 0.04 and 0.06 ng/mL at QS2, MB and SB, respectively, Data S3).

Mixtures of up to five neonicotinoid insecticides were chronically present in water at low concentrations (Data S3). Thiacloprid was detected in 100% of samples collected from all three sites. Moreover, acetamiprid and imidacloprid were detected in 100% and thiamethoxam was detected in 85% and 94% of samples from SB and QS2, respectively (Data S3). Clothianidin was only detected in five water samples overall but is the product of thiamethoxam metabolism in organisms [47]. This is consistent with the observation that only clothianidin was measured in the spiders and not thiamethoxam. The water concentrations of these two neonicotinoids were thus plotted together in Fig. 2 B. Overall, mixtures of neonicotinoids accounted for between 13% and 100% of total insecticide concentrations in each sample across all three sites. Furthermore, both the 50th and 90th percentiles of the individual neonicotinoid concentrations measured in the present study were considerably lower than what has been reported recently for global surface water concentrations (Table 1)).

Rainwater deposited small amounts of between 28 and 41 pesticides (Data S4). This included fourteen of the pesticides detected in the spiders collected from the least impacted sampling sites, KB and EB (Data S1 and S4). However, amounts of pesticides deposited via rainfall were extremely small during the sampling period (0.002–0.6, 8×10^{-5} to 0.3 and 8×10^{-5} to 0.04 ng/cm^2 for individual fungicides, herbicides and insecticides, respectively) and therefore do not provide a likely explanation for high concentrations of especially neonicotinoids in spider samples (Data S4).

3.2. *Aquatic-terrestrial pathway of pesticides via emerging insects to spiders*

Eleven fungicides, eight herbicides and eight insecticides were detected in at least one emerging insect sample (Fig. 3; Fig. S3; Data S5). Individual concentrations of fungicides, herbicides and insecticides ranged from 0.02 to 3.7, 0.05–1.9 and 0.02–23.2 ng/g, respectively (Fig. S3). The majority, 83%, of concentrations were below 1 ng/g, but two neonicotinoids, thiacloprid and imidacloprid, had consistently higher concentrations, which were up to 23.2 and 6.7 ng/g, respectively (Fig. S3). The neonicotinoids, acetamiprid and thiacloprid were the most frequently detected pesticides, found in 90–100% of all samples from each site. Of the 19 fungicides and herbicides detected in the emerging insects, 11–16 were detected in the aquatic leaf litter and sediment samples (Data S5). However, only two of the insecticides detected in the emerging insects were also detected in the aquatic leaf litter and sediment; water samples contained the pesticides detected in the insects but not in sediment or leaf litter (Data S5). Biota-water accumulation factors (BWAF) for the emerging insects covered a range of approximately 1.8 – 12300, with 84% of the values lying between 10 and 1000 (Fig. 3). The neonicotinoid, thiacloprid, had the highest BWAFs (up to 12300, Fig. 3).

Emerging dipterans made the greatest contribution to the flux of insect biomass (70–90%) and pesticides (94–96%) at both sampling sites (Table S1). Dipterans also had significantly higher BWAFs for two insecticides, namely thiacloprid and dimethoate, compared to the other insect orders (Fig. S4). Average total pesticide concentrations were the lowest in trichopterans at both sites compared to the other two orders (Table S2). Thus, despite contributing approximately 10–25% to the emerging biomass (Table S1), they contributed similarly to the weekly pesticide flux as the ephemeropterans, which contributed only approximately 1–5% of the biomass. The concentrations and prevalence of fungicides and most herbicides were greater in emerging insects than in spiders, whereas the opposite was true for insecticides (Data S5).

Biomagnification in spiders was observed for three neonicotinoids and one herbicide (Fig. 4). Significantly higher concentrations (factor of 6–15) of the neonicotinoids acetamiprid, imidacloprid and thiacloprid, were observed in female spiders compared to the emerging insect samples collected from SB. Male spiders from this site had concentrations that were a factor of 3–5 times higher than found in the emerging insects, although not statistically significant. Acetamiprid concentrations were significantly higher (factor 15–32) in both spider sexes at MB, as well as for the herbicide propyzamide in male spiders (by factor 7).

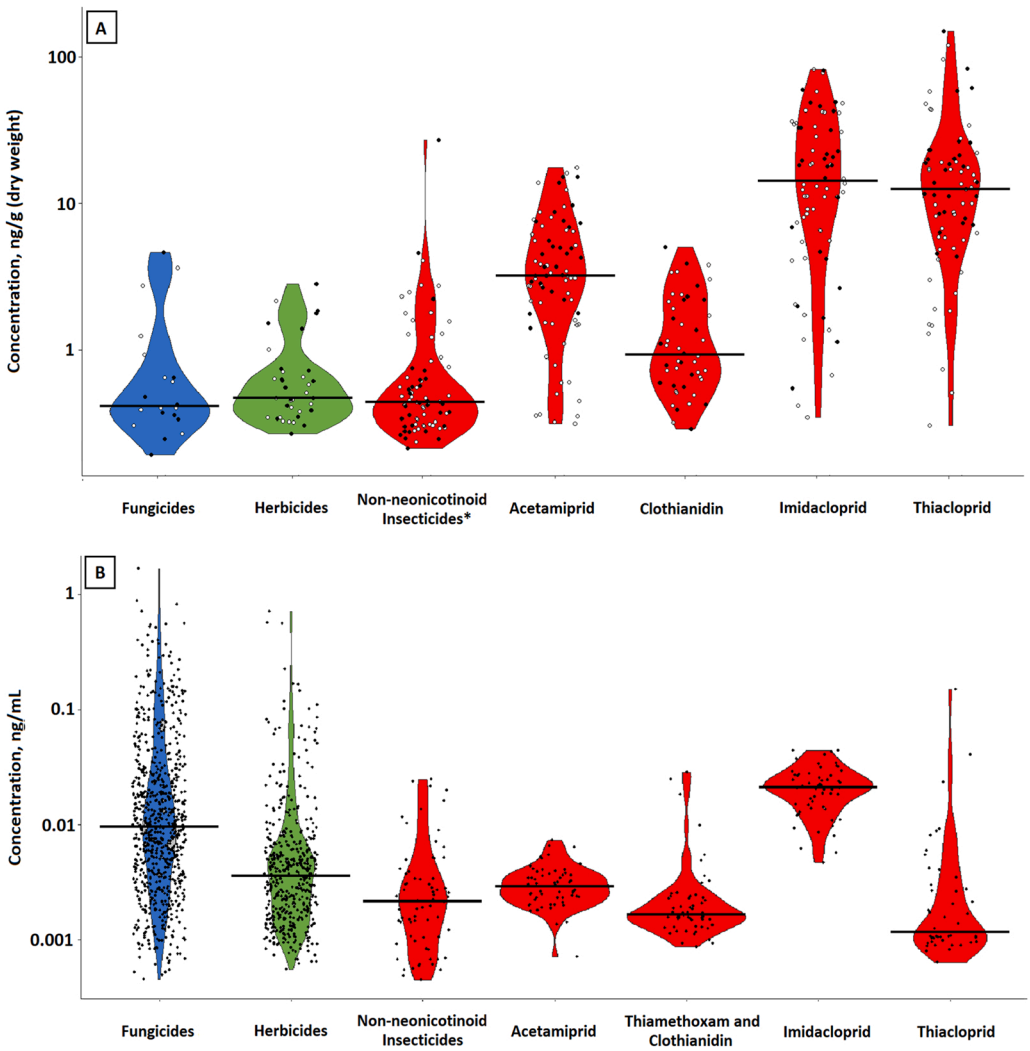

Fig. 2. Pesticide concentrations in spider and water samples from stream sites. (A) Pesticide concentrations are reported for individual spider samples (n = 79 samples) collected from ten stream sites. Points indicate individual detections above the analytical limits of quantification for fungicides (n = 20 detections), herbicides (n = 38 detections), other (non-neonicotinoid) insecticides (n = 77 detections) and the neonicotinoid insecticides; acetamiprid (n = 77 detections), clothianidin (n = 49 detections), imidacloprid (n = 74 detections) and thiacloprid (n = 76 detections). Spider samples collected from the three sites where water samples were also collected are indicated with solid black points. (B) Pesticide concentrations in daily water samples from QS2 and weekly water samples from MB and SB (n = 71 pooled from all three sites). Horizontal lines indicate the median concentrations. Violin plot colours indicate the pesticide class (blue – fungicides, green – herbicides and red – insecticides). *Non-neonicotinoid insecticides in spider samples includes concentrations for fipronil's sulfone metabolite.

Table 1
Concentrations of neonicotinoids in water samples compared to global values. Global values are calculated from data in a recent meta-analysis by Stehle et al. [30].

Neonicotinoid insecticide	50th percentile concentration (ng/L) in field samples	Equivalent global concentration percentile	90th percentile concentration (ng/L) in field samples	Equivalent global concentration percentile	Number of field measurements	Number of reference measurements
Acetamiprid	2.2	22.5	3.6	30.7	61	272
Clothianidin	5.2	29.8	10.0	44.3	6	951
Imidacloprid	15.0	47.6	27.0	60.6	62	1305
Thiacloprid	0.9	10.0	8.4	66.5	47	246
Thiamethoxam	1.2	8.8	2.3	19.2	55	785

Principle component analysis of eight parameters associated with physicochemical properties, toxicity and stability of 18 pesticides (Table S3), which were grouped according to their frequency of detection in abiotic and biotic compartments (Data S7) did not yield a separation of groups (Fig. S5, Table S4).

4. Discussion

4.1. Pesticides in riparian spiders and the adjacent aquatic environment

Sum average concentrations (SACs) of insecticides in spiders were four orders of magnitude greater than fungicides and herbicides (Fig. 1). Furthermore, the insecticide SACs were composed of 78–100% by four neonicotinoids, out of a total of eleven insecticides detected in the spiders (Fig. 2 A, Fig. S1, Data S1), revealing a selective bioaccumulation. Insecticide SACs in the present study (Fig. 1 C) were at least a factor of approximately 10–20 times lower than what has been reported for sum per- and polyfluorinated alkyl substances (PFAS) and sum polychlorinated biphenyls (PCBs) in tetragnathid spiders feeding on emerging insects, but similar to concentrations of pharmaceuticals and endocrine disrupting substances [16,48,49]. As far as the authors are aware, no data on pesticide concentrations in riparian spiders exists. Due to their neurotoxic mode of action and potential for synergistic and cumulative toxicity, mixtures of neonicotinoids may have a high potential to disrupt food webs through sublethal effects even when accumulated at lower concentrations compared to more bioaccumulative

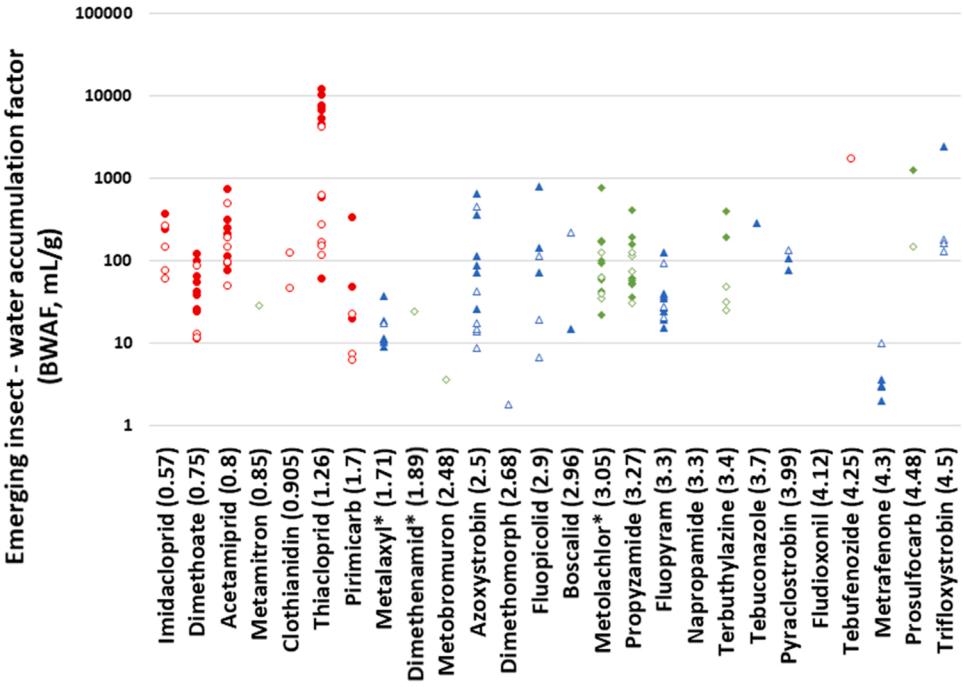

Fig. 3. Biota-water accumulation factors (BWAF) for pesticides in emerging aquatic insects. Values shown for fungicides (blue triangles), herbicides (green diamond) and insecticides (red circles) in samples of emerging insects (including Diptera, Ephemeroptera and Trichoptera) collected from two stream sites affected by agricultural land use, namely the Modenbach (MB, solid shapes) and the Spiegelbach (SB, outlined shapes). Pesticides are arranged from left to right in order of increasing lipophilicity ($logK_{ow}$ values are provided in brackets). *BWAFs are reported for the sum of isomers.

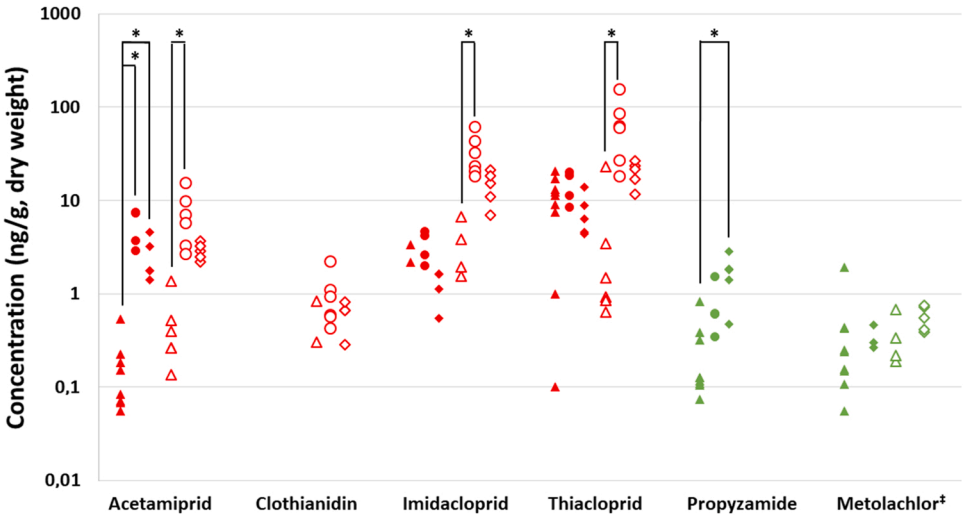

Fig. 4. Pesticide concentrations in emerging insects and riparian spiders (adult *Tetragnatha* spp.) Samples were collected from two stream sites impacted by agricultural activities (Modenbach, MB – solid black shapes and Spiegelbach, SB – outlined shapes). Pesticide concentrations in emerging aquatic insect samples, comprised of dipterans, ephemeropterans and trichopterans, are indicated by triangles, while concentrations in female and male spiders are indicated by circles and diamonds, respectively. Insecticide concentrations are shown in red and herbicides in green. Asterisks indicate a significant difference in concentrations between groups (Kruskal-Wallis rank sum test with post hoc Dunn's test using Bonferroni correction, $p < 0.05$). ‡Concentrations are reported as the sum of isomers.

chemical classes [35,36,50].

Mixtures of neonicotinoids in water samples were chronically present at low concentrations, which are typically exceeded in agricultural surface waters worldwide (Fig. 2 B, Table 1 and Data S3). In fact, the 90th percentiles of individual neonicotinoid concentrations detected in the present study corresponded with the 19th to 67th percentiles of neonicotinoid concentrations detected globally (Table 1). This hydrophilic class of insecticides (log K_{ow}: 0.57–1.26) represented a substantial proportion of insecticide usage (approximately 20%, contributed primarily by thiacloprid and acetamiprid) in Germany during the period 2019–2021 [51]. Their ubiquitous presence at the sampling sites likely results from their preemptive use, high solubility and resulting high mobility in soils [36]. It should also be noted that the neonicotinoids clothianidin, thiamethoxam and imidacloprid were banned for outdoor use in the entire EU by the end of 2018 [52–54], yet were found in water and spiders collected in 2021. This implies that either the half-life times in the environment are longer than those used during the regulatory risk assessment [55], considerably higher concentrations were present until the end of 2018 or illegal pesticide use took place. The fungicides and herbicides measured were all more lipophilic ($logK_{ow}$: 1.7–4.5) compared to the neonicotinoids, which correlated with their higher prevalence and concentrations in aquatic sediment and leaf litter (Fig. 1 A and B). Chronic low to medium concentrations of many different fungicides are common in aquatic environments globally, due to their prophylactic but mixed applications to prevent outbreaks while controlling for pest resistance [29].

4.2. *Aquatic-terrestrial transfer via emerging insects to spiders*

Emerging insects contained a broad range of pesticides, including those found in the spiders and were the mediators between low insecticide concentrations in the aquatic environment and high concentrations in riparian spiders. Fungicides, in contrast, were more prevalent and showed higher concentrations in emerging insects compared to

spiders (Data S5). In the present study, developmental stages of aquatic insects were exposed to insecticides with low lipophilicity ($logK_{ow} < 1.7$) at very low concentrations primarily through water, while other pesticide classes with higher lipophilicity ($logK_{ow}$ 1.7–4.5) had higher concentrations in water, sediment and leaf litter (Data S5). However, despite an exposure- and lipophilicity-gradient, BWAFs remained within a similar range for 84% of measurements (Fig. 3). This might be explained by a combination of bioaccumulation and retention processes over the emerging insect life cycle. Under laboratory conditions, bioconcentration of low to medium-polarity pesticides ($logK_{ow}$: 2–5) generally increases with pesticide lipophilicity in aquatic larvae [56], thus favouring fungicides and herbicides in the present study. Retention of accumulated pesticides across metamorphosis, however, generally follows the opposite pattern, decreasing with increasing lipophilicity [8,20], and thus favours the hydrophilic insecticides. Moreover, the rate of pesticide-specific elimination by developmental stages can determine concentrations in the adults. For example, a slow elimination rate was responsible for the selective transport of the neonicotinoid, thiacloprid, over more rapidly eliminated insecticides by emerging insects in a previous laboratory study [21]. The results from the present study indicate that this is also true for other neonicotinoids. Thus, chronic sub-lethal concentrations of neonicotinoids in the aquatic environment have a higher than expected propensity to be retained and transported to riparian spiders by emerging aquatic insects. The generally more lipophilic fungicides and herbicides, however, appear to be more easily eliminated by the emerging insects, resulting in lower concentrations in adult insects even when exposure takes place at persistently higher concentrations compared to the neonicotinoids.

Individual neonicotinoids share a propensity to be transported by emerging insects and bioaccumulated by riparian spiders. The tendency for pesticides to be transported by emerging insects could, however, not be explained by evaluation of their physicochemical properties, toxicity or persistence (Fig. S5, Table S4). The majority of pesticides measured in the present study have systemic properties strongly linked to their solubility, which facilitates their dispersal and movement through plants and insects [57]. It is therefore unsurprising that differences in the physicochemical properties of pesticides were not sufficient to differentiate between those pesticides with a high or a low propensity to be transported by emerging insects and detected in spiders. A similar selective bioaccumulation of neonicotinoids in the presence of complex mixtures of pesticides, as in the present study, has been reported in earthworms under laboratory conditions [58]. The neonicotinoids used in Chevillot et al. [58] as well as those from the present study, are first- and second-generation neonicotinoids, which share a common structural backbone and stearic conformations that are essential to their systemic behaviour and mode of toxic action [59,60]. Specific binding of neonicotinoids to proteins or other large biomolecules has been put forward by several authors to explain the differences between the predicted and measured toxicokinetics of neonicotinoids in aquatic crustaceans [38,61]. A mechanism involving specific binding is further supported by the enantioselective bioaccumulation rates in earthworms reported for dinotefuran, the only neonicotinoid containing a stereocenter [62]. Furthermore, flupyradifurone, which is a newer generation butenolide insecticide [63] structurally related to the neonicotinoids, was frequently detected at low concentrations in spiders in the present study. It was, however, not detected in the water or emerging insects (Data S1 and S5). The volume of this insecticide applied was < 1% of the total neonicotinoids applied during the sampling period [51], which could potentially have resulted in these concentrations lying below the analytical detection limits [64]. A mechanism of biomagnification similar to the neonicotinoids could explain the results in spiders, it can, however, only be speculated from the current data.

The bioaccumulation and biomagnification of neurotoxic insecticides across the aquatic-terrestrial ecosystem boundary have potential negative impacts on terrestrial food webs. Emerging dipterans contributed the most to both the overall pesticide flux and transport of specific insecticides in the present study (Fig. 4). This result is relevant for the exposure of terrestrial insectivores considering that dipterans have a very wide emergence window, subsiding only during the coldest months of the year [65]. Furthermore, communities of emerging aquatic insects disturbed by agricultural activities shift toward dominance by more tolerant dipterans, often with an increase in overall emergence biomass [4]. This implies the potential for a near-constant flux of neurotoxic insecticides from contaminated surface waters. Moreover, this could potentially include sites with very low insecticide input, as seen at the upstream forested sites in the present study (Data S1), where atmospheric deposition is assumed to contribute to concentrations in the aquatic environment (Data S4). Furthermore, spiders are fairly tolerant towards neonicotinoids in comparison to insects [66] and could create a reservoir for these insecticides in the food web. Both emerging aquatic insects and web-building riparian spiders can serve as prey for small birds and bats, the latter of which can consume 25–100% of their body weight in a single night especially in times of peak energy requirement (e.g. reproduction) [48,67]. Dietary exposure to neurotoxic insecticides can cause several sublethal effects in vertebrates [68,69]. These sublethal effects include, for example, reduced fecundity, raised stress hormone concentrations, reduced immune response, disorientation and other behavioural effects [37,68], which may threaten insectivores in riparian food webs.

5. Conclusion

Our results provide new evidence for the transfer of a broad range of neonicotinoids by aquatic insects emerging from agriculturally impacted surface waters to web-building riparian spiders preying on these insects, and the persistence of neonicotinoids at this higher trophic level. The results are based on the measurement of a large number of pesticides in a large number of insect and spider samples, despite biomass often being a limiting factor in similar studies [19]. The results do, however, carry some uncertainties because the study comprises only a restricted number of ten sites from a small geographic region and the entire set of water, emerging insect and spider samples were only collected at two sites. However, neonicotinoids are one of the most used groups of insecticides worldwide [30] and the patterns of neonicotinoid concentrations in water samples were compared to a large global data set. The results of this comparison show that neonicotinoid concentrations detected in the present study were at the lower end of neonicotinoid levels reported in the published literature. Furthermore, the emerging insects and web-building riparian spiders are not endemic to this particular region, but are widely studied in similar studies internationally [26]. We therefore assume that the results from this study and most notably, the transfer of neonicotinoids to riparian spiders is a relevant pathway in many other impacted surface waters worldwide. Future studies need to evaluate the importance of the pesticide biomagnification reported here for the viability of populations of terrestrial predators. A more detailed mechanistic study of molecule characteristics is also needed to further elucidate the mechanisms involved in the observed biomagnification of neonicotinoids. Overall, the importance of systemic insecticides, particularly neonicotinoids, for terrestrial consumers preying on emerging insects or riparian spiders and the ecological integrity of exposed riparian ecosystems as a whole requires further attention.

Environmental implication

Statement of environmental implication in support of the submission of an original research paper with the title Neonicotinoid insecticides at low concentrations in surface waters are mediated by emerging insects to high concentrations in riparian spiders. Neonicotinoid insecticides are in terms of use the most important group of insecticides in agriculture. They have a high potential to negatively impact food webs due to their invertebrate toxicity. Furthermore, sub lethal effects of neonicotinoid

exposure have been reported for vertebrate insectivores, such as birds and bats. Neonicotinoids are, however, not considered bioaccumulative in organisms, although their bioaccumulation in earth worms has recently been reported. Our results, which document the bioaccumulation and biomagnification of neonicotinoids across the aquatic-terrestrial ecosystem boundary, therefore provide new information on the risks associated with surface water contamination by these insecticides.

CRediT authorship contribution statement

Alexis P. Roodt: Conceptualization, Methodology, Investigation, Formal analyses, Visualization, Writing – original draft. **Maike Huszarik:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Martin Entling:** Funding acquisition, Conceptualization, Supervision, Writing – review & editing. **Ralf Schulz:** Funding acquisition, Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alexis Pieter Roodt reports financial support was provided by German Research Foundation. Maike Huszarik reports financial support was provided by German Research Foundation.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.131635.

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Chapter 6: Synthesis and Outlook

Maike Huszarik

Effect of chemical stream pollution on riparian predators

Characteristics of studied stream pollution

We found that the chemical stream pollution at the studied stream sites was a combination of agricultural and wastewater pollution, with similar gradients of pesticide toxicity and wastewater detections across the study area (Huszarik et al. 2023). We also found that chemical pollution was related to higher concentrations of dissolved nutrients, higher water temperature and a lower concentration of dissolved oxygen in the streams. Thus, the more polluted streams in our study region were not only exposed to chemical contaminants, but also had lower water quality than less polluted streams, characteristics which were also acting on the emergent insect community. Indeed, streams may be exposed to multiple stressors in addition to chemical pollution. For example, wastewater effluents can raise salinity and nutrient concentrations and decrease the concentration of dissolved oxygen downstream, which decreases the overall water quality (Stalter et al. 2013). These changes in the stream can interact with pollutants and cause different effects on the stream community (Birk et al. 2020), and should also be considered when evaluating individual stressors in the field.

A case of subsidies driving exposure?

We did not find evidence for a decrease in insect emergence at more polluted streams in the field studies. Instead, we tended to observe the opposite effect in both riparian insectivores, which hinted towards an increase in prey availability at polluted sites. For example, bat activity and hunting rates of the more specialized *Myotis* spp. were higher at more polluted sites (Huszarik et al. 2023), which indicates that they captured more prey at these streams. Similarly, the overall proportion of aquatic prey in *Tetragnatha montana* individuals' diet remained consistent between sites with different levels of chemical pollution, as had been found by Graf et al. (2020). However, with DNA metabarcoding we were able to confirm that *T. montana* consumed significantly more Chironomidae and fewer other aquatic Diptera families at more polluted streams (Huszarik et al. *in prep*). Results from both Chapters 3 and 4 indicate that, although toxic effects of chemical contaminants could be expected, chemical pollution did not result in an overall reduction in insect emergence abundance, i.e., "exposure driving subsidy" (Kraus 2019).

Although there was no clear effect of pesticide or wastewater contaminants on the overall abundance of emergent insects, "exposure driving subsidy" may apply in more subtle ways. For example, the shift in *T. montana* diet suggests that chironomids were more available at

more polluted sites (Huszarik et al. *in prep*). In addition, Plecoptera were not detected at the streams with the highest pesticide toxicity. These results imply that there was a shift from a more sensitive toward a more tolerant insect community at polluted stream sites. Such a shift in stream community is known to occur when the concentration of pollutants is not high enough to cause mortality in the most tolerant species (Cuffney et al. 1984; Liess and Von Der Ohe 2005; Liess et al. 2013). The in-stream toxicity measured at the streams in our studies was certainly high enough to negatively affect insects (Liess et al. 2021), but may only have caused mortality in sensitive species. In addition, the interaction between increased water temperature and dissolved nutrients may have given an advantage to tolerant species in the streams through increased stream productivity (Greig et al. 2012; Birk et al. 2020), masking the negative effects of chemical pollutants. Further ways that the contaminants could have acted was a possible temporal (Ohler et al. 2023; Roodt et al. 2023b), size (Stenroth et al. 2015), or nutrient quality shift in the prey (Jonsson and Stenroth 2016; Pietz et al. 2023). Any of these shifts could result in a de-coupling of the aquatic-terrestrial linkage where the terrestrial insectivores do not receive the nutrient input at the correct time or in the correct amount. Such a de-coupling would also be a form of chemical pollutants driving subsidy, but has rarely been considered.

Without a clear decrease in insect emergence, the studied chemical stream pollution could either have resulted in no detrimental effect, or in an increased dietary contaminant exposure for riparian insectivores (Kraus 2019; Kraus et al. 2021b). This depends on the potential for the chemical contaminants found in the stream to accumulate in emergent insects and be retained after metamorphosis into flying adults. Increasing evidence shows that emergent insects can accumulate and retain certain pesticides and pharmaceuticals, which has been proven both experimentally (Previšić et al. 2021; Roodt et al. 2022, 2023b) and in the field (Richmond et al. 2018; Kraus et al. 2021a). In Chapter 5, we confirmed that emergent insects in the study area accumulated a variety of pesticides, and that a subset of these biomagnified in *Tetragnatha* spp. (Roodt et al. 2023a). This evidence for emergent insect transfer of pollutants, coupled with the increased bat hunting and sustained aquatic diet of *T. montana* at polluted streams in Chapters 3 and 4, strongly suggest that riparian insectivores in our study were exposed to chemical contaminants from the streams. Thus, we can conclude that chemical stream pollution, in the context of the studied streams, leads to dietary exposure of riparian insectivores rather than an overall reduction of available prey, and that the "subsidy driving exposure" hypothesis is the most relevant outcome.

Open questions

Although we saw no overall effect of chemical toxicity reducing insect emergence, as described above, the chemical pollution in the streams likely acted negatively on more sensitive groups and may have temporally or nutritionally de-coupled the aquatic-terrestrial linkages at the streams. The studies in this dissertation focused on overall differences between sites along a pollution gradient and did not include these effects. It would be interesting to conduct similar field studies which focus on temporal shifts, for example, to evaluate if the observed results are constant or whether chemical stream pollution has differing seasonal implications for riparian consumers. For instance, bat activity and the aquatic proportion in *T. montana* diet may be higher at polluted sites in the spring if there is a higher peak of Diptera and Ephemeroptera emergence at this time (Ohler et al. 2023). In addition, changes in the diet of other arthropod predators across a stream pollution gradient such as beetles or wolf spiders could be examined using the combination of DNA metabarcoding and mass-sampling methods which was tested in Chapter 2 (Huszarik et al. *in prep*). Although wolf spiders may be less affected in our study area due to their seemingly lower reliance on aquatic prey, the use of DNA metabarcoding can provide more taxonomically detailed results for changes in trophic interactions in other regions.

The results of this dissertation also lend importance to open questions regarding the direct transfer of contaminants into riparian areas via emergent insects. This has only begun to be investigated for emerging contaminants, including pesticides and pharmaceuticals (Bundschuh et al. 2022), and more knowledge about the potential transfer of contaminants is needed to be able to predict the relevance of this exposure route to riparian insectivores. This knowledge is also particularly important for vulnerable bats (Browning et al. 2021). A future field study could address this by further investigating the pathway from the stream to riparian insectivores via emergent insects and establishing the risk of dietary exposure. This could be done in several ways. Firstly, determining which species of insects successfully emerge, and which are then consumed by bats at differently polluted streams would reveal exactly which taxa may play a role in transporting stream contaminants. DNA metabarcoding of bulk emergence samples (Piper et al. 2019), as well as guano of bats captured along the streams (Galan et al. 2018) is an effective approach for determining collected and consumed taxa. Secondly, directly quantifying the pollution pathway to riparian bats would determine the actual uptake of pollutants in bats. This could be done by measuring the concentration of contaminants at all stages of the food web: in the stream, the emergent insects, and riparian bats captured at the

stream sites. This approach ideally uses non-invasive methods such as bat hair (Hooper et al. 2022) or guano (Martín et al. 2023) to avoid unnecessary killing of bats or bias from using only bats found moribund or dead. Finally, continuing to evaluate the bioaccumulation potential of untested pesticides as well as pharmaceuticals and other emerging contaminants, either experimentally or through field observation, is imperative to better understand which contaminants are likely to accumulate and cause effects in the riparian food web. This includes determining possible lethal or sublethal effects occurring in riparian insectivore populations, which have rarely been investigated.

It is important to note that the field studies in this dissertation have been conducted in southwestern Germany over two years. We have likely not captured all variation in the local context, and including data from several years would be beneficial to strengthen our knowledge of the observed effects. While there is a history of measuring in-stream contaminants in the study area (Bereswill et al. 2012; Halbach et al. 2021), knowledge about yearly variation in their effects on riparian bats and spiders is limited. On a larger scale, although all studies in this dissertation focused on European species and ecosystems, the results and conclusions are likely applicable to other global regions. Similar pollution scenarios in temperate climates are likely to have comparable outcomes to our studies, but it is also important to test how the effects of chemical pollution change with different severities in the dominance of toxic effects. A more toxic effect of contaminants may result in a more dominant "exposure driving subsidy" effect, though there still may be an export of contaminants from the stream in the case of high bioaccumulation (Kraus et al. 2021a, 2021b). Furthermore, much of the bat and stream pollution research has been focused on North America and Europe, whereas ecosystems of the highest biodiversity are found in tropical regions of South America, Africa, and Asia (Frick et al. 2020; Brauns et al. 2022). Including a wider variety of climates and ecosystems in future studies would determine whether the hypotheses and findings developed so far are also applicable to other areas, and supply knowledge for effective conservation of riparian areas in different global ecosystems.

Implications for the riparian food web

The work included in this dissertation has only investigated effects of chemical stream pollution on the first level of the aquatic-terrestrial linkage: the riparian insectivores consuming emergent insects. However, the effects of chemical stream pollution may propagate further into the terrestrial component of the riparian food web.

In terms of contaminants, substances which bioaccumulate may be transferred to terrestrial consumers and possibly magnify throughout the food chain. Spiders, for example, are prey to other consumers such as birds and bats (Vallejo et al. 2019; Beaubien et al. 2020). These terrestrial consumers at higher trophic levels may, in this way, be exposed to higher concentrations than the initial predators of emergent insects (Bartrons et al. 2015). In addition, the decline of many terrestrial insects (Hallmann et al. 2017; Van Klink et al. 2020) may increase the reliance of riparian predators on emergent insects, exacerbating this potential problem. Although the contribution of streams as a source of contaminants is likely low in most cases, this should be ascertained as biomagnification of toxic compounds could have drastic effects. For example, it has been suggested that concentrations of pharmaceuticals could accumulate to relevant levels in riparian insectivores (Richmond et al. 2018), and changes in the microbiomes of bats (Mehl et al. 2021) and spiders (Millar et al. 2022) have been reported downstream from wastewater treatment plants. In extreme cases where toxic and accumulative chemicals are present in streams, this transportation route could threaten a collapse of the highest trophic levels in the riparian food web, such as the drastic population declines of bald eagles and other raptors due to DDT (Grier 1982; Rodríguez-Jorquera et al. 2017).

In addition to the transfer of contaminants, further food web effects likely take place at polluted sites. The increase in bat foraging may put additional predation pressure on prey which do not profit from stream pollution. The negative relationship between Daubenton's bat and flying Ephemeroptera, Plecoptera and Trichoptera (EPT) seen in the field study may have been due to such apparent competition (Huszarik et al. 2023). In the case of *T. montana*, the shift in diet may release other prey from predation at sites with more chironomid emergence (Graf et al. 2017; Recalde et al. 2020). The terrestrial and aquatic food web are complex systems that are intimately linked, and alterations in nutrient flows can have reverberating effects throughout, as demonstrated by Collins et al. (2020) and Osakpolor et al. (2023).

Improving protection for streams and riparian insectivores

In response to the degradation of surface waters, legislation like the European Union's Water Framework Directive have been established to protect their quality. Nevertheless, most European waterways have not reached a "good" ecological status (Grizzetti et al. 2017), in part due to chemical pollution (Grizzetti et al. 2017; Posthuma et al. 2020). Stream restoration is a major goal for the improvement of stream habitat quality. This includes adding meanders, and improving in-stream and riparian habitats to support a more diverse emergent insect community. However, it is important that stream restoration also reduces upstream input of chemical contaminants, otherwise sensitive species will not return. For example, the emergence of *Hexagenia* spp. mayflies in the Great Lakes region of North America once consisted of up to one trillion individuals, but suffered drastic declines in the past. Improved habitat and water quality of Lake Erie was associated with a partial recovery of the population (Krieger et al. 1996), but effects of neonicotinoids and other pollutants have caused the declines to resume (Stepanian et al. 2020). Reduction of pesticides and pharmaceuticals in streams can be achieved by improving wastewater treatment to include an advanced treatment step which removes more contaminants, though this can be costly (Eniola et al. 2022). In addition, agricultural and other non-point sources of chemical pollutants can be mitigated by ensuring that streams are surrounded by riparian buffer strips and riparian forests to filter run-off before it enters the stream, and continuing to monitor contaminant concentrations (Bunzel et al. 2014).

Conclusions

The chemical stream pollution studied within this dissertation resulted in an increased risk for riparian insectivores of contaminant exposure, rather than a decrease in emergent insect prey. This risk could be applicable for streams with similar contamination profiles, i.e., inputs of agricultural pesticides and wastewater contaminants that can accumulate in insects but are not toxic enough to reduce the overall insect emergence. It is important to further to investigate which drivers act on aquatic-terrestrial linkages under different pollution and stressor scenarios, and to quantify the risk that this exposure route presents to riparian ecosystems. Additionally, continuing to investigate dietary changes in riparian consumers at more detailed levels will aid in understanding the complex trophic interactions occurring in these food webs.

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Appendix

Status and author contributions of publications included in the dissertation

Author affiliations

Curriculum vitae

Declaration

Supplementary Information for Chapters 2, 3 and 4

**The location of any digital sections of the supplementary information is available online at figshare. https://figshare.com/s/2bf1249dc721dfb88f46*

Status and author contributions of publications included in the dissertation

Chapter 2 (published)

Huszarik M, Röder N, Eberhardt L, Kennedy S, Krehenwinkel H, Schwenk K, Entling MH. External DNA contamination and efficiency of bleach decontamination for arthropod diet analysis. Environmental DNA. 2023;5(3):540–50. https://doi.org/10.1002/edn3.410

See Chapter 2 for author contribution statements.

Chapter 3 (unsubmitted)

Huszarik M, Roodt AP, Wernicke T, Wollscheid L, Link M, Lima-Fernandes E, Åhlén D, Schreiner V, Schulz R, Hambäck P, Entling MH. Shift in diet composition of Tetragnatha montana along a chemical stream pollution gradient. *In preparation.*

See Chapter 3 for author contribution statements.

Chapter 4 (published)

Huszarik M, Roodt AP, Wernicke T, Chávez F, Metz A, Link M, Lima-Fernandes E, Schulz R, Entling MH. Increased bat hunting at polluted streams suggests chemical exposure rather than prey shortage. Science of The Total Environment. 2023;167080. https://doi.org/10.1016/j.scitotenv.2023.167080

See Chapter 4 for author contribution statements.

Chapter 5 (published)

Roodt AP, Huszarik M, Entling MH, Schulz R. Aquatic-terrestrial transfer of neonicotinoid insecticides in riparian food webs. Journal of Hazardous Materials. 2023;455:131635. https://doi.org/10.1016/j.jhazmat.2023.131635

See Chapter 5 for author contribution statements.

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In prep:	**Huszarik M**, Roodt AP, Wollscheid L, Wernicke T, Åhlén D, Schreiner V, Schulz R, Hambäck P, Entling M. Indirect effects of chemical stream pollution on the diet of riparian *Tetragnatha montana*. (*in preparation*)

Posters and scientific presentations

April 2023- Onsite presentation	**Huszarik M**, Roodt AP, Wernicke T, Chávez F, Metz A, Lima-Fernandes E, Link M, Schulz R, Entling MH. „Indirekte Effekte von chemischer Verschmutzung in Fließgewässern auf Fledermausaktivität und -jagdverhalten“. 15. Tagung des Bundesfachauschusses Fledermaüse 2023.
December 2022- Onsite presentation	**Huszarik M**, Roodt AP, Metz A, Lima-Fernandes E, Link M, Schulz R, Entling MH. „Effekte von Fließgewässerverschmutzung auf das Nahrungsnetz von Fledermäusen“. NABU RLP Tagung: "Fledermausschutz in Rheinland-Pfalz"2022.
November 2022- Onsite presentation	**Huszarik M**, Roodt AP, Metz A, Lima-Fernandes E, Link M, Schulz R, Entling MH. „ Indirect effects of stream pollution on riparian food webs: Activity and hunting behaviour of terrestrial insectivores “. SFE2, GfÖ & EEF Joint Meeting, International Conference on Ecological Sciences 2022.
August 2022- Onsite presentation	**Huszarik M**, Roodt AP, Metz A, Lima-Fernandes E, Link M, Schulz R, Entling MH. „ Indirect effects of stream pollution on riparian food webs: Activity and hunting behaviour of terrestrial insectivores “. 36th Congress of the Society of Limnology 2022.
December 2021- Online poster	**Huszarik M**, Roodt AP, Lima-Fernandes E, Metz A, Wernicke T, Schirmel J, Link M, Schulz R, Entling MH. „Indirect effects of pesticide and wastewater pollution in streams on riparian bat activity and hunting behaviour”. British Ecological Society Ecology Across Borders 2021.
May 2021- Online poster	**Huszarik M**, Roodt AP, Metz A, Lima-Fernandes E, Link M, Schulz R, Entling MH. „ Do chemical contaminants in streams decrease the activity and foraging behaviour of riparian bats?”. SETAC Europe 31st annual meeting 2021.

Declaration according to §8 of the Promotionsordnung des Fachbereichs 7: Natur- und Umweltwissenschaften der Rheinland-Pfälzische Technische Universität Kaiserslautern-Landau, Campus Landau vom 14.06.2013 i.d.F. vom 19.08.2014

I, the author of this work, hereby declare that I independently conducted the work presented in this thesis entitled "*Indirect effects of chemical stream pollution on the riparian food web*".

All used assistances are mentioned and involved contributors are either co-authors of or are acknowledged in the respective publication.

This thesis has never been submitted elsewhere for an examination, as a thesis or for evaluation in a similar context to any department of this university or any scientific institution. I am aware that a violation of the aforementioned conditions can have legal consequences including the withdrawal of the doctoral degree.

Erklärung der Doktorandin darüber,

dass sie die eingereichte Dissertation selbstständig verfasst hat und alle von ihr für die Arbeit benutzten Hilfsmittel und Quellen in der Arbeit angegeben sowie die Anteile etwaig beteiligter Mitarbeiterinnen oder Mitarbeiter sowie anderer Autorinnen oder Autoren klar gekennzeichnet sind;

dass sie nicht die entgeltliche Hilfe von Vermittlungs- oder Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen hat;

dass sie die Dissertation nicht in gleicher oder ähnlicher Form als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung im In- oder Ausland eingereicht hat;

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dass ihr bewusst ist, dass ein Verstoß gegen einen der vorgenannten Punkte den Entzug des Doktortitels bedeuten und ggf. auch weitere rechtliche Konsequenzen haben kann

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Place, date Signature

Chapter 2 Supplementary Information

Supporting Information:

"External DNA contamination and efficiency of bleach decontamination for arthropod diet analysis"

Table S1: Coordinates of spider sampling sites, pitfall traps and sampling dates

Event	Stream (If applicable)	Location	Date
Vineyard for hand sampling		49.2038456, 8.0950493	August 19th, 2019
Forest for simulated pitfall trap		approx. 49.2046261, 8.1060383	
Riparian forests for sampling	Michelsbach	49.1848960, 8.3514450	
	Neuer Tiefer Graben	49.2191616, 8.3205017	May-July 2019 (Hand
	Queich	49.2197106, 8.3233606	sampling June 2019)
	Otterbach	49.0935382, 8.2609278	

Table S2: High salt extraction protocol used for DNA extraction from whole spiders, which had been dried and ground into a powder.

Step	Proceedure
1.	Add 450 µL SEB and 100 µL SDS to dried and ground samples
2.	Add 5 µL Proteinase K and vortex
3.	Incubate 1 hr at 60 °C in a 400-rpm shaker
4.	Add 350 µL NaCl (5M) and vortex
5.	Centrifuge at 16200 x g for 30 minutes
6.	Transfer 600 µL supernatant to new tube and add 600 µL ice-cold isopropanol. Mix briefly
7.	Freeze at -80 °C for 20 minutes
8.	Centrifuge at 4 °C for 20 minutes (16200 x g)
9.	Discard supernatant, careful not to disturb pellet. Add 200 µL ice-cold 70% ethanol
10.	Centrifuge at 4 °C for 10 minutes (16200 x g)
11.	Discard supernatant, careful not to disturb pellet. Dry tube with pellet in heat block at 60 °C
12.	Elute in 25 µL 1x TE buffer

Table S3: Pairs of forward and reverse primers used for selective amplification of prey DNA in wolf spider gut content with a multiplex PCR step, as used by *Krehenwinkel et al. (2019)*. The barcode portion of the indexing primers is shown in bold red.

Primer Type	Primer Name	Sequence (5' to 3')
Multiplex forward 1 (18SS)	18SrDNA F1046	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTCTGGTTRATTCCGRTAACGAA
Multiplex forward 2 (18SL)	18SrDNA F985	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCTCTTTCTYGATTCRGTGGGT
Multiplex reverse 1&2	18SrDNA R1238	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCACAGACCTGTTATTGCTCAA
Multiplex forward 3 (28S)	28SrDNA F1020	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCGTCTTGAAACACGGACCA
Multiplex reverse 3	28SrDNA R1333	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGWCCTCCATCAGGGTTTCCC
Index forward	F_01	AATGATACGGCGACCACCGAGATCTACAC**TAGATCGC**ACACTCTTTCCCTACACGA
Index forward	F_02	AATGATACGGCGACCACCGAGATCTACAC**CTCTCTAT**ACACTCTTTCCCTACACGA
Index forward	F_03	AATGATACGGCGACCACCGAGATCTACAC**TATCCTCT**ACACTCTTTCCCTACACGA
Index forward	F_04	AATGATACGGCGACCACCGAGATCTACAC**AGAGTAGA**ACACTCTTTCCCTACACGA
Index forward	F_05	AATGATACGGCGACCACCGAGATCTACAC**GTAAGGAG**ACACTCTTTCCCTACACGA
Index forward	F_06	AATGATACGGCGACCACCGAGATCTACAC**ACTGCATA**ACACTCTTTCCCTACACGA
Index forward	F_07	AATGATACGGCGACCACCGAGATCTACAC**AAGGAGTA**ACACTCTTTCCCTACACGA
Index forward	F_08	AATGATACGGCGACCACCGAGATCTACAC**CTAAGCCT**ACACTCTTTCCCTACACGA
Index reverse	R_01	CAAGCAGAAGACGGCATACGAGAT**ATTGGTCA**GTGACTGGAGTTCAGACGTG
Index reverse	R_02	CAAGCAGAAGACGGCATACGAGAT**TAAAAATG**GTGACTGGAGTTCAGACGTG
Index reverse	R_03	CAAGCAGAAGACGGCATACGAGAT**ATCACTGT**GTGACTGGAGTTCAGACGTG
Index reverse	R_04	CAAGCAGAAGACGGCATACGAGAT**TATTTCAC**GTGACTGGAGTTCAGACGTG
Index reverse	R_05	CAAGCAGAAGACGGCATACGAGAT**ATATTGGC**GTGACTGGAGTTCAGACGTG
Index reverse	R_06	CAAGCAGAAGACGGCATACGAGAT**TATACAAG**GTGACTGGAGTTCAGACGTG
Index reverse	R_07	CAAGCAGAAGACGGCATACGAGAT**ATGATCTG**GTGACTGGAGTTCAGACGTG
Index reverse	R_08	CAAGCAGAAGACGGCATACGAGAT**TACTCTAC**GTGACTGGAGTTCAGACGTG
Index reverse	R_09	CAAGCAGAAGACGGCATACGAGAT**ATAAGCTA**GTGACTGGAGTTCAGACGTG
Index reverse	R_10	CAAGCAGAAGACGGCATACGAGAT**TAGTATAG**GTGACTGGAGTTCAGACGTG
Index reverse	R_11	CAAGCAGAAGACGGCATACGAGAT**ATTACAAG**GTGACTGGAGTTCAGACGTG
Index reverse	R_12	CAAGCAGAAGACGGCATACGAGAT**TAATTGGC**GTGACTGGAGTTCAGACGTG

Table S4: Components of the multiplex PCR master mix to selectively amplify prey DNA from wolf spider samples

Ingredient	Volume (µL)
18S_F1046	0.42
18S_F985	0.54
18S_R1238	0.96
28S_F1020	0.75
28S_R1333	0.75
Multiplex PCR Master Mix (QIAGEN)	7.5
H_20	2.58
Master Mix total	***13.5***
Isolated DNA	***1.5***

Table S5: Timings and temperatures for multiplex and index PCR steps to extract prey DNA from wolf spider samples

	Temperature (°C)	Time (mm:ss)
Step 1	95	15:00
Step 2	94	0:30
Step 3	55	1:30
Step 4	72	1:30
Step 5	*Repeat steps 2-4, 34 times (multiplex) or 5 times (index)*	
Step 6	72	10:00
Step 7	12	infinite hold

Table S6: Components of the indexing PCR master mix to selectively amplify prey DNA from wolf spider samples

Ingredient	Volume (µL)
Multiplex PCR Master Mix (QIAGEN)	5
H_20	3.5
Indexing Master Mix total	***8.5***
Multiplex PCR product	***0.5***
Indexing primer (forward + reverse)	***0.5 + 0.5***

Table S7: Count of sequence reads obtained from spider diet analysis using three primers. Target reads were non-spider non-crustacean arthropods.

Primer	18SL	18SS	28S
Number ASVs total	274	593	327
Number target ASVs	82	125	82
Number reads total	52,080	157,628	137,259
Average reads bleached	567	1604	1961
Average reads control	753	2473	1586
Number target reads	14,926	50,963	11,228
Average reads bleached	134	395	53
Average reads control	282	1049	279
Spiders with no reads	3 (all bleached)	0	0
Spiders with no target reads	26 (4 control)	10 (2 control)	33 (6 control)

Chapter 3 Supplementary Information

Supplementary information for

"Shift in diet composition of Tetragnata montana along a chemical stream pollution gradient"

Maike Huszarik, Alexis P. Roodt, Teagan Wernicke, Moritz Link, Eva Lima-Fernandes, Verena Schreiner, Ralf Schulz, Peter Hambäck, Martin H. Entling

9 Pages, 3 Excel Sheets, 4 Figures, 9 Tables

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****Tables S4, S5, and S6 can be found in Excel file Huszarik2023SpiderDiet_SI.xslx, available at figshare: https://figshare.com/s/2bf1249dc721dfb88f46

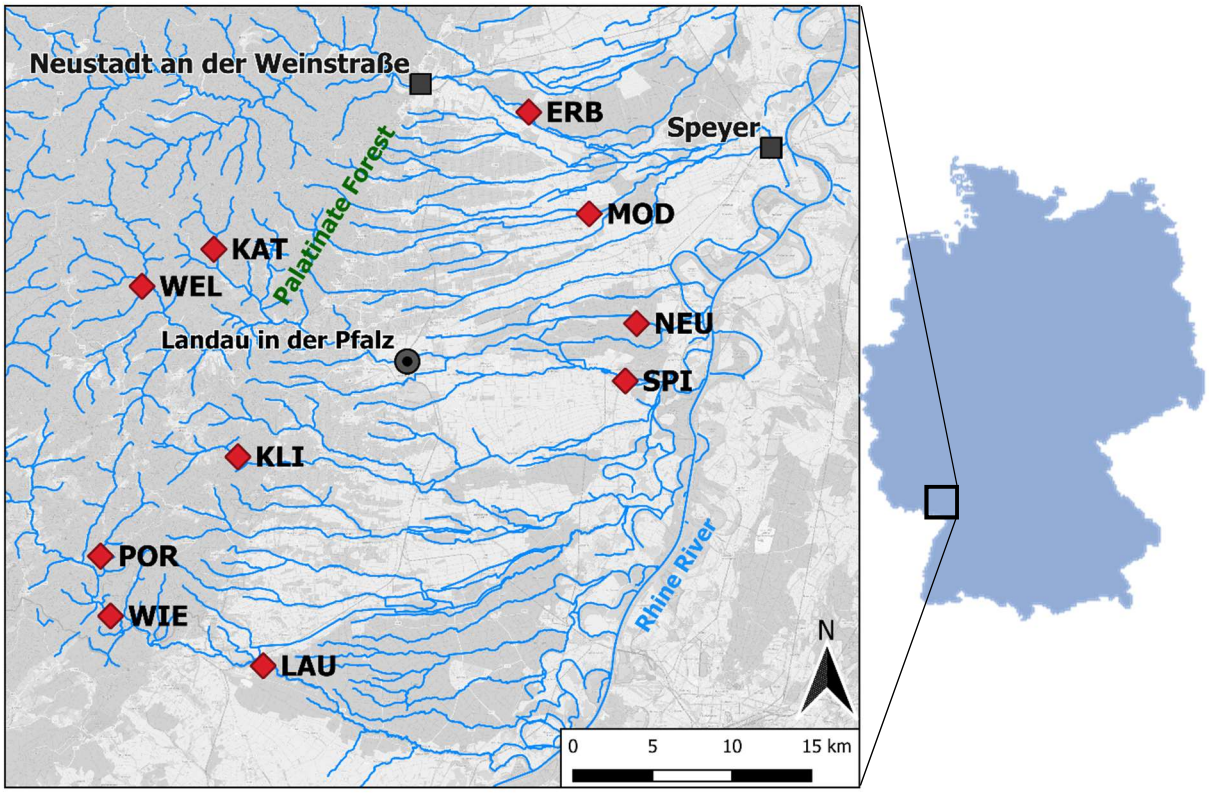

Figure S1: Location of study area in Germany (right), and map of study sites (left). Sites were located along streams, represented by the blue lines. Three cities are depicted with dark grey icons, and the Palatinate Forest and Rhine are labelled. The inset of Germany is adapted from that by "Antman", http://en.wikipedia.org/wiki/Image:Map_Germany_Blank.png (CC BY-SA 3.0). The basemap is OpenStreetMap, available under the Open Database Licence (CC BY-SA 2.0). The stream layer is "Gewässernetz 2017", available from WWV RLP (CC BY 4.0). The map was created in QGIS v3.12.1-București.

Table S1: Stream study site names, catchment sizes, German working group on water issues (LAWA) stream types, coordinates and location/protected area descriptors. FFH: Flora-Fauna-Habitate (Habitats Directive), and VSG: Vogelschutzgebiete (Birds Directive).

Site ID	Stream Name	Site Coordinates	Catchment size (km²)	Stream Type (LAWA)	German Nature Conservation Area (Naturschutz-gebiet)	International Protected Area Type (IUCN, Natura 2000)	Forest Name
ERB	Urerbsengraben	49.337496, 8.229486	7.7	NA	No	VSG	Ordenswald/Grauwald
KAT	Katzenbach	49.260855, 7.959262	10.5	5.1	No	FFH	Pfälzerwald
KLI	Klingbach	49.1445697, 7.9798149	17	5.1	No	None	Pfälzerwald
LAU	Lauter	49.02721, 8.00154	44.1	9	Yes	FFH, VSG	Bienwald
MOD	Modenbach	49.280672, 8.281392	41.6	19	No	FFH, VSG	Modenbachniederung
NEU	Neuer Tiefer Graben	49.2192816, 8.3221335	10.5	9	No	FFH, VSG	Bellheimer Wald
POR	Portzbach	49.08884, 7.86190	15.2	5.1	No	None	Pfälzerwald
SPI	Spiegelbach	49.1873981, 8.3113842	62.9	19	Yes	FFH, VSG	Eichtal-Brand
WEL	Wellbach	49.2401133, 7.8975234	41.3	5.1	No	FFH	Pfälzerwald
WIE	Wießlauter	49.05517, 7.87057	250.3	5.1	No	FFH	Pfälzerwald

Table S2: Classification scale for shrub separation of forest along shore of stream sites.

Score	Name	Definition
0	Open	>5 m between shrubs
1	Moderately open	2-5 m between shrubs
2	Moderately dense	1-2 m between shrubs
3	Dense	<1 m between shrubs

Table S3: Classification scale of objects (plants, debris, or branches) cluttering/breaking the water surface of streams at field sites.

Score	Name	Definition
0	Open	0-10 % surface covered by occasional low plants, branches or debris
1	Moderately open	10-25 % surface covered
2	Moderately cluttered	25-50 % surface covered
3	Cluttered	50-75 % surface covered, plants are high and/or branching over the water, taking up surface area
4	Inaccessible	>75 % water surface covered by plants or branches hanging low over water surface, or debris out of water blocking easy access

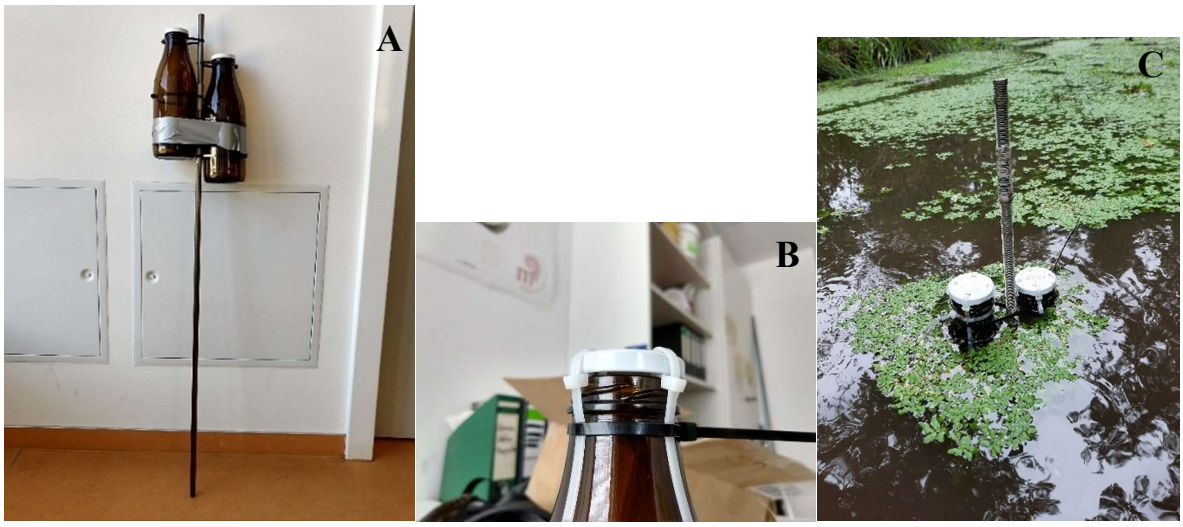

Figure S2: Event samplers for collecting water samples from streams during high-water events caused by rain and run-off entering the streams. A) The whole sampler with two glass bottles taped at different heights to a stake. B) The cap was attached to the bottle with zip-ties so that there was a small gap between the cap and bottle rim to allow water to enter. C) The typical positioning of the sampler so that bottles were slightly above the normal stream water level.

Figure S3: A Sea-Land-Air-Malaise trap (SLAM) hanging above the shore of a stream. The openings are orientated parallel to the stream.

Section S1: DNA extraction following modified protocol OBT_M6399_KDuo_100uL_v1.1 (developed by Omega Bio-Tek, Inc.)

To be used with buffers included in the Mag-Bind® Blood & Tissue DNA HDQ 96 kit (Omega Bio-Tek, Inc., Norcross, GA, USA) and prepared spider tissue.

1. Add 300 µL TL Buffer to 1.5 mL tube containing spider material and use a sterile pestle to homogenize the spider tissue until no large pieces are visible.
2. Add 20 µL Proteinase K solution, vortex for 10 s, and incubate tube overnight at 55 °C.
3. Add 5 µL of 10 mg/mL RNAse, mix thoroughly by pipette and vortex for one second, then incubate at room temperature for 15 minutes.
4. During incubation time, prepare the rows of wells in plate as follows:
    a. Row A: Add 290 µL AL Buffer to each well
    b. Row B: Add 500 µL VHB Buffer to each well
    c. Row C: Add 500 µL VHB Buffer to each well
    d. Row D: Add 500 µL SPM Buffer to each well
    e. Row E: Add tip comb
    f. Row F: Empty
    g. Row G: Empty
    h. Row H: Empty
    i. Elution strip: add 100 µL Elution Buffer to each well
5. Centrifuge samples for 10 minutes at 40x100g.

6. Carefully transfer 250 µL of supernatant (lysate) to wells in Row A of the plate and mix thoroughly by pipetting (one sample per well).
7. Add 400 µL of HDQ Buffer and 20 µL of vortexed HDQ Mag-Bind® Magnetic Particles (vortexed briefly to re-suspend after every 4 samples) to each sample well in Row A and mix thoroughly by pipetting.
8. Place plate and elution strip in instrument, and run the OBT_M6399_KFDuo_100uL_v1.1 script.

Table S7: Tagged forward and reverse primers used for selective amplification of prey DNA from spider samples. The 8-base-pair tags are shown in bold red, located at the 5'-end of the primer sequences. NoAranR was developed by Hambäck et al. (2021, "More intraguild prey than pest species in arachnid diets may compromise biological control in apple orchards", doi: https://doi.org/10.1016/j.baae.2021.09.006) and LCO1490 was developed by Folmer et al. (1994, "DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates", Molecular Marine Biology and Biotechnology 3(5), 294-299).

Primer type	Primer name	Sequence (5'-3')
Tagged forward, LCO1490	LCO T01	**GCATGCAC**GGTCAACAAATCATAAAGATATTGG
	LCO T03	**GTGCATGC**GGTCAACAAATCATAAAGATATTGG
	LCO T04	**GATGCATC**GGTCAACAAATCATAAAGATATTGG
	LCO T05	**GGACTGAC**GGTCAACAAATCATAAAGATATTGG
	LCO T07	**GAGTCAGC**GGTCAACAAATCATAAAGATATTGG
	LCO T08	**GCTAGCTC**GGTCAACAAATCATAAAGATATTGG
	LCO T09	**GTAGCTAC**GGTCAACAAATCATAAAGATATTGG
	LCO T11	**GCGATCGC**GGTCAACAAATCATAAAGATATTGG
Tagged reverse, NoAranR	NoAran T01	**GCATGCAG**TGTTCATCCDGTNCCWG
	NoAran T03	**GTGCATGG**TGTTCATCCDGTNCCWG
	NoAran T04	**GATGCATG**TGTTCATCCDGTNCCWG
	NoAran T05	**GGACTGAG**TGTTCATCCDGTNCCWG
	NoAran T07	**GAGTCAGG**TGTTCATCCDGTNCCWG
	NoAran T08	**GCTAGCTG**TGTTCATCCDGTNCCWG
	NoAran T09	**GTAGCTAG**TGTTCATCCDGTNCCWG
	NoAran T11	**GCGATCGG**TGTTCATCCDGTNCCWG

Table S8: Timing and temperatures for the single indexing step tagging PCR protocol to amplify prey DNA extracted from spiders.

Step	Temperature (°C)	Time (mm:ss)
0.	Heat lid 110	-
1.	95	15:00
2.	92.5	00:30
3.	53 (decrease temperature by 0.5 °C for this step for each subsequent repetition, final repetition = 47 °C)	00:30
4.	72	01:00
5.	*Repeat steps 2-4 13 times, with a decrease in step 3 temperature each repetition.*	
6.	92.5	00:30
7.	50	00:30
8.	72	01:00
9.	*Repeat steps 6-8 25 times*	-
10.	74	10:00

Section S2: PCR-Free phosphorylation and Illumina adapter-ligation protocol

Materials:

- Volume of sample pool containing 300 ng DNA (vortexed)
- T4 Polynucleotide Kinase (T4 PNK Kit; Thermo Fischer Scientific)
- 10X Reaction Buffer A (T4 PNK Kit; Thermo Fischer Scientific)
- 10mM ATP (Thermo Fischer Scientific)
- Nuclease-free water
- Illumina TruSeq® DNA Single Index tagged adaptors
- T4 DNA Ligase 5U/µL (Thermo Fischer Scientific)
- 50% PEG 4000 solution (Thermo Fischer Scientific)
- 10X T4 DNA Ligase buffer (Thermo Fischer Scientific)

DNA Phosphorylation (Following Thermo Scientific protocol):

1. Combine volume of sample pool containing 300 ng DNA with 2 µL reaction buffer A, 2 µL ATP, 1 µL T4 Polynucleotide Kinase, and enough water to give a total volume of 20 µL.
2. Mix thoroughly and spin down.
3. Incubate at 37 °C for 20 minutes, then 10 °C for 10 minutes

Adapter ligation (Following Thermo Scientific protocol):

1. Combine 14 µL of phosphorylated DNA with 1 µL tagged Illumina adaptors (different tags for each pool), 2 µL Ligase Buffer, 2 µL PEG 4000 solution, and 1 µL T4 DNA Ligase for a total volume of 20 µL.
2. Mix thoroughly and spin down.
3. Incubate at 22 °C for 1 hour, then 65 °C for 10 minutes.

Table S9: Effects of pesticide toxicity in streams (sumTU), the average stream width (m), and the percent of tree canopy cover (%) on insects. Results and test statistics of generalized linear model testing (above) and permanova testing (below). Df represents the degrees of freedom. *P*-values in bold are significant ($p < 0.05$).

Response variable	**Model type**	**Explanatory variable**	**Estimate**	***p*-value**
Total number of flying insects *	Gaussian	Intercept	1207.6	**0.049**
		sumTU	0.07	0.069
		Stream width	0.03	0.393
		Canopy cover	- 0.01	0.064
Number of aquatic flying insects *	Gaussian	Intercept	110.2	0.714
		sumTU	-34.39	0.154
		Stream width	9.83	0.547
		Canopy cover	-0.48	0.872
Proportion of aquatic flying insects *	Gaussian	Intercept	-0.224	0.500
		sumTU	-0.02	0.573
		Stream width	0.03	0.308
		Canopy cover	0.01	0.185
Proportion of aquatic insects in spider diet *	Gaussian	Intercept	0.71	0.137
		sumTU	0.01	0.381
		Stream width	0.01	0.555
		Canopy cover	-0.001	0.766
Number of prey detections in spider diet *	Gaussian	Intercept	3.74	0.138
		sumTU	-0.09	0.684
		Stream width	0.04	0.440
		Canopy cover	-0.04	0.148
Response variable	**Model type**	**Explanatory variable (df)**	**Sum of squares**	***p*-value**
Composition of taxonomic groups of flying insects	Permanova (Adonis)	sumTU (1)	26482	0.238
		Stream width (1)	8322	0.697
		Canopy cover (1)	39054	0.121
		Residual (6)	105644	-
		Total (9)	179502	-
Composition of taxonomic groups in spider diet	Permanova (Adonis)	sumTU (1)	0.30	0.096
		Stream width (1)	0.03	0.814
		Canopy cover (1)	0.02	0.894
		Residual (5)	0.51	-
		Total (8)	0.87	-
Composition of most common species in spider diet	Permanova (Adonis)	sumTU (1)	0.05	0.252
		Stream width (1)	0.05	0.235
		Canopy cover (1)	0.03	0.624
		Residual (5)	0.21	-
		Total (8)	0.35	-

* GLM was permuted to obtain *p*-value

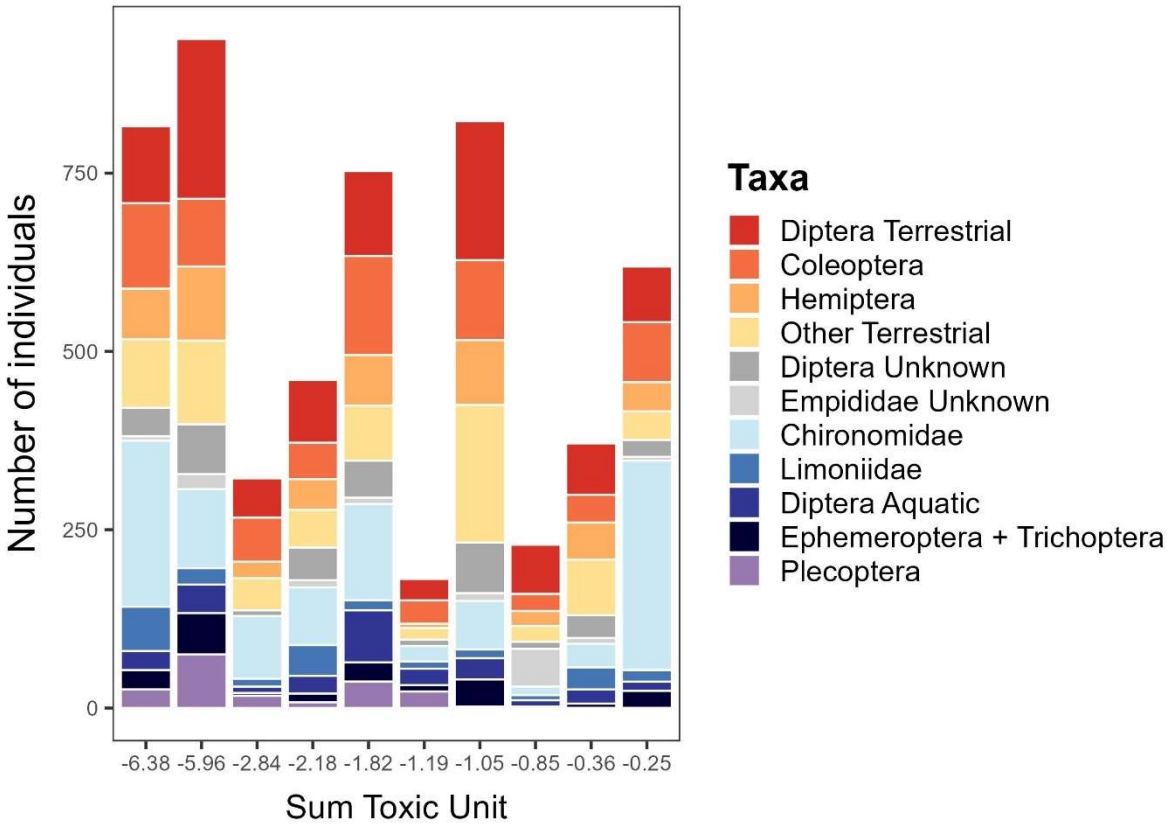

Figure S4: Composition of flying insects sampled at different stream sites in riparian forests, stacked by absolute abundance per site. Stream sites were located along a pollution gradient and are labelled by their average in-stream pesticide toxicity value (sum toxic unit) on the x axis (increasing toxicity). A sum toxic unit closer to zero represents a higher toxicity, whereas a more negative value represents a lower toxicity. The sum toxic unit is on a logarithmic scale. The taxa are represented by the number of individuals of each group captured by malaise traps (B). Taxa coloured in red are terrestrial and in blue are aquatic. It should be noted that a few individuals of aquatic species included in Coleoptera and that Empididae were not separated into aquatic and terrestrial.

Chapter 4 Supplementary Information

SUPPLEMENTARY INFORMATION FOR

"Increased bat hunting at polluted streams suggests chemical exposure rather than prey shortage"

Authors: Maike Huszarik[a]*, Alexis P. Roodt[a], Teagan Wernicke[a], Fernanda Chávez[a], Annika Metz[a], Moritz Link[a], Eva Lima-Fernandes[a], Ralf Schulz[a,b], Martin H. Entling[a]

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8 Pages, 15 Excel Sheets, 5 Figures, 18 Tables

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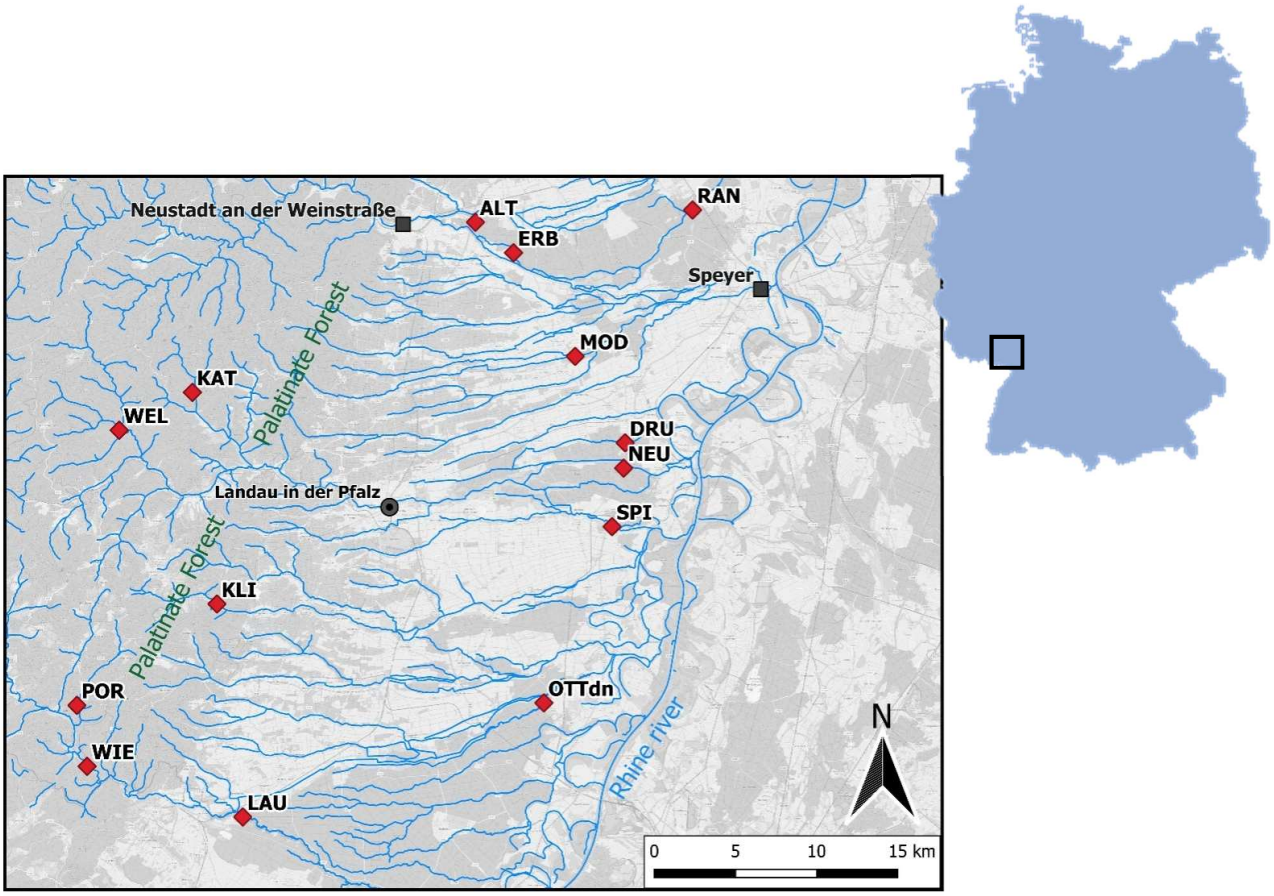

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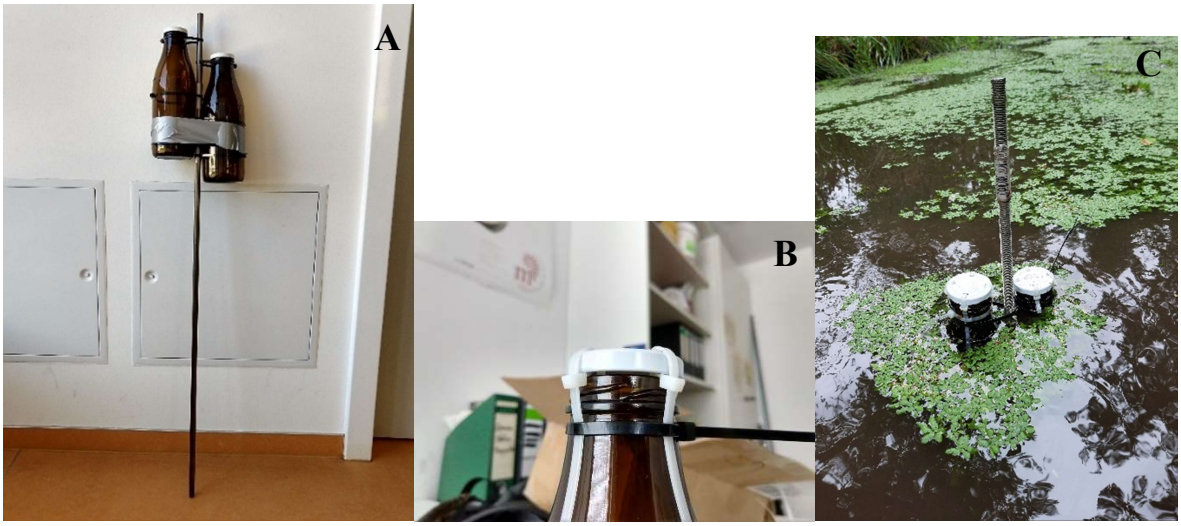

Figure S2: Event samplers for collecting water samples from streams during high-water events caused by rain and run-off entering the streams. A) The whole sampler with two glass bottles taped at different heights to a stake. B) The cap was attached to the bottle with zip-ties so that there was a small gap between the cap and bottle rim to allow water to enter. C) The typical positioning of the sampler so that bottles were slightly above the normal stream water level.

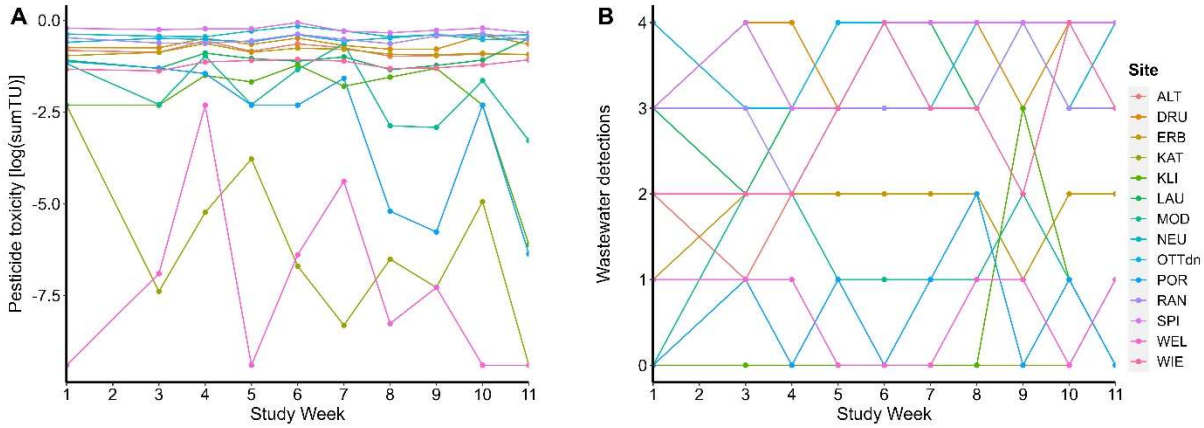

Figure S3: Change in the pesticide sum toxicity (A) and the number of wastewater indicator detections (B) in weekly stream water samples taken over the course of the field study. Each line represents samples collected from the same site.

SectionS1: Details describing the preparation of stream water samples and analysis of pollutants with HPLC-ESI-MS/MS

S1.1: Details of solid phase extraction of analytes from stream water

Chemical pollution analytes were extracted from stream water samples using solid phase extraction (SPE), following the method of Machado et al. (2016). After settling at 4 °C for 24 hours, water samples were decanted into 1 L glass dropping funnels containing glass wool as a filter. Bottles were weighed before and after pouring water to measure the exact volume of each sample extracted (±0.01 g, PCB 2500-2; Kern & Sohn GmbH, Germany). The funnels were connected to Oasis® HLB 6cc 500 mg SPE cartridges (Waters Corporation, Milford, MA, USA), conditioned with one aliquot of 5 mL of methanol (HPLC grade,Sigma-Aldrich Chemie GmbH, Germany) and two aliquots of 5 mL ultrapure water. Samples were passed through the cartridges at a flowrate of 8 mL/min, using a peristaltic pump (IPC, Ismatec®, Cole-Parmer GmbH, Germany). Following extraction, cartridges were dried under a gentle stream of nitrogen gas (NGM 11, cmc Instruments GmbH, Germany).

Analytes were eluted from the cartridges into glass vials using two 2.5 mL aliquots of methanol (HPLC grade,Sigma-Aldrich Chemie GmbH, Germany) and one of 3 mL acetonitrile (MS grade; Sigma-Aldrich Chemie GmbH, Germany). A solvent blank (n = 5) consisting only of methanol and acetonitrile was run through an empty cartridge alongside the samples. The vials were covered in aluminium foil to protect from light and eluates were dried completely under nitrogen gas. The analytes were then re-suspended in 500 µL of a 70:30% (v/v) mixture of water with 0.1% (v/v) formic acid and MeOH (MS grade; Sigma-Aldrich Chemie GmbH, Germany). The extracts were subsequently vortexed, centrifuged, transferred into 2 mL brown glass HPLC vials and stored at -20 °C prior to quantification. Two blanks of the water-methanol solution were included.

S1.2: Details of analyte quality assurance and normalisation procedures

Analytical standards (Sigma-Aldrich Chemie GmbH, Germany; LGC Standards, LGC Limited, UK; Restek GmbH, Germany) were prepared in 70:30% (v/v) ultra-pure water and methanol with 0.1% formic acid at seven concentrations: 0.025, 0.05, 0.5, 1.0, 5.0, 10.0 and 50.0 µg/L. The limits of detection (LODs) and limits of quantification (LOQs) were calculated for each

analyte based on the calibration curves using the equations: LOD = 3.3s/m and LOQ = 10s/m, where s is the standard error of the linear regression and m is the slope (ICH, 2005). The LODs and LOQs for each analyte are provided in Table S5. Furthermore, five replicate 1 L samples of high purity water containing a mixture of all the measured analytes at a concentration of 100 ng/L were extracted and measured in order to determine the accuracy and reproductivity of the extraction method. Analytes with recoveries between 70 – 120% and relative standard deviations between replicates (RSD) of 15% or less were quantified in the samples (Table S5). Any analytes (n = 23) which did not meet these standards were only considered qualitatively and not included in the toxicity calculations. However, fipronil, which had a recovery of 50%, was quantified as an exception due to its high ecotoxicological relevance and frequent occurrence in analysed samples.

Caffeine and boscalid both had low but quantifiable levels of contamination in SPE blanks (Table S12).To compensate for this, we increased the LOQ above the highest measured concentration andwe did not consider detections for concentrationsfalling below the LOQ. For remaining analytes which had been detected but not quantified in blanks, we subtracted half of the LOQ concentration of the respective analytes from all samples extracted in the same week as the contaminated blanks.

Normalisation of measured analyte concentrations for HPLC-ESI-MS/MS

Concentrations of analytes obtained from HPLC-ESI-MS/MS were normalized to account for the actual volume of water extracted with solid phase extraction (SPE) and the volume of sample used for the analysis:

$$normalised\ concentration\ [ng \cdot L^{-1}] = \frac{1000}{mv} \cdot mc \cdot 2$$

Where mv is the measured volume of water used for the SPE in mL, and mc is the measured concentration obtained from the LC-MS/MS output in µg/L. The assumed volume used for the measured concentrations was 1000 mL, and the result was multiplied by 2 to account for the 500 µL sample volume used for the HPLC-ESI-MS/MS analysis.

References:

ICH Expert Working Group (ICH)., 2005. ICH Harmonised Tripartite Guideline. Validation of analytical procedures: text and methodology Q2 (R1).

Machado, K.C., Grassi, M.T., Vidal, C., Pescara, I.C., Jardim, W.F., Fernandes, A.N., Sodré, F.F., Almeida, F.V., Santana, J.S., Canela, M.C., Nunes, C.R.O., Bichinho, K.M, Severo, F.J.R., 2016. A preliminary nationwide survey of the presence of emerging contaminants in drinking and source waters in Brazil. Science of the Total Environment 572, 138–146. https://doi.org/10.1016/j.scitotenv.2016.07.210

Section S2: Description of the manual validation procedure for identifying bat calls

Manual verification and identification of bat calls was done using the following identification guides:

- Barataud, M., 2020. Acoustic ecology of European bats: Species identification, study of their habitatis and foraging behaviour, 2nd ed. Biotope éditions, Mèze - Muséum nationale d'Histoire naturelle, Paris.
- Russ, J. (ed.), 2021. Bat Calls of Britain and Europe: A Guide to Species Indentification. Pelagic Publishing.

With the assistance of descriptions and specific information from the following references:

- Bayrisches Landesamt für Umwelt., 2020. Bestimmung von Fledermausrufaufnahmen und Kriterien für die Wertung von akustischen Artnachweisen: Teil 1. Fledermausschutz in Bayern.
- Skiba, R., 2003. Europäische fledermäuse. Westarp Wissenschaften, Hohenwarsleben.
- Lewanzik, D., Goerlitz, H.R., 2021. Task-dependent vocal adjustments to optimize biosonar-based information acquisition. Journal of Experimental Biology 224, jeb234815. https://doi.org/10.1242/jeb.234815
- Pfalzer, G., 2007. Verwechslungsmöglichkeiten bei der akustischen Artbestimmung von Fledermäusen anhand ihrer Ortungs-und Sozialrufe. Nyctalus 12, 3–14.
- Pfalzer, G., Kusch, J., 2003. Structure and variability of bat social calls: implications for specificity and individual recognition. Journal of Zoology 261, 21–33. https://doi.org/10.1017/S0952836903003935
- Russo, D., Ancillotto, L., Cistrone, L., Korine, C., 2016. The buzz of drinking on the wing in echolocating bats. Ethology 122, 226–235. https://doi.org/10.1111/eth.12460
- Griffiths, S.R., 2013. Echolocating bats emit terminal phase buzz calls while drinking on the wing. Behavioural Processes. 98, 58–60.https://doi.org/10.1016/j.beproc.2013.05.007

To perform the manual validation and identification, each minute which had been recognized by Kaleidoscope as containing a bat call was examined individually. We classified the calls into 11 phonic groups, as call identification to species level is not reliable in all cases due to overlapping echolocation parameters. Each phonic group (listed below in Table S2.1) present in the minute was identified using the resources listed above. Social calls and habitat-specific contexts (i.e., more or less cluttered forest vegetation at streams) were also taken into account to assist with identification. If a call series could not be attributed to a specific group, the phonic group was defined as "Bat species". Call sequences within the minute required at least one call identified by Kaleidoscope (i.e. labelled with zero-cross points) to be considered for manual identification, and each phonic group was only recorded once per minute, regardless of how many call sequences they emitted within the minute.

Table S2.1: List of bat phonic groups identified and grouped manually from recordings. Although the descriptions in identification guides (listed above in main text) were the main resources used for identification, specific requirements and thresholds used for each group are highlighted here. FM stands for frequency modulated call types, and QCF for quasi-constant frequency call types.

Phonic group	Bat species included	Specific identification requirements
MYODAU	*Myotis daubentonii*	"S"-shaped sigmoid myotis curve, rarely higher than 85 kHz, interference patterns in call from water surface, strength equally distributed across call
MYOBRAMYS	*Myotis brandtii and Myotis mystacinus, considered Myotis* cf. *brandtii*	Slightly sigmoid, but steeper and sometimes higher than MYODAU, with strength concentrated in lower part of call
MYOsp	*Myotis bechsteinii, Myotis emarginatus, Myotis nattereri*	Other FM Myotis calls
MYOMYO	*Myotis myotis*	Relatively low and long FM calls, sometimes with small wiggles. Strength of call closest to lowest frequency
BARBAR	*Barbastella barbastellus*	Quiet calls with alternative call types, convex and concave (both must be present for ID)
PIPPIP	*Pipistrellus pipistrellus*	Between 41 and 51 kHz, sequences with calls under 40 kHz are PIPNAT and over 52 are PIPPYG
PIPPYG	*Pipistrellus pygmaeus*	Over 51 kHz
PIPNAT	*Pipistrellus nathusii*	Under 41 kHz
NYCNOC	*Nyctalus noctula*	Two call types "plip" and "plop" may be visible, with at least 2 kHz difference. Flat QCF should be under 20 kHz
Nyctaloid	*Nyctalus leisleri, Eptesicus serotinus, Eptesicus nilsonii, Vespertillio murinus*	Other Nyctaloid-type QCF calls
PLEsp	*Plecotus auritus, Plecotus austriacus*	Quiet, nasal sound with strong second harmonic

Feeding buzzes were identified within each minute and tallied, producing a total number of feeding buzzes per stream. This was used in the feeding rate calculation for each phonic group. Feeding buzzes (as demonstrated in Russ 2021) were defined as call sequences containing an approach phase with a decreasing inter-call interval, a first buzz phase of rapid calls, and a final buzz phase with rapid calls decreasing in frequency (Figure S3). A feeding buzz was only counted if a call prior to the approach phase (within 20 calls prior to a ½

decrease in inter-call interval time) or a call within 10 calls after the final buzz phase was identified by Kaleidoscope, to avoid counting feeding buzzes of bats further away from the stream. Feeding buzzes without the final buzz (i.e., likely unsuccessful) were not counted.

We also identified drinking buzzes, to avoid mislabelling them as feeding buzzes. Drinking buzzes are emitted as bats approach a water surface to drink, and were defined as feeding buzzes without the final buzz phase, a long pause after the buzz and a long and steady approach phase (Griffiths, 2013; Russo 2016; Figure S4). These were also tallied for each minute and assigned to phonic groups with the same requirements as feeding buzzes.

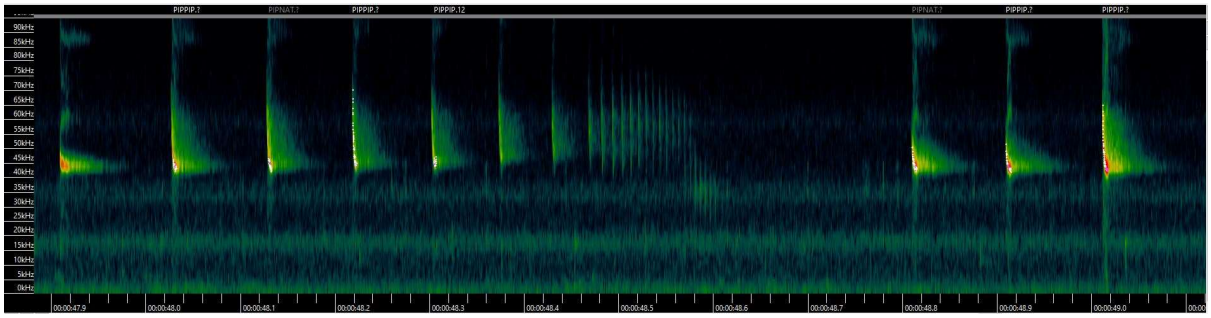

Figure S3: A feeding buzz emitted by a common pipistrelle (*Pipistrellus pipistrellus*) flying above a stream. The approach phase, Buzz I and Buzz II (lower frequency) of the terminal phase are clearly visible. The search-phase calls continue after the feeding buzz finishes.

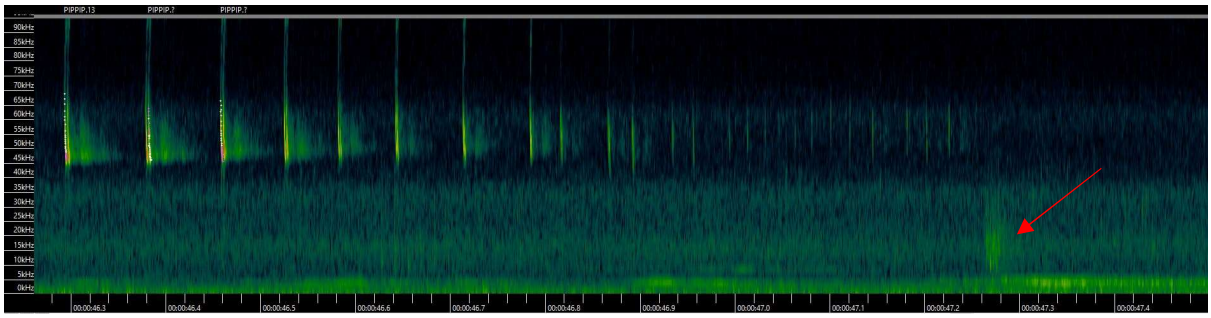

Figure S4: A drinking buzz emitted by a common pipistrelle (*Pipistrellus pipistrellus*) drinking from a stream. The approach phase and Buzz I phase are visible, but the lower-frequency terminal buzz characteristic of feeding buzzes is missing. An audible water splash is shown by the red arrow. There is a longer pause between the buzz and the next search-phase calls (not visible).