Micropollutants as Stressors in Aquatic and Terrestrial Systems: Their Spatial Distribution and Effects on Non-target Organisms, Biodiversity and Ecosystem Functions

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> “Now I realise that there is no righteous path, it’s just people trying to do their best in a world where it is far too easy to do your worst.”
>
> − Castiel, *Supernatural*

LIST OF PUBLICATIONS

The present thesis is a cumulative dissertation based on the following peer-reviewed publications:

1. **Mauser, K.M.**, Wolfram, J., Spaak, J.W., Honert, C., & Brühl, C.A. (2025). Current-use pesticides in vegetation, topsoil and water reveal contaminated landscapes of the Upper Rhine Valley, Germany. *Communications Earth & Environment*, *6*(1), 166. https://doi.org/10.1038/s43247-025-02118-2
2. Honert, C., **Mauser, K.M.**, Jäger, U., & Brühl, C.A. (2025). Exposure of insects to current use pesticide residues in soil and vegetation along spatial and temporal distribution in agricultural sites. *Scientific Reports*, *15*(1), 1817. https://doi.org/10.1038/s41598-024-84811-4
3. **Mauser, K.M.,** Dekker, T., Spaak, J.W., Elsässer, T. & Brühl, C.A., (2026). Current-use pesticide exposure of riparian ecosystems – stream buffer or non-target habitat? *Science of The Total Environment* 1019, 181503. https://doi.org/10.1016/j.scitotenv.2026.181503
4. **Mauser, K.M.,** Paudel, S., Sigmund, O., Entling, M.H., Ott, J. & Brühl, C.A., (2025). Effects of chemical and hydrological stress on the wing morphology of a damselfly. *Environmental Entomology* 54, 1335–1347. https://doi.org/10.1093/ee/nvaf112
5. **Mauser, K.M.**, Baumeyer, J., Eshghi, S., Gorb, S.N., Manfrin, A., & Brühl, C.A. (2025). Short-term mating success in relation to fluctuating wing asymmetry in the male azure damselfly *Coenagrion puella*. *Basic and Applied Ecology*, *86*, 55–65. https://doi.org/10.1016/j.baae.2025.05.002
6. Mutel, S., Entling, M.H., **Mauser, K.M.,** Spani, F., Thomas, C., Ogbeide, C., Bundschuh, M., Fiolka, F., Middendorf, F., Schulz, R., Manfrin, A., (2026). Flooding affects fluctuating asymmetry but not growth of a riparian orbweaving spider. *Ecological Entomology* een.70061. https://doi.org/10.1111/een.70061

TABLE OF CONTENT

ABBREVATION LIST

Bti	*Bacillus thuringiensis israelensis*
CUP	Current-use pesticide
DT_{50}	Dissipation time, half-life
FA	Fluctuating asymmetry
HQ	Hazard quotient
LC_{50}	Lethal concentration 50%
NOEC	No observed effect concentration
NRMSE	Normalized root mean squared error
MRQ	Mixture risk quotient
PNEC	Predicted no-effect concentration
RQ	Risk quotient
RNBZ	Riparian non-target buffer zone

THESIS SUMMARY

English abstract

Agricultural intensification and climate change increase pressure on biodiversity, with pesticides dispersing beyond treated fields into non-target areas. This thesis investigated three major stressors relevant to aquatic-terrestrial ecosystems: pesticide contamination, altered hydrological regimes, and the mosquito control agent *Bacillus thuringiensis israelensis* (Bti). It addresses the lack of data on the environmental presence and spatio-temporal dynamics of pesticide contamination and on how different environmental stressors influence trait development in aquatic-terrestrial organisms, with potential consequences for reproduction and higher trophic levels. This thesis combines multi-scale mapping of pesticide residues with computer-vision analysis of morphological traits of a damselfly under controlled exposures to each stressor. It uses a tiered approach linking field observations, experiments, and validation across scales for mechanistic and ecological insight. Large-scale pesticide monitoring across the Upper Rhine Valley in Germany revealed pronounced spatial gradients and pervasive background contamination. Prediction maps identified regional hotspots of mixture complexity, and contamination of conservation areas far from agricultural land. Temporal monitoring demonstrated chronic contamination in soils and seasonal peaks in vegetation during periods of high biological activity. At finer scales, riparian non-target buffer zone assessment showed high contamination regardless of buffer width, challenging their function as effective buffers or refuges for non-target organisms. Together, these studies showed pesticide mixtures to be a widespread and persistent characteristic in non-target habitats. Experimental exposures of damselfly larvae to an insecticide, Bti, and altered hydrology produced distinct trait signatures in wing morphology, including size, structure and asymmetry indicating changes in developmental stability. In two damselfly populations, morphological predictors of short-term mating success varied between sites, indicating that trait-fitness relationships are environmentally mediated. Furthermore, changes in emerging insect prey quality influenced higher trophic levels by affecting the developmental stability in riparian spiders. This research reveals that environmental stressors can alter morphological traits in aquatic organisms that are linked to important terrestrial processes such as mating success and predator-prey interactions. The widespread detection of pesticide mixtures in non-target areas suggests exposure pathways that current regulatory frameworks may not adequately address. The findings also emphasize the need for integrated management approaches that consider cross-ecosystem connectivity when assessing the ecological implications of micropollutants and climate change.

German abstract (Zusammenfassung)

Die Intensivierung der Landwirtschaft und der Klimawandel setzen die Biodiversität unter Druck, wobei sich Pestizide über behandelte Felder hinaus in Nicht-Zielgebiete ausbreiten. Diese Dissertation untersuchte drei Hauptstressoren für aquatisch-terrestrische Ökosysteme: Pestizidkontamination, veränderte hydrologische Regime und das Stechmücken-Bekämpfungsmittel *Bacillus thuringiensis israelensis* (Bti). Sie adressiert den Mangel an Daten zum Umweltvorkommen und zur räumlich-zeitlichen Dynamik von Pestiziden sowie zu den Auswirkungen verschiedener Stressoren auf die Merkmalsausprägung aquatisch-terrestrischer Organismen mit möglichen Folgen für Fortpflanzung und höhere Nahrungsebenen. Die Arbeit verbindet mehrskalige Erfassung von Pestizidrückständen mit computergestützter Analyse morphologischer Merkmale einer Kleinlibelle unter kontrollierten Expositionen. Ein mehrteiliger Ansatz verknüpft Feldbeobachtungen, Experimente und Validierung über verschiedene Skalen für mechanistische und ökologische Erkenntnisse. Großflächiges Pestizid-Monitoring im Oberrheintal deckte ausgeprägte räumliche Gradienten und weiträumige Hintergrundkontamination auf. Vorhersagekarten identifizierten regionale Hotspots komplexer Pestizidmischungen und belastete Schutzgebiete fernab der Landwirtschaft. Das zeitliche Monitoring zeigte chronische Bodenkontamination und saisonale Belastungshöhepunkte in der Vegetation während Perioden biologisch hoher Aktivität. Detailanalysen von Gewässerrandstreifen ergaben hohe Belastungen unabhängig von ihrer Breite und stellten ihre Wirksamkeit als Puffer oder Habitat in Frage. Die Studien belegten, dass Pestizidmischungen weit verbreitet und dauerhaft in Nicht-Zielhabitaten vorkommen. Die experimentelle Exposition von Kleinlibellenlarven gegenüber einem Insektizid, Bti und veränderten hydrologischen Regimen führten zu unterschiedlichen Veränderungen der Flügelmorphologie in Größe, Struktur und Asymmetrie, die auf beeinflusste Entwicklungsstabilität hinweisen. In zwei Libellenpopulationen variierten morphologische Indikatoren für den Paarungserfolg zwischen den Standorten, was zeigt, dass Merkmal-Fitness-Beziehungen umweltabhängig sein können. Qualitätsveränderungen emergierender Insekten wirkten sich auf höhere Nahrungsebenen aus und beeinträchtigten die Entwicklungsstabilität von Spinnen. Die Dissertation zeigt, dass Stressoren morphologische Merkmale aquatischer Organismen verändern können, die für wichtige terrestrische Prozesse wie Paarungserfolg und Räuber-Beute-Beziehungen relevant sind. Der Nachweis von weitverbreiteten Pestizidmischungen in Nicht-Zielgebieten weist auf Belastungspfade hin, die in der aktuellen Regulierung möglicherweise unzureichend berücksichtigt werden. Die Ergebnisse unterstreichen die Notwendigkeit integrierter Managementansätze, die bei der Bewertung ökologischer

Auswirkungen von Schadstoffen und Klimawandel die Vernetzung zwischen Ökosystemen einbeziehen.

1 INTRODUCTION

Global assessments report alarming declines in insect populations, raising concerns for biodiversity and ecosystem services (Sánchez-Bayo & Wyckhuys, 2021). In Germany, a long-term study found that flying insect biomass in protected areas dropped by more than 75% over 27 years (Hallmann et al., 2017). Such widespread insect decline is a major ecological crisis, and understanding its causes is a key scientific and societal challenge (Dicks et al., 2021). These declines are linked to many human-caused factors, including climate change, habitat fragmentation, and intensive agriculture with high pesticide use (Wagner, 2020). Pesticides do not remain confined to treated fields but instead spread into surrounding areas via spray drift, run-off, and atmospheric transport (Reichenberger et al., 2007). Long-range atmospheric transport can carry these contaminants far beyond their application areas, as evidenced by pesticide detections in remote regions (Vorkamp & Rigét, 2014). Consequently, pesticide residues are frequently found in off-field "non-target" areas (Brühl et al., 2024; Linhart et al., 2021). Residue studies of soils across Europe, detected pesticides even years after application, underlining this widespread and long-term environmental contamination (Silva et al., 2019). Pesticides have been detected in the ethanol of Malaise trap insect samples from protected areas, underscoring their broad reach into ecosystems intended for conservation (Brühl et al., 2021).

There is growing evidence that pesticide exposure contributes significantly to biodiversity loss and can be directly harming beneficial insects and other wildlife (Geiger et al., 2010). A global expert review identified agricultural pesticide use as a key driver of pollinator declines, alongside other stressors (Dicks et al., 2021). An additional concern is that wild organisms are exposed not just to single chemicals but to complex mixtures of many pesticide residues. For example, a study in France detected dozens of co-occurring pesticide compounds in agricultural soils, revealing that pesticides occurring individually are rather the exception (Froger et al., 2023). Such mixtures can pose risks through synergistic interactions even if individual chemicals are at low concentrations (Gandara et al., 2024). However, current regulatory risk assessments do not adequately address these realistic exposure scenarios (Brühl & Zaller, 2019). Typically, pesticides are evaluated and regulated one by one, and the combined toxicity of pesticide cocktails is largely ignored (Weisner et al., 2021). Moreover, some important exposure pathways are overlooked: for instance, terrestrial contamination of soil and wild vegetation is usually not accounted for in insect risk assessments, even though many insects come into contact with these contaminated matrices (Uhl & Brühl, 2019) exposing pronounced, often overlooked species (Sedlmeier et al., 2025). In response to such gaps, international agencies have called for expanded environmental monitoring of pesticides after approval and better consideration of chronic and mixture exposures (UNEP, 2022). Realistic, landscape-scale studies are urgently needed to quantify how much pesticide residue wildlife is actually exposed to and how this realistic exposure affects organisms (Brühl et al., 2024). In response, this thesis established three field studies that measure real-world exposure across non-target zones in the Upper Rhine Valley: a large-scale assessment (Paper 1), a year-round temporal study (Paper 2), and an in-depth analysis of small-scale gradients in riparian zones and adjacent streams (Paper 3).

Beside terrestrial ecosystems, pesticide commonly enter streams, ponds, and other water bodies adjacent to fields through processes like run-off, drainage, spray drift and erosion (Prosser et al., 2020). In intensively farmed regions, water bodies can contain numerous pesticide residues, and insecticide pollution has become a widespread threat to freshwater biodiversity (Stehle & Schulz, 2015). Riparian non-target buffer zones, as an interface between land and water, are meant to serve as buffers that protect streams from agricultural contaminants, but they are often too narrow or ineffective to fully prevent pesticide entry (Vormeier et al., 2023). At the same time, they are important habitats for aquatic-terrestrial organisms (Lind et al., 2019). Pesticide contamination in streams, however, can reduce the emergence of aquatic insects, thereby limiting food availability for riparian insectivores and potentially triggering trophic cascades in this ecosystems (Bundschuh et al., 2022).

At the same time, pesticides are not the only aquatic stressor: global climate change is altering hydrological regimes in Europe and flood magnitudes have shifted over the past decades, with regionally increasing or decreasing peak discharges (Blöschl et al., 2019). Globally, more frequent and intense droughts and floods are expected in many regions (Intergovernmental Panel On Climate Change (IPCC), 2023). In floodplain wetlands, reduced water levels during droughts can initially concentrate aquatic organisms, but prolonged drying severely diminishes aquatic insect populations and other sensitive species (Herbst et al., 2019). Conversely, extreme flooding can suddenly change water quality and connectivity, modifying how pollutants spread and how aquatic communities function (Gmitrowicz-Iwan et al., 2020; Milner et al., 2018). The effects of future hydrological alterations in aquatic-terrestrial environments on non-target organisms, and the extent to which they add to pesticide-related stress, remain largely unknown (Arenas-Sánchez et al., 2016).

In addition to synthetic-chemical pesticides and climate change, the use of biocides represents a further challenge. An example is the large-scale application of *Bacillus thuringiensis israelensis* (Bti) in seasonally flooded wetlands in the Upper Rhine Valley, Germany, where mosquito breeding sites are treated repeatedly between April and September by backpack sprayers or by aerial application via helicopter (Becker et al., 2018). Historically, mosquito control shifted in Germany from promoting natural predators such as dragonflies and fish (Lamborn, 1890) to petroleum oils in the 1920s-1930s, synthetic pesticides in the 1950s-1960s, and finally to Bti from the 1980s onward (Becker, 1997). Looking at the history of Mosquito control, it represents a textbook full circle example for different ways of managing insect populations, from enhancing natural pest control to synthetic pesticides and biocides. Although Bti is labelled as highly selective for mosquito larvae, a growing body of evidence shows that it can inadvertently reduce non-target midge larvae (Chironomidae) in addition to the target mosquitoes, thereby lowering prey availability for higher trophic levels such as dragonflies and damselflies (Brühl et al., 2020). A recent mesocosm experiment with a stressor-combination of Bti and altered hydrological regimes showed that the effects of Bti on aquatic and riparian insects can depended on flooding regimes, highlighting that climate change may also modulate Bti's non-target impacts (Bauspiess et al., 2025). Understanding these interactions is even more important in the light of diseases that can be transmitted by mosquitoes (malaria, dengue fever), as these are likely to become more widespread because of climate change (Zhang et al., 2024) and must therefore be taken into account in mosquito management strategies. In summary, aquatic organisms in agricultural landscapes face multiple simultaneous stressors such as pesticides, nutrient enrichment, hydrological extremes, and mosquito control agents. These stressors may together impact biodiversity, which motivates the organism-focused work that follows on cross-ecosystem linkages.

Crucially, impacts on aquatic insects can carry over to affect terrestrial ecosystems due to their coupling (Bundschuh et al., 2022). Aquatic and terrestrial ecosystems are tightly linked via reciprocal resource fluxes (e.g. emergence of aquatic insects and terrestrial leaf litter input), meaning that disturbances in one can propagate to the other (Bartels et al., 2012). Riparian zones support a high diversity and abundance of semi-aquatic organisms, such as dragonflies (Nagy et al., 2019) and amphibians (Crawford & Semlitsch, 2007), while also providing habitat for specialized terrestrial organisms including ants, spiders, bats, and birds (Carrasco-Rueda & Loiselle, 2019; Larsen et al., 2010; Ramey & Richardson, 2017). Dragonflies and damselflies (order Odonata) are a particularly relevant group for examining such cross-ecosystem impacts

(Samways et al., 2025). Odonates have an amphibious life cycle: they spend their juvenile stages as aquatic larvae in freshwater, but transition into terrestrial flying adults. Because of this biphasic life cycle, sublethal stress experienced during the aquatic larval stage can carry over to influence the individual's fitness in the terrestrial adult (Tüzün & Stoks, 2018). One common approach to quantify this sublethal stress is by measuring fluctuating asymmetry (FA) which are the small random differences between left and right bilateral structures of an organism (Benítez et al., 2020). FA is widely used as an indicator of developmental stability, with the idea that higher asymmetry reflects greater stress or developmental instability (Benítez et al., 2020). Field studies have shown that insects living under stress exhibit increased FA in traits like wing shape or size (Friedli et al., 2020; Hierlmeier et al., 2022). In damselflies, for example, some experiments have demonstrated that stressed larvae can develop more asymmetrical wings compared to controls (Hardersen & Frampton, 1999; Stoks, 2001) although some other studies did not find this relationship (Campero et al., 2008; Ryazanova & Polygalov, 2013). In summary environmental stressors in freshwater, including pesticides or changes in habitat quality, were shown to affect odonate larval development and physiology (Dinh et al., 2016; Stahl & Johansson, 2024). However, comprehensive synthesis remains limited and cross-study comparability of FA is hindered by heterogeneous protocols, trait definitions, and error treatment (Palme & Strobeck, 2003). Therefore, a study was set up to investigate the physiological response of a damselfly and its wing development, under aquatic chemical stress (insecticide and Bti) and hydrological stress reflecting a climate-change scenario (Paper 4), using consistent and modern computer-vision tools.

After metamorphosis, adult dragonflies and damselflies act as top predators of aerial insect prey and are highly mobile in the terrestrial environment (Combes et al., 2012). Flight in Odonata depends critically on specialised wing features such as corrugation, compliant venation, and hinge architecture (i.e. ridged wing surface, flexible veins and mobile joints) that enable aerodynamic efficiency and stability (Bomphrey & Walker, 2022). These adaptations support essential behaviours such as prey capture and mating (Carchini et al., 2005; Combes et al., 2012). This raises the question of whether larval stress that leads to even subtle wing deformities or weakness would impair an individual's flight performance, with potential consequences for its feeding efficiency and mating success. In this context, fluctuating asymmetry may correlate with other impairments in wing physiology that affect aerodynamics and mechanical stability, and it may also constitute a morphological deformity that compromises flight. However, linking sublethal physiological effects directly to fitness outcomes is complex. The relationship between FA and actual reproductive success after emergence is not straightforward and led to mixed results when investigated (Banks & Thompson, 1985; Carchini et al., 2000, 2005). Since the importance of wing morphology for mating success may vary with species-specific mating strategies such as territorial defence or non-territorial searching behaviour (Fincke et al., 1997) and with habitat features (Outomuro et al., 2016), a comprehensive understanding of this relationship is still lacking. To benefit of comparability, a further study on wing morphology of a damselfly and male mating success was conducted with the same computer-vision wing assessment (Paper 5). To additionally link the quality of emergent aquatic insects to terrestrial food webs and to test FA in another trait-stressor context, we analysed how altered hydrology changed prey emergence and the developmental stability of a predatory spider (Paper 6).

In summary, aquatic-terrestrial systems are characterised by pervasive micropollutant residues, mixture exposure, and concurrent stressors from biocontrol and changing hydrology, yet assessment still proceeds in isolation. What is needed is a clearer link from micropollutant exposure to larval developmental stability, adult performance, and terrestrial outcomes such as mating success. It should also extend to food-web effects driven by changes in the quality of emerging aquatic prey for terrestrial predators as well as the changes in emerging predator performance on terrestrial prey. The next chapters outline how this thesis addresses these needs through Papers 1 to 6, detailing the study area, study species, objectives, structure, and methods.

1.1 Study area

As described in Paper 1, the Upper Rhine Valley in Germany ranges from Basel in Switzerland to Bingen in Germany for around 270 km length. It is framed by the Palatinate Forest and Odenwald in the north and Vosges Mountains and Black Forest in the south. The Upper Rhine Valley is home to over six million inhabitants (Deutsch-Französisch-Schweizerische Oberrheinkonferenz, 2022) and features a polycentric network of cities concentrated in the valley lowlands and connected by dense transport corridors (Scholze et al., 2020). The valley shows a mean air temperature of 8.6 °C, mean annual precipitation of 735 mm and 15 large cities with more than 100,000 people (Uehlinger et al., 2009). The region is identified as one of Germany’s main climate-change hotspots, with mid-century projections showing above-average increases in heat-wave days, tropical-night frequency and summer drought intensity compared to other German regions (Crespi et al., 2023). Agriculture is a dominant land-use, especially in the Southern Palatinate (Figure 1, Blickensdörfer et al., 2021), shaping the landscape structure and visual character in addition to biodiversity patterns and habitat fragmentation (Schnitzler et al., 2005). The region including bordering low mountain ranges hosts a dense mosaic of protected areas, including Natura 2000 sites (FFH and special protection areas for birds), nature conservation areas, national parks, biosphere reserves, nature parks and landscape protection areas (Bundesamt für Naturschutz, 2025). As one of the most intensively used river basins in Europe, the Rhine Valley reflects a long legacy of hydromorphological alterations, high population pressure and land-use intensification, with ongoing ecological restoration efforts (Uehlinger et al., 2009). The transboundary Upper Rhine aquifer (estimated 135 billion m^3) is Western Europe's largest groundwater reservoir (Association de droit local (Bas-Rhin, Haut-Rhin et Moselle), 2025). Accordingly, since the 1990s the region has seen cross-border cooperation among German, French and Swiss authorities to monitor and mitigate pollution in shared waters and ecosystems (Association de droit local (Bas-Rhin, Haut-Rhin et Moselle), 2025). Agricultural activities define regional identity and the region's scenic and especially viticulture drives local tourism (Tafel et al., 2023). Thus, the Upper Rhine Valley's combination of intensive agricultural use, high environmental heterogeneity and conservational challenges make it an especially suitable case-study region for investigating agriculture-driven environmental pressures and their impacts on biodiversity.

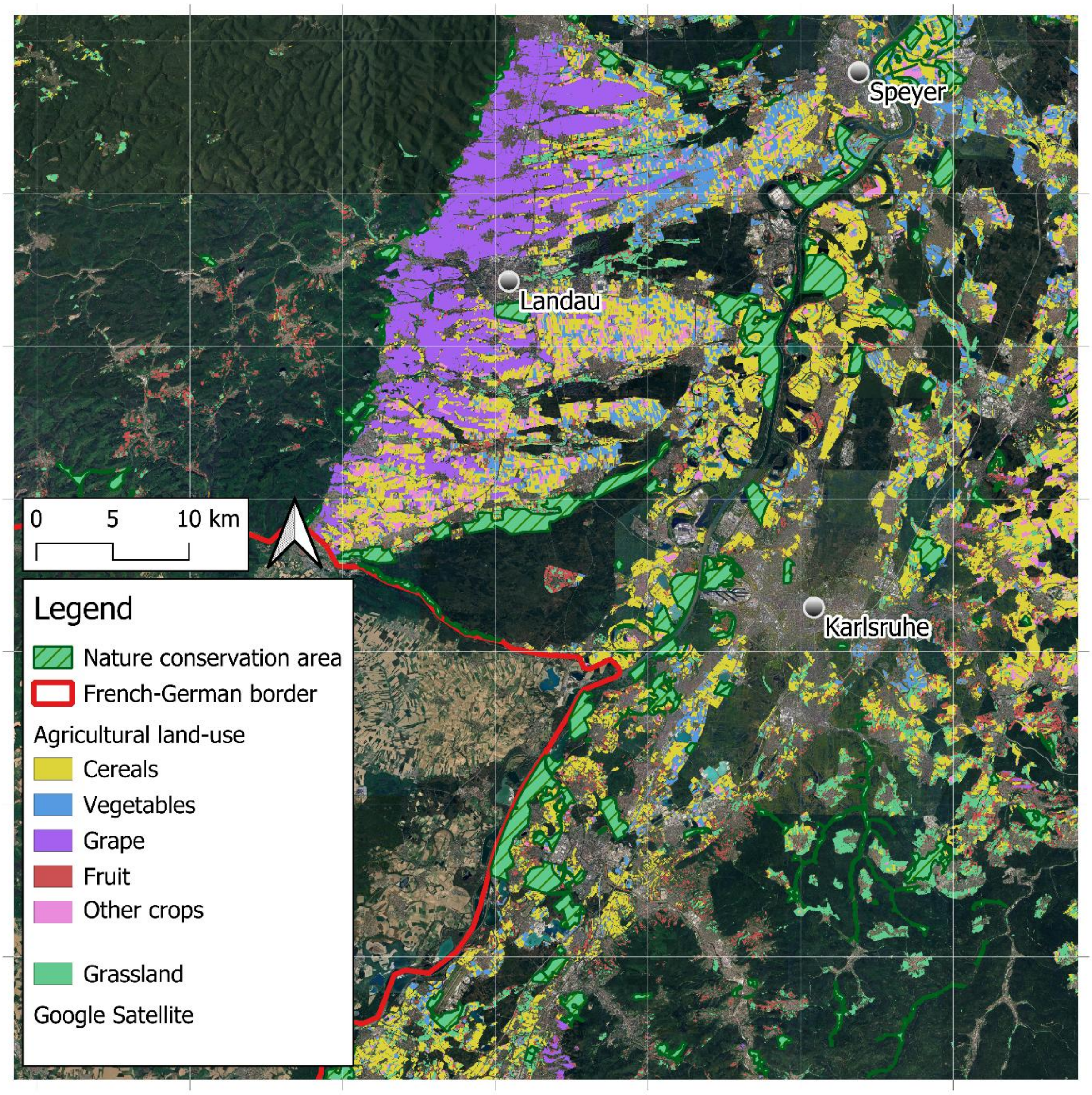

Figure 1. Aerial view with the agricultural land-use in a section of the Upper Rhine Valley around Landau, Germany, where a major part of the studies of this thesis took place. The map features grassland (green), cereals (yellow), vegetables (blue), grape (purple) and fruit (red). Several nature conservation areas (green with stripes) can be found in the region. The map shows the exceptional proportion of grape at the foot of the Palatinate Forest, which merge into mixed crops towards the Rhine in the east. Land-use from Blickensdörfer et al., (2021). Aerial view: © Google & TerraMetrics, 2025.

1.2 Species profile of *Coenagrion puella* (Azure Damselfly)

The representative damselfly examined in this dissertation was *Coenagrion puella* (Linnaeus 1758). As described in Paper 4 and 5, *C. puella* of the suborder Zygoptera and family of Coenagrionidae is a widespread damselfly in Central Europe, generally univoltine, and inhabits a broad range of habitats from natural still waters to agricultural and urban environments. (Wildermuth & Martens, 2019). It has a larval length of 17 to 21 mm during the last instar and an adult body length of 33-35 mm. *C. puella* larvae hatch between mid-July and late August, overwinter in instars 6 to 9, and emerge the following summer (Waringer & Humpesch, 1984). Adults can be observed between the beginning of May to the beginning of August in central Europe (Wildermuth & Martens, 2019). Male adults show a black pattern on a blue head and thorax, whereas females share a base colour of usually yellow-green, less commonly blue (Wildermuth & Martens, 2019). Due to its generalism, high local abundance, broad distribution, and well-documented life history, *C. puella* meets important criteria for effective bioindicators, including cost-efficient sampling and consistent population-level responses to environmental changes (Holt & Miller, 2010) e.g. for various anthropogenic stressors in freshwater ecosystems. In terms of behaviour, males of *C. puella* are non-territorial and characterized by scrambling competition, actively looking for partners (Fincke et al., 1997). After mating, males try to improve their success by remaining in tandem with females to prevent them from disturbances by rival males (Cordero et al., 1995).

Figure 2. Male azure damselfly *Coenagrion puella*, photographed on the 18th of May 2024 in the “Hördter Rheinaue” a riparian nature conservation area in the study area of this thesis. The individual shows a damaged right hindwing.

2 OBJECTIVES AND THESIS STRUCTURE

This thesis assessed the distribution and gradients of pesticide contamination across aquatic-terrestrial boundaries and investigated how micropollutants and hydrological stressors in aquatic systems affect trait-mediated ecological responses in non-target organisms (Figure 3).

The first objective was to characterise aquatic-terrestrial pesticide contamination and its spatial and temporal gradients. Surface water, topsoil and vegetation of non-target areas in the Upper Rhine Valley and the adjacent mountain regions were systematically sampled at a large landscape-scale and prediction maps were created based on the surrounding land-use (Paper 1). In addition, samples of topsoil and vegetation across three different cultivation types were collected monthly over a year to investigate local gradients and temporal fluctuations (Paper 2). Furthermore, samples and local gradients across the riparian non-target buffer zone were investigated and mixture patterns were analysed (Paper 3). The detected number of CUPs and their sum concentration were discussed with regard to possible effects on the aquatic and terrestrial environment.

The second objective was to investigate how aquatic stressors affect organisms in the aquatic system by examining traits that are also carried over to the terrestrial stage through emergence (Paper 4). The scrambling damselfly *C. puella* was exposed to the insecticide chlorantraniliprole, the mosquito control agent Bti was introduced to trigger changes in aquatic food webs and hydrological regimes were altered to simulate climate change. After emergence, the wing morphology and asymmetry was assessed via modern computer vision and effects of stressors were evaluated (Paper 4). Wing morphology was used as a proxy for developmental stability during the larval stage. Reduced developmental stability may impair the functional traits of aquatic insects and thereby influence trophic interactions, for example by altering vulnerability to aquatic predators or competitive abilities within the aquatic food web.

The third objective was to investigate the terrestrial trait-fitness linkage in emerging *C. puella*, using mating success as a model context, and to assess how changes in prey availability and quality affect terrestrial consumers (Paper 5 & 6). First, short-term mating success was related to morphological traits in *C. puella*, comparing two populations from contrasting land-use contexts (Paper 5). Second, spider developmental stability was analysed as an indicator of energetic condition in response to changes in the abundance of emergent aquatic prey caused by altered hydrological regimes (Paper 6). Trait data across studies was discussed to evaluate potential implications for population dynamics, cross-ecosystem linkages and effects on the terrestrial food web. The relevance for ecological monitoring and conservation was discussed.

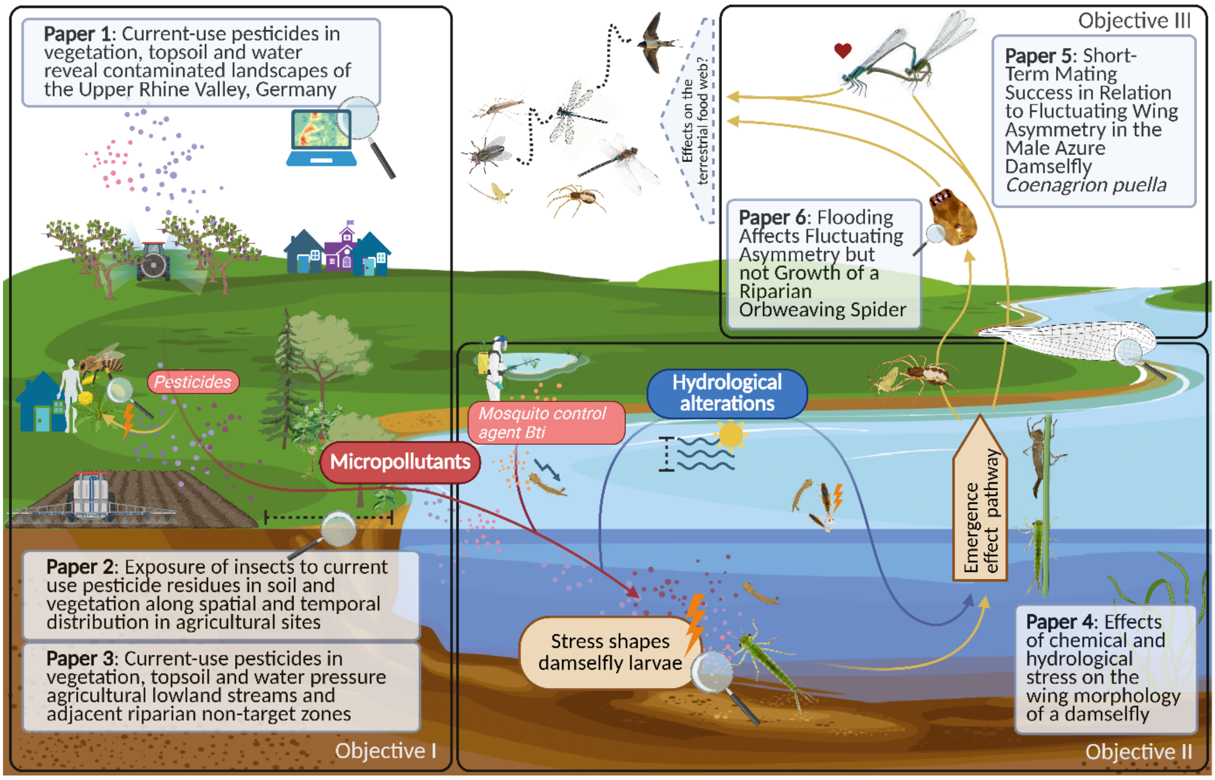

Figure 3. Conceptual overview of the thesis structure, including Objective I - III. A cross-ecosystem framework linking aquatic micropollutant exposure and hydrological alterations to trait-mediated effects in organisms of the aquatic-terrestrial interface and their consequences for terrestrial reproduction and food webs. Created with biorender.com

3 METHODS OVERVIEW

3.1 Experimental framework across spatial scales

A core concept of the underlying project SystemLink was to use different unique experimental facilities and to perform an integrative analysis across multiple spatial scales (Manfrin et al., 2023). Environmental studies face the challenge of disentangling the effects of multiple possible interacting variables within complex ecosystems. This dissertation addressed this complexity through a stepwise approach across spatial and experimental scales (Figure 4). Field-scale studies were first used to assess real-world anthropogenic pressures in aquatic and terrestrial systems, focusing on the occurrence and composition of pesticide mixtures (Paper 1, 2 & 3). These observations enabled the identification and contextualisation of contamination patterns, placed those patterns in the context of comparable European field studies and informed the selection of a representative pesticide for the subsequent laboratory experiment. To isolate specific effects under controlled conditions, a batch-scale laboratory experiment was conducted in which damselfly larvae were exposed to a selected insecticide (Paper 4). This reduction of environmental complexity enabled methodological refinement in quantifying wing asymmetry and linking trait shifts directly to a defined chemical stressor. Building on this, the experimental system was transferred to the site-scale using a flood plain mesocosm (Paper 4) where additional stressors such as Bti and hydrological alterations were introduced under semi-natural conditions. To widen the view, a now again field-based mating study examined whether previously identified morphological traits influenced short-term mating success in two damselfly populations (Paper 5). Complementary, at the site-scale in a riparian stream mesocosm, it was investigated how altered hydrological regimes influenced the number of emerging insects and therefore developmental stability of spiders, extending the trait-based approach of fluctuating asymmetry across taxa (Paper 6). This tiered approach moved from field observations to controlled experiments and returned to field validation, enabling both mechanistic understanding and ecological relevance.

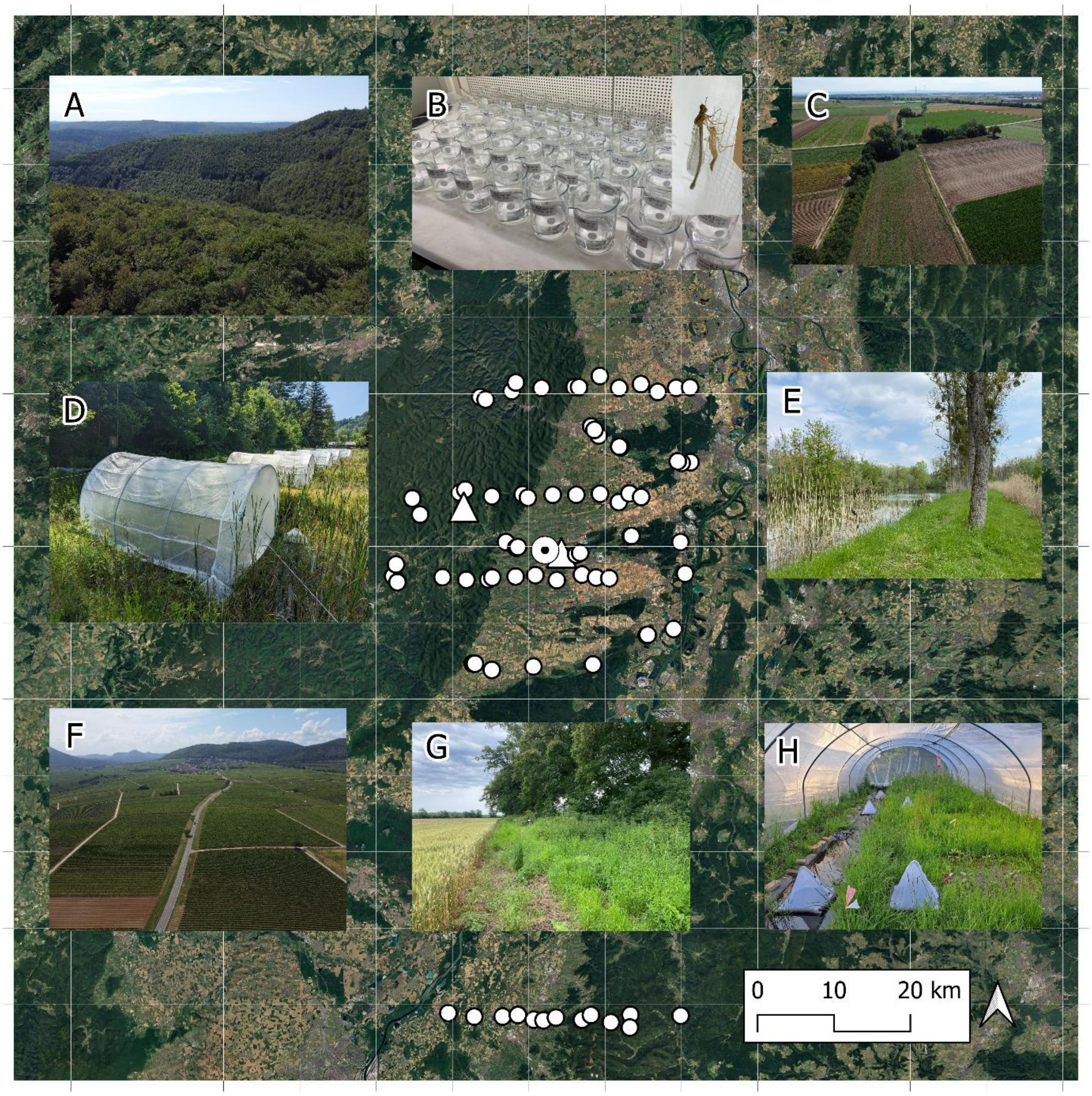

Figure 4. Map section of the Southern Palatinate in Germany and selected sampling locations of the studies conducted within this dissertation. These included laboratory experiments at batch scale (white with black dot), mesocosm experiments at site scale (white triangles), and field investigations at multiple sites across the region (white dots). Additional studies were conducted further south along the Upper Rhine Valley and in the Black Forest. Panel A shows a typical landscape of the Palatinate Forest, where contamination was assessed. Panel B illustrates the rearing of damselfly larvae in individual beakers in the laboratory and an emerging damselfly. Panel C shows an agricultural crop type mosaic as found to towards the Rhine. Panel D shows the floodplain mesocosms and emergence tents used for the Bti experiment. Panel E presents an example of a pond in Sondernheim, where mating success of C. puella was studied. Panel F shows dense viticulture at the mountain foot of the Palatinate Forest, where non-target areas are scarce. Panel G illustrates a representative riparian non-target buffer zone that was sampled for pesticide residues. Panel H displays the riparian stream mesocosms where spider asymmetry was assessed under altered hydrology (taken from Paper 6).

3.2 Landscape contamination

3.2.1 Landscape-wide assessment of pesticide contamination (Field scale)

This methodological approach follows the description in Paper 1. Between June 15th and July 11th 2022 topsoil, vegetation and water were sampled along six line transects with west/east orientation and 30 km length with 13 sampling sites each (total of 78 sites) in the Upper Rhine Valley in Germany between Staufen im Breisgau (47.88321°N 7.73293°E) in the south and Ludwigshafen (49.47041°N 8.43816°E) in the north. For each transect, six non-target, off-crop sites were placed in the valley, one close to the mountain foot and six in the mountain region. Composite topsoil samples consisting of 25 subsamples were taken from a grassland area of 4 x 4 m per sample point in a depth of (0-5 cm). Vegetation was handpicked representative of the sample site in the same 4 x 4 area. If a puddle, stream or river was present in the immediate proximity, a water sample was taken. Vegetation and topsoil samples were analysed for 93 CUPs using an acetonitrile-based extraction in combination with a HPLC-ESI-MS/MS (Bakanov et al., 2023). Water was analysed for 74 CUPs using a direct-injection HPLC-ESI-MS/MS method (Roodt et al., 2023). The sample sites were analysed for their surrounding land-use composition, including crop types, forest cover and urban areas. Assuming that similar land-use types result in similar CUP applications, a two-dimensional Gaussian kernel was applied to weigh each land-use raster cell according to its distance to the sampling site, varying kernel widths between 50 m and 5000 m. Then, the combination of kernel widths for each land-use category that maximised the explained variance (R^2) in generalised linear models predicting the number of CUPs and their sum concentration was identified. Using the resulting land-use weights, CUP contamination across a 11,000 km² area between Worms and Basel in Germany was predicted, covering the study transects and surrounding regions with comparable land-use. Predictions were generated at a resolution of 1000 x 1000 m for CUP number and concentration in topsoil and vegetation.

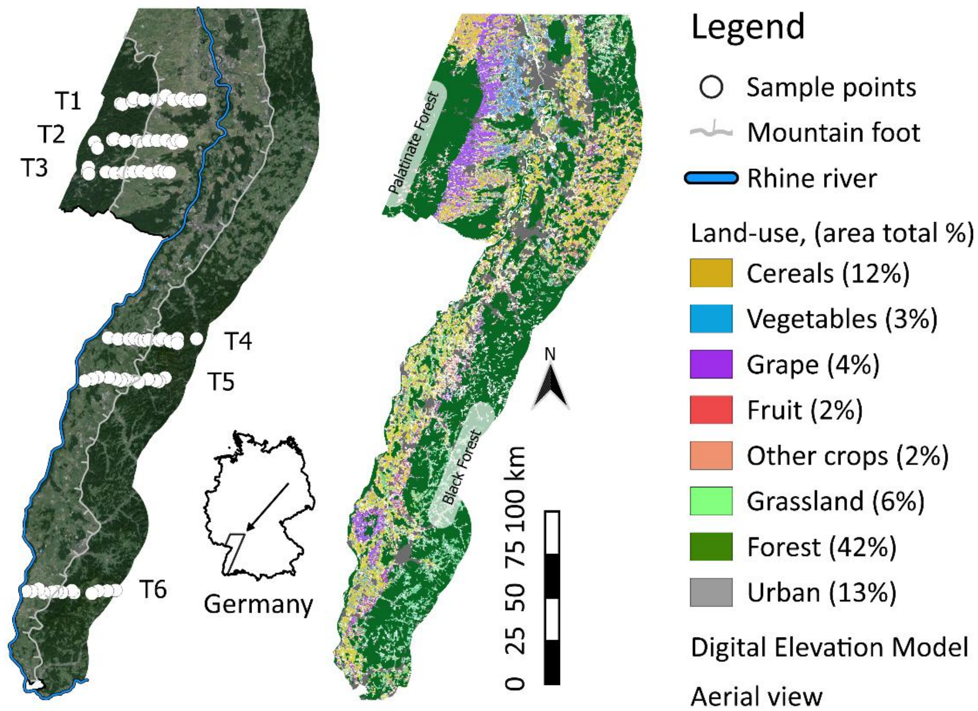

Figure 5. Aerial view of the study region in the Upper Rhine Valley, Germany. Left: location of the 78 sampling sites (white dots) along six west–east transects (T1–T6, north to south), classified as valley or mountain sites depending on their position relative to the mountain foot. Right: land-use composition of the study area. Digital Elevation Model and forest/urban land cover from European Environment Agency (EEA), (2019); remaining land use categories from Blickensdörfer et al., (2021) of the year 2019. Aerial view: © 2025 Google, TerraMetrics. Figure taken from Paper 1.

3.2.2 Temporal and spatial assessment of pesticide contamination (Field scale)

This methodological approach follows the description in Paper 2. Between February 2021 and February 2022, nine fields with adjacent meadows downwind of the main wind direction (SW) in Southern Rhineland-Palatinate in Germany were sampled monthly for topsoil and vegetation. The nine fields with common and realistic conventional pesticide management included three replicates each of arable crops, vegetables and vineyards. The number of fields ensured both high-quality sampling and good comparability due to similar site conditions. Arable farming involved long-term crops with pesticides usually applied in 2D spraying, whereas vegetable cultivation was characterised by quicker crop rotations also involving 2D spraying. Viticulture however, involved a permanent crop usually with 3D spraying. At each of the nine sites, four distances were sampled with 20 m inside the field (target area) as well as 1, 5 and 20 m from the field margin into the meadow (non-target area, Figure 6). Each sample consisted of three subsamples taken 5 m apart along the same distance line from the field margin. Topsoil was sampled in a depth of (0-5 cm) and vegetation was handpicked representative of the field or meadow. Vegetation and topsoil samples were analysed for 93 CUPs using a acetonitrile-based extraction in combination with a HPLC-ESI-MS/MS (Bakanov et al., 2023).

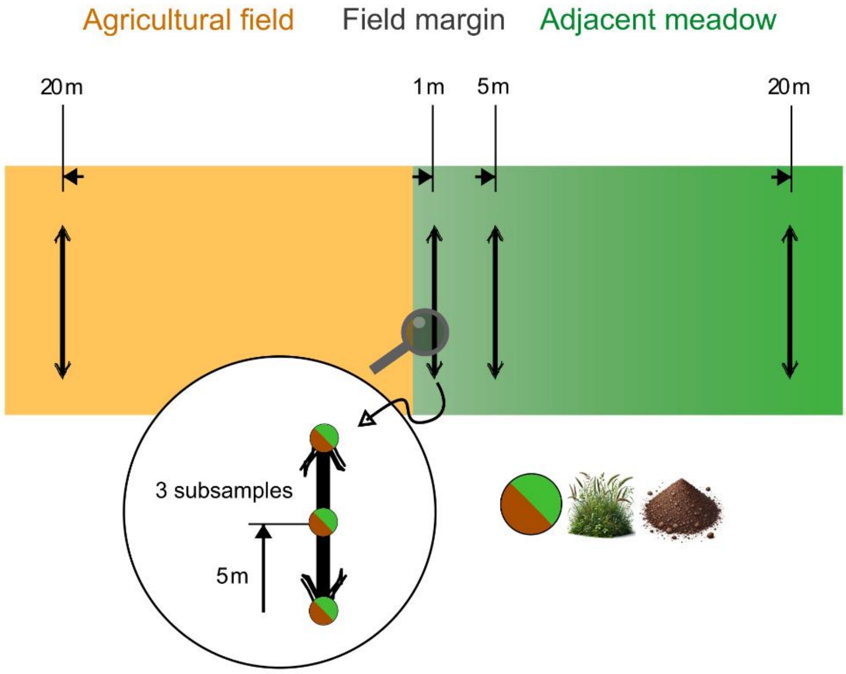

Figure 6. Sampling design of agricultural fields and adjacent meadows at the nine study sites. At each site, topsoil and vegetation were sampled monthly at four distances: 20 m inside the field and 1, 5, and 20 m from the field margin into the meadow. Each sample consisted of three subsamples spaced 5 m apart, resulting in four samples per site and month. Figure adapted from Paper 2.

Based on the measured topsoil concentrations, a conservative approach was used to calculate the cumulative chronic risk for earthworms (*Eisenia fetida*) and collembola (*Folsomia candida*), as well as a contact-based hazard quotient (HQ) for wild bees. Only soil contact exposure was considered for all groups. For earthworms and collembola, risk quotients (RQ) were derived from the difference between the predicted no-effect concentrations (PNECs) calculated from chronic NOEC or LC_{50} values divided by established assessment factors and the measured environmental concentrations. For bees, HQs were estimated using honey bee LD_{50} values, applying a surrogate correction factor ($LD_{50}/10$) to account for higher sensitivity of solitary ground-nesting bees. Soil contact exposure was modelled as 2.23 g soil per bee based on nest construction estimates, and HQs were summed per sample to obtain mixture HQs. Mixture RQs and HQs were categorised into risk classes to evaluate potential impacts on soil fauna and pollinators from current-use pesticide residues in topsoil. In addition, the effect of distance and to the field margin on the number of CUPs and their sum concentration was modelled using exponential decay functions. Temporal fluctuations throughout the year were assessed and compared.

3.2.3 Riparian transmission of pesticides (Field scale)

This methodological approach follows the description in Paper 3. The study investigated the riparian non-target buffer zone (RNBZ) along the streams Speyerbach (49°21'01.0"N 8°11'02.8"E), Queich (49°12'27.7"N 8°03'43.9"E) and Otterbach (49°04'09.0"N 7°59'21.4"E) in southern Rhineland-Palatinate, Germany. Between 15th and 29th of June 2024, 24 sites (8 per stream) were selected to represent a gradient from mountain foot to valley and to maximise variability in RNBZ width (10 - 260 m) and land-use. At each site, CUP contamination was assessed (Figure 7) in water, topsoil, and vegetation along a transect perpendicular to the stream. Topsoil and vegetation samples were collected from the field (F0) and 1, 2, and 5 metres into the RNBZ (F1-F5), mirrored by equivalent samples from the stream side (S1-S5), with a water sample collected directly from the stream (S0). Topsoil samples (0-5 cm) were pooled from five subsamples 1 m apart. Vegetation was handpicked from the same transect line. Vegetation and topsoil samples were analysed for 93 CUPs using an acetonitrile-based extraction in combination with a HPLC-ESI-MS/MS (Bakanov et al., 2023). Water was analysed for 75 CUPs using a direct-injection HPLC-ESI-MS/MS method (Roodt et al., 2023). The influence of RNBZ width and distance downstream on CUP contamination was evaluated additionally to the field-to-RNBZ and stream-to-RNBZ CUP gradients per site. To analyse CUP mixture composition across matrices and position inside the RNBZ, Jaccard dissimilarity indices were calculated based on presence-absence data. Water-specific models were used to investigate the difference between streams and the effect of distance downstream on CUP contamination.

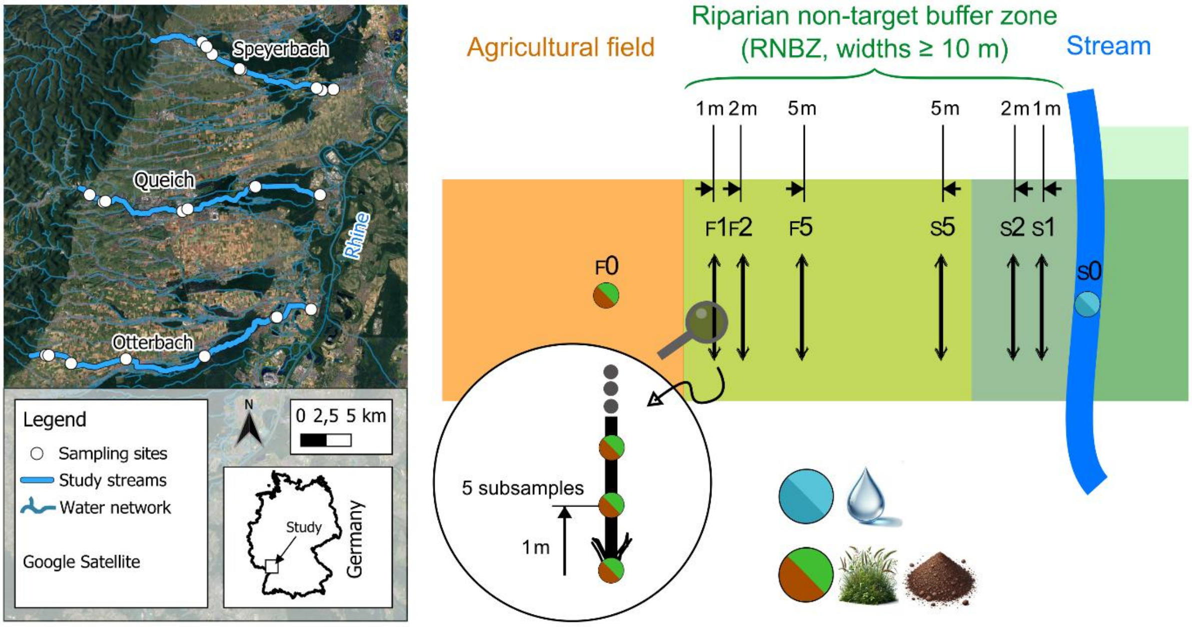

Figure 7. Study map (left) and sampling design (right) at each of the 24 sampling sites. Topsoil and vegetation samples were taken within the RNBZ at three distances from the stream: 1 meter (S1), 2 meters (S2), and 5 meters (S5). Additionally, topsoil and vegetation samples were taken from the adjacent agricultural field, with a reference sample collected inside the field (F0) and additional samples at 1 meter (F1), 2 meters (F2), and 5 meters (F5) into the RNBZ. A water sample was also collected (S0). Figure taken from Paper 3.

3.3 Experimental exposure to aquatic stressors

3.3.1 Insecticide climate chamber experiment (Batch scale)

This methodological approach follows the description in Paper 4. On the 13th of April 2023, larvae of *Coenagrion puella* were collected from the LIBELLULA ponds in Trippstadt in the Palatinate Forest (49.35832°N 7.74351°E) and individually reared in 250 mL glass beakers filled with aerated tap water. The experiment tested the effects of the neurotoxic insecticide chlorantraniliprole, applied in four concentrations (0, 0.0025, 0.01, 0.04 mg/L). Stock solutions were prepared in acetone and diluted with tap water, ensuring a constant acetone solvent concentration (<100 µL/L) across treatments. Beakers were sealed with gauze and equipped with plastic strips as ladders to allow emergence. Each larva was fed daily with *Artemia salina* nauplii and water levels were regularly maintained. All individuals were kept in a climate chamber at 23 °C, 80% relative humidity, and a 16:8 h light-dark cycle. The exposure lasted 14 days and reflected environmentally relevant exposure periods during spring in agricultural regions. Therefore, most of the larvae already were in the final larval instar (F-0) at the start of the exposure. After the exposure period, the beakers with the individuals were maintained under the same conditions until emergence. Emerged individuals were weighed and stored at −20 °C until further analysis, which is described later in this chapter.

3.3.2 Bti application in a floodplain pond mesocosm (Site scale)

This methodological approach follows the description in Paper 4. The experiment took place in twelve floodplain mesocosms (FPMs) at the Eußerthal Ecosystem Research Station (49.25356°N 7.96194°E; RPTU Kaiserslautern-Landau). The twelve FPMs (176 m² each; 23.5 × 7.5 m) were constructed in 2017 along the Sulzbach stream to allow natural colonisation (Manfrin et al., 2023). Each unit has steep banks at three sides and a flat water-land floodplain riparian area at the inflow and has a 1:20 bed slope towards the outflow. The mesocosms are inhabited by diverse merolimnic insect communities (e.g. Diptera, Ephemeroptera, Trichoptera, Odonata), amphibians, and terrestrial arthropods (Stehle et al., 2022). Since 2020, Bti was applied in six of the twelve FPMs (Figure 8) at maximum field rates (2.88 × 109 ITU/ha, Gerstle et al., 2023) annually in April, May and June once a month using a VectoBac® WDG solution (Valent BioSciences Corporation, Illinois, USA). To mimic a realistic Bti application, the FPM units were flooded three times by increasing the water level from 30 to 50 cm from mid-April to the end of May (only in the years 2020 and 2021, see Gerstle et al., 2023), before the implementation of the hydrological regime 2022). In 2023, the application rate was intensified, to increase hunger stress in the system, to seven biweekly applications starting from the 14th of April 2023 using the same field rates (Schöndorfer et al., 2025). Emergence tents (Greenhouse 4.5 x 3.0 x 2.0 m, vidaXL, Netherlands) were placed in the water-land floodplain area of the FPMs from mid-May to mid-August 2023. Adult *C. puella* were collected on the 13th, 14th and 15th of June 2023 with an insect net (mesh size = 1 mm). Damselflies were selected depending on availability in the units and approximation of equal shares between treatments and sexes. Individuals were weighed and stored at −20 °C until further analysis, which is described later in the methods chapter.

3.3.3 Hydrological alterations in a floodplain pond mesocosm (Site scale)

This methodological approach follows the description in Paper 4. In 2022, one year prior to the damselfly sampling, a modified hydrological regime was implemented in six out of twelve previously described FPMs to simulate a climate change scenario (Figure 8). These units received altered flooding frequency and base water levels during both hydrological winter (February-April) and summer (May-August), while the remaining units served as controls. At lower water levels (e.g. 20 cm), only the deeper half of the FPM retained water, leaving the inflow area dry. At higher levels (e.g. 70 cm), the entire pond including the shallow inflow area was submerged.

During the hydrological winter period, both treatments were set to a standard water level of 40 cm, measured at the deepest point of each FPM, opposite of the inflow area. While control units were flooded once every four weeks (total of four floodings), the altered hydrology units experienced weekly floodings (total of eleven floodings), mimicking wetter winters under climate change. Each flooding raised water levels to 70 cm for a duration of 4-5 days.

In the hydrological summer (May to August), baseline water levels in altered hydrology FPMs were reduced to 20 cm, mimicking reduced water availability under climate change. Control units retained the 40 cm standard and floodings every four weeks (four floodings in total). For the altered hydrology FPMs, the flooding frequency was matched to the control ponds by changing from weekly to every four weeks, also resulting in four floodings. For both control and altered hydrology FPMs, summer floodings involved a rise in water level to 70 cm for 4-5 days.

This setup ensured an increase in the frequency of flooding in hydrological winter (without a change in standard water level) and a reduced standard water level (without a change in flooding frequency) in hydrological summer compared to the control. As previously described, Emergence tents in the water-land floodplain area of the FPMs from mid-May to mid-August 2023 enabled collecting of emerged *C. puella* on the 13th, 14th and 15th of June 2023. Individuals were weighed and stored at -20 °C until further analysis, which is described later in the methods chapter.

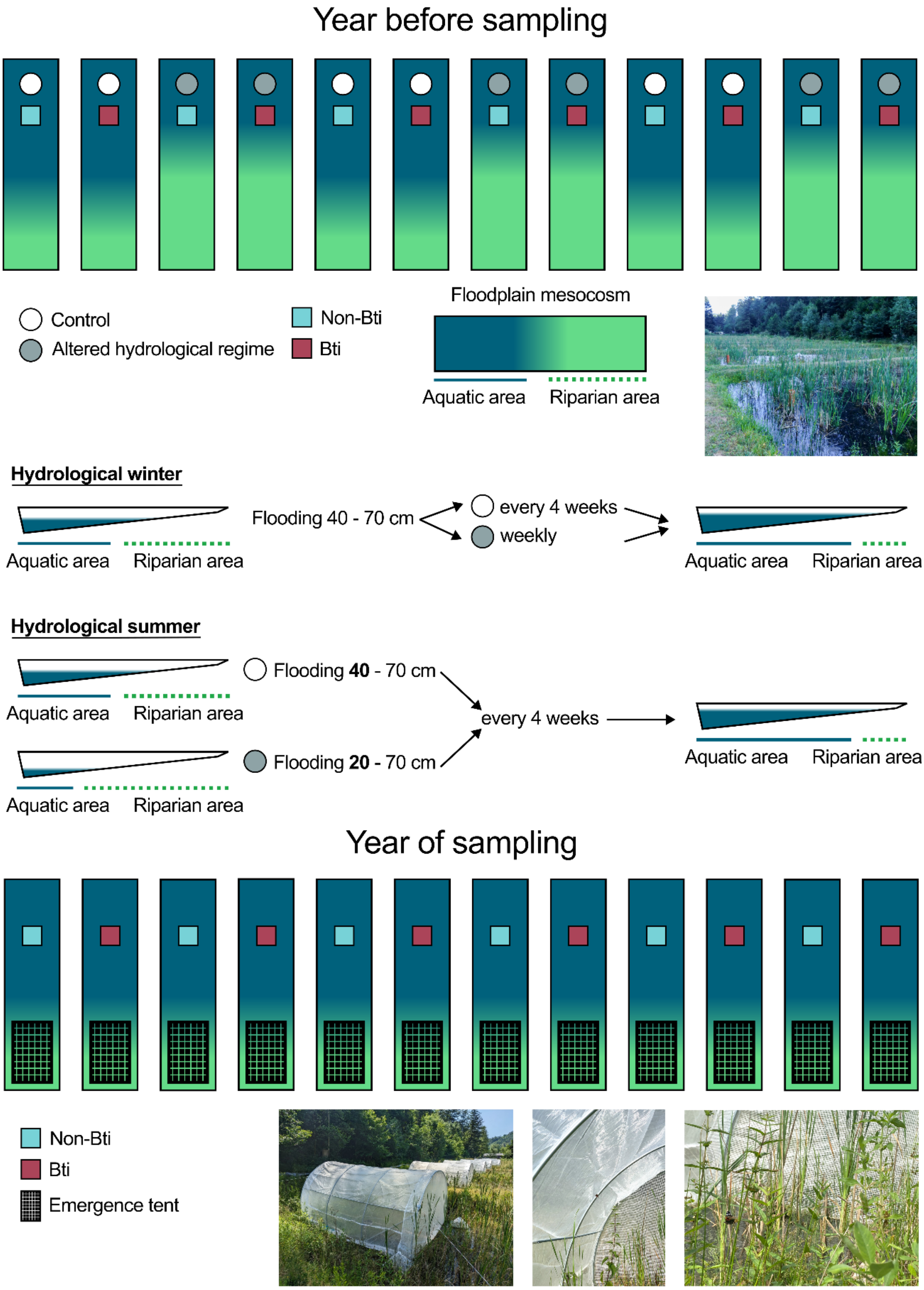

Figure 8. Schematic overview of the twelve floodplain pond mesocosms (FPMs). Each unit included an aquatic (blue) and a riparian area (green). In the year before sampling (2022), six FPMs were assigned to a climate change scenario with altered hydrology (grey dots), while six remained as controls (white dots). During hydrological winter, only flooding frequency differed (weekly vs. monthly), while hydrological summer manipulations involved reduced baseline water levels (20 cm vs. 40 cm) with equivalent flooding frequency. Floodings temporarily raised water levels to 70 cm for 4-5 days (upper picture). Half of the FPMs additionally received Bti treatment (red = with, blue = without). In the year of sampling (2023), half of the FPMs received Bti treatment (red = with, blue = without). Damselfly were caught in the riparian area using emergence tents (black box, lower pictures). For more information see Paper 4. Figure adapted from Bauspiess et al. (2025) and Paper 4.

3.4 Short-term mating success in two populations (Field scale)

This methodological approach follows the description in Paper 5. To investigate short-term mating success in *C. puella*, adult males were collected from two contrasting sites: fishing ponds near Sondernheim in the agricultural Rhine Valley (49.17859°N 8.35995°E) and at ponds at the forested Eußerthal Ecosystem Research Station in the Palatinate Forest (49.25356°N 7.96194°E). Sampling took place from May 18th until May 26th 2023 (Rhine Valley) and June 5th until June 11th 2023 (Palatinate Forest). Individuals were classified either as “mating males”, observed in copula (copulation wheel), or as “chasing males”, which were actively pursuing females or tandem pairs but not copulating at the time of capture. At each site, individuals were caught using hand nets between 10:00 and 16:00 h under stable weather conditions. A total of 80 males (40 per site; 20 per behavioural group) were sampled. Immediately after capture, individuals were placed in 15 ml glass tubes and stored in a cooling box for transport. At the end of each sampling day, all collected specimens were frozen at -20 °C until further analysis which is described later in this chapter. To characterise environmental conditions at the study ponds, 250 ml water samples were collected on the last sampling day at each site. Samples were frozen at -20 °C and later analysed for nitrate, phosphate, and copper using photometric test kits (NANOCOLOR® Nitrate 8, Phosphate 5 total, Copper 5, Macherey-Nagel GmbH & Co. KG). Additionally, pH and conductivity, and temperature were measured using a handheld multiparameter analyser (Multi 340i, WTW, Weilheim, Germany).

3.5 Hydrological alterations in a riparian stream mesocosm (Site scale)

This methodological approach follows the description in Paper 6. The experiment was conducted at the Riparian Stream Mesocosm (RSM) facility in Landau in Germany (49.20100°N 8.13902°E) using eight outdoor mesocosms simulating common temperate riparian streams (Figure 9). Each mesocosm consists of an artificial stream (0.7 x 15 m; 1% slope) and adjacent riparian zone (3.7 m wide along one side of the stream). The streams include six alternating gravel bars and get their water (1.5 L/s) from the nearby stream Queich. Each mesocosm is enclosed with a large mesh tent (1 mm). Before the experiment, aquatic macroinvertebrates were introduced using colonized brooms from the nearby River Queich to simulate natural aquatic-to-terrestrial food web dynamics.

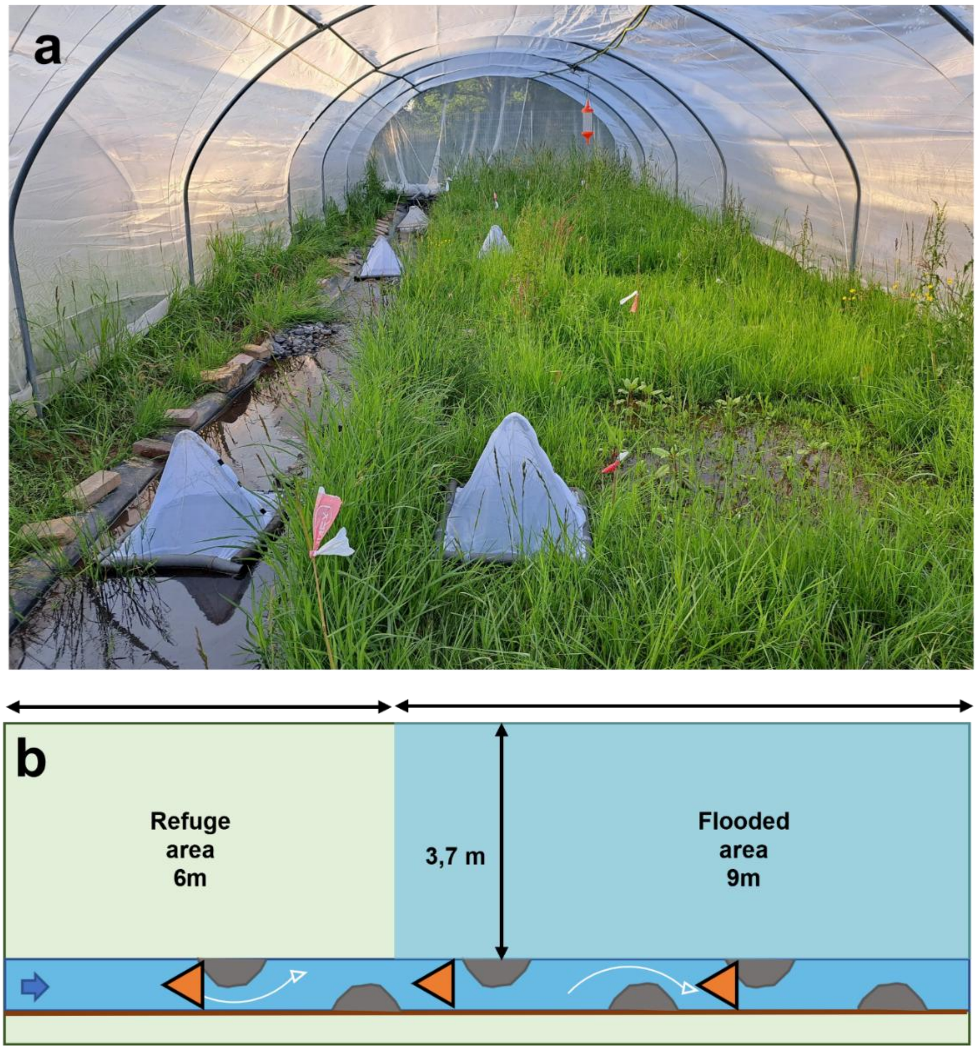

Figure 9. The Riparian Stream Mesocosm (RSM), Landau, Germany. Panel a: Interior view of one mesocosm, covered by a 1 mm mesh gauze and equipped with an emergence trap. Panel b: Schematic of a single unit, consisting of a stream (blue), alternating gravel bars (grey), an adjacent riparian grassland with upstream refuge area (light green), and a downstream area subject to flooding (light blue). Orange triangles indicate the position of emergence traps. Figure taken from Paper 6.

Four mesocosms were exposed to repeated 14-day floodings in May, June, August and September 2023, while four served as non-flooded controls. Flooding covered up to two-thirds of the terrestrial area with a water depth reaching 10 cm downstream. Emerging aquatic insects were sampled using pyramidal emergence traps during each flooding event. Traps were placed along the stream channel and sampled every few days from three days before to four days after flooding. Insects were collected, frozen at −20 °C, and later counted and identified to family level. Chironomids made up 98% of all captured aquatic insects and were therefore used for subsequent analyses. Riparian spiders (*Tetragnatha extensa*) were collected manually along the stream banks following flood 1 (May) and flood 4 (September). From each mesocosm, twelve individuals were hand-picked and kept in individual containers for 24 h prior to freezing at −20 °C. Only female spiders were selected for morphometric analysis to compare individuals that developed before experimental manipulations (flood 1) with those exposed throughout the flooding treatment (flood 4).

3.6 Body and wing trait evaluation

3.6.1 Damselfly body and wing features

This methodological approach follows the description in Paper 4 & 5. Wet body weight was measured to the nearest 0.01 mg before wing dissection. Wing images of front and hind wings were captured using a stereomicroscope and camera setup (Stemi 508, Carl Zeiss AG, Oberkochen, Germany, objective magnification: 0.63x, internal zoom: 1.0x, camera adapter magnification: 0.5x, resolution 3840 x 2160 pixels), optimizing contrast for cell visibility. For the individuals of the climate chamber, Bti and altered hydrology experiment (Paper 4), twelve anatomical landmarks (Figure 10A) were set on each wing using IdentiFly (version 1.5, Tofilski, 2023), with prior training on 60 images. Image brightness and resolution were standardized for reliable processing. Wing shape asymmetry was assessed using landmark-based geometric morphometrics. A Procrustes fit was applied with separate models for females and males. Fluctuating asymmetry (FA) was extracted using bilateral symmetry models and summarized into an FA-score per individual which represents classical value for the overall wing shape asymmetry.

In addition, wing morphology (Figure 10B) of all individuals was analysed using the novel computer-vision software WingAnalogy 4.0 (Eshghi et al., 2024). For each pair of left and right wings, wing area, length, width and perimeter were extracted as basic size variables. They were also used to calculate wing size asymmetry between the left and right wing in addition to differences in the number of cells and junctions (“subtract values”). Cell- and junction-based features were assessed to quantify cell shape and cell position asymmetry (Figure 10C). Cell shape asymmetry was derived from the normalized root mean square error (NRMSE) of cell size, length, width, and circularity (Figure 11). Cell position asymmetry was quantified as the mean distance between homologous cell centroids and vein junctions across left and right wings.

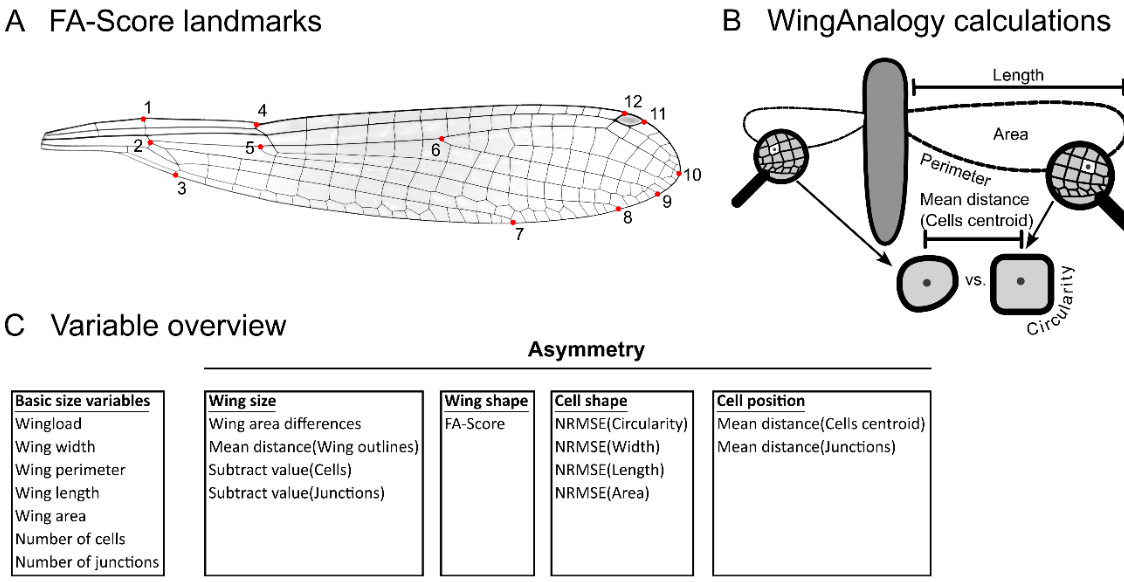

Figure 10. Calculation of geometric features. (A) Landmark placement to calculate the shape FA-Score indicated in red. (B) WingAnalogy calculation of geometric features. The overall wing area, length, width and perimeter were compared between the left and right wing. In addition, several cells feature like the circularity were compared between the two sister cells between the left and right wing. The distance between cells centroid of homologous cells added another cell position asymmetry parameter. (C) Overview of all wing variables, including basic size variables and asymmetry variables. Figure taken from Paper 4.

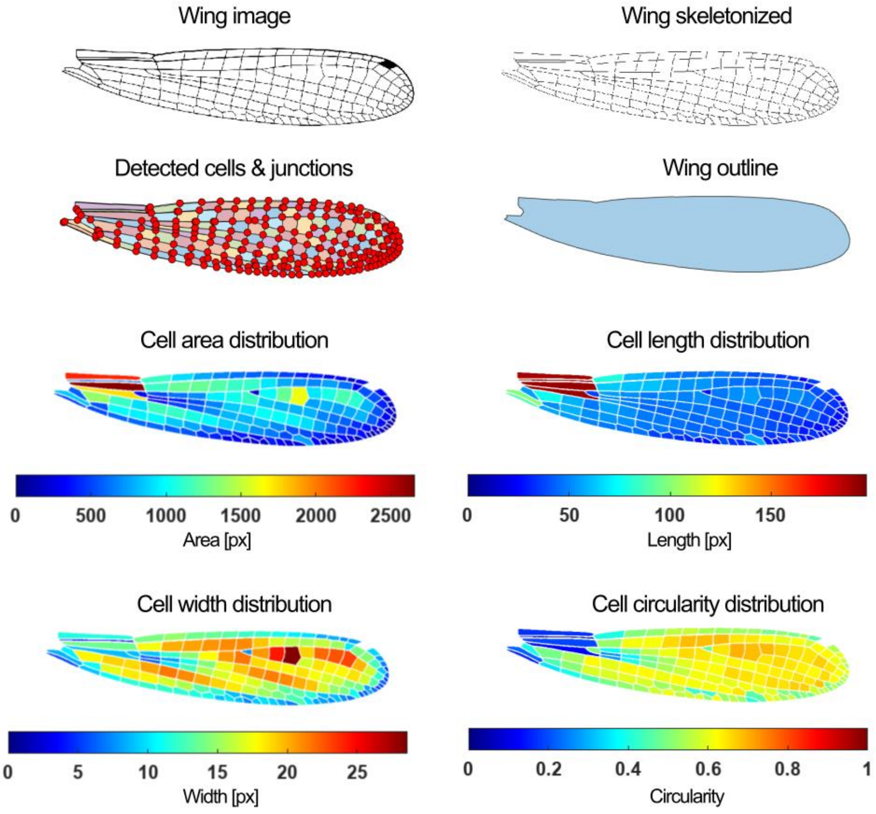

Figure 11. Example output from the WingAnalogy software illustrating automated extraction of damselfly wing traits. Top row: original wing image and skeletonized venation. Second row: detected wing cells with junctions and extracted wing outline. Lower rows: spatial distribution of cell area, length, width, and circularity across the wing, with corresponding colour scales.

Wing load was calculated by dividing wet body weight by wing length. The normal distribution and symmetry of traits were checked to rule out directional or anti-symmetry. Trait values were then used in linear models to test for treatment effects.

3.6.2 Spider prosoma features

This methodological approach follows the description in Paper 6. For each spider, collected in the RSM, the prosoma was prepared for photography by removing legs and the opisthosoma to ensure stable dorsal imaging. Photographs were taken with a stereomicroscope (Stemi 508, Carl Zeiss AG, Oberkochen, Germany, objective magnification: 0.63x, internal zoom set at 4.0x, resolution 2160 x 3840 pixels), and 18 homologous landmarks (Figure 12) were placed on the prosoma with tpsDig2 (version 2.32, Rohlf, 2023).

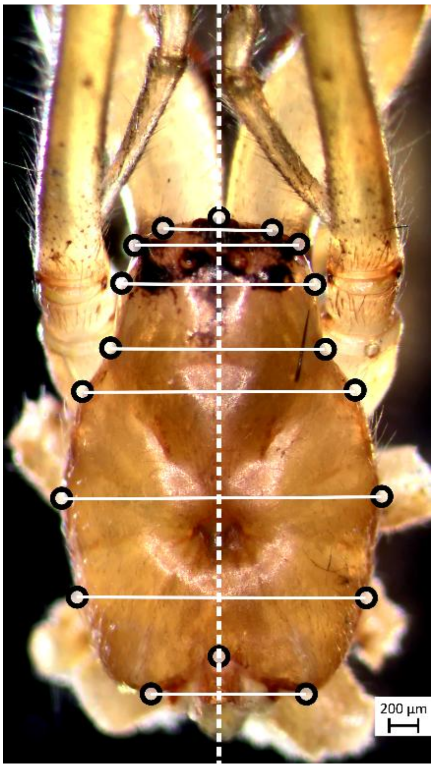

Figure 12. *Tetragnatha extensa*, dorsal view with 18 landmarks (dots) to capture the heads object symmetry. White dashed line represents the axis of symmetry. Paired landmarks connected by white lines. Picture adapted from Paper 6.

4 AQUATIC-TERRESTRIAL CONTAMINATION (OBJECTIVE I)

4.1 Mixture complexity of CUPs

The three complementary studies with focus on the aquatic-terrestrial contamination examined CUP occurrence across 93 compounds in agricultural and adjacent mountainous landscapes of the Upper Rhine Valley. The studies used standardised HPLC-ESI-MS/MS analytical protocols achieving detection limits of 0.008-19.11 ng g^{-1} for topsoil, 0.01-21.5 ng g^{-1} for vegetation, and 0.006-0.01 ng g^{-1} for water samples. The use of optimized high-throughput extraction and measurement methods (Bakanov et al., 2023; Roodt et al., 2023) enabled detection of contamination at concentrations up to 1000 times lower than concentrations previously reported in European monitoring studies (Silva et al., 2019). The three studies included a landscape contamination study (78 sites, Paper 1), a temporal monitoring study (nine sites over 13 months, Paper 2), and a riparian non-target buffer zone (RNBZ) study (24 sites, Paper 3). The landscape study documented pesticide occurrence in 97% of all 78 vegetation samples, 97% of all 78 topsoil samples and 83% of all 30 surface water samples from non-target areas during the spraying season. Using land-use categories as predictors, this dataset also enabled spatial extrapolation of CUP concentrations and compound numbers across the region (Figure 13), providing a first landscape-scale estimate of off-field pesticide exposure in this region. The year-round temporal monitoring detected at least one CUP in 100% of all 468 topsoil samples and 97% of all 442 vegetation samples collected from meadows adjacent to agricultural fields and the fields itself. The RNBZ study revealed pesticide presence in 100% of samples across riparian topsoil (n = 166), vegetation (n = 166), and water matrices (n = 24) within designated non-target zones during the spraying season. These detection rates and the resulting prediction map of the first study (Figure 13) alone underscore that CUP contamination is a defining characteristic of modern agricultural landscapes rather than localised contamination events. This pattern is broadly consistent with other European studies where pesticide residues were detected in 98% of all non-target vegetation samples and 59% of all non-target topsoil in an Italian mountainous landscape (Brühl et al., 2024) or 98% in French target and non-target topsoils (Froger et al., 2023). In the present three studies, the vast majority of samples contained not just single compounds but mixtures of multiple CUPs.

All studies documented high mixture diversity that fundamentally challenges conventional risk assessments that mostly focus on single substance testing. The landscape contamination study detected 140 different mixtures with ≥2 components whereas the temporal monitoring detected almost 350 distinct mixture combinations across all matrices. The RNBZ study revealed 281 unique mixtures within the non-target zone alone and streams. This exceptional mixture diversity reveals site-specific CUP contamination communities rather than reoccurring standard combinations. The most frequent mixture combination in the temporal study appeared in only 11 topsoil samples and 7 vegetation samples across hundreds of samples, representing very few occurrences of the same mixture even when the same fields and adjacent meadows are sampled repetitively. Therefore, organisms encounter spatially and temporarily heterogeneous chemical environments characterised by location-specific mixture compositions rather than predictable patterns. In addition, the detected mixture complexity represents conservative estimates given that the analytical scope targeted 93 compounds whilst 282 active substances are currently authorised in Germany (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, 2025) and 422 across Europe (European Commission, 2025). Fungicides consistently dominated CUP detections across all three studies in the Upper Rhine Valley, representing the largest compound class regardless of study design or environmental matrix (analysis for 93 CUPs consisted of 36 fungicides, 36 herbicides and 21 insecticides). The temporal study detected 30 fungicides among 66 total CUPs in topsoil and 31 fungicides among 62 total compounds in vegetation. The RNBZ study identified 30 fungicides among 65 total compounds across all matrices whereas the landscape contamination study detected 29 fungicides among 63 total compounds across all matrices. One fungicide was especially common in non-target areas: fluopyram is a broad-spectrum fungicide for use as a foliar application and as a seed treatment to control various diseases but also has nematicide activity (PPDB, 2024). It was demonstrating the highest detection frequencies across studies with 94% of all samples in the landscape contamination study, 83% in all samples of the temporal monitoring and 99.7% in all samples of the riparian assessment. This consistency reveals that specific compounds can achieve near-universal distribution in agricultural landscapes. Several other frequently detected substances like fluopyram have per- and polyfluoroalkyl substance (PFAS) characteristics, including cyflufenamid and fluopicolide. PFAS-type pesticides represent 12% of synthetic pesticides authorised in the European Union and their sales tripled in France between 2008-2021 to reach 2,332 tonnes in 2021 (PAN Europe, 2023). These compounds exhibit exceptional environmental persistence with soil half-lives exceeding 300 days and potential for long-term accumulation. Fluopyram degrades into trifluoroacetate (TFA), a highly persistent and mobile contaminant that represents a long-term threat to water resources (UBA, 2022). Although the EU is committed to phasing out PFAS, PFAS-based pesticides have so far been exempt from the proposed group ban and continue to be authorised under existing pesticide regulation (PAN Europe, 2025). Notably, PFAS are intended to be banned in applications like electronics and lubricants to prevent accidental environmental contamination, yet their deliberate release via pesticides remains exempt from this restriction.

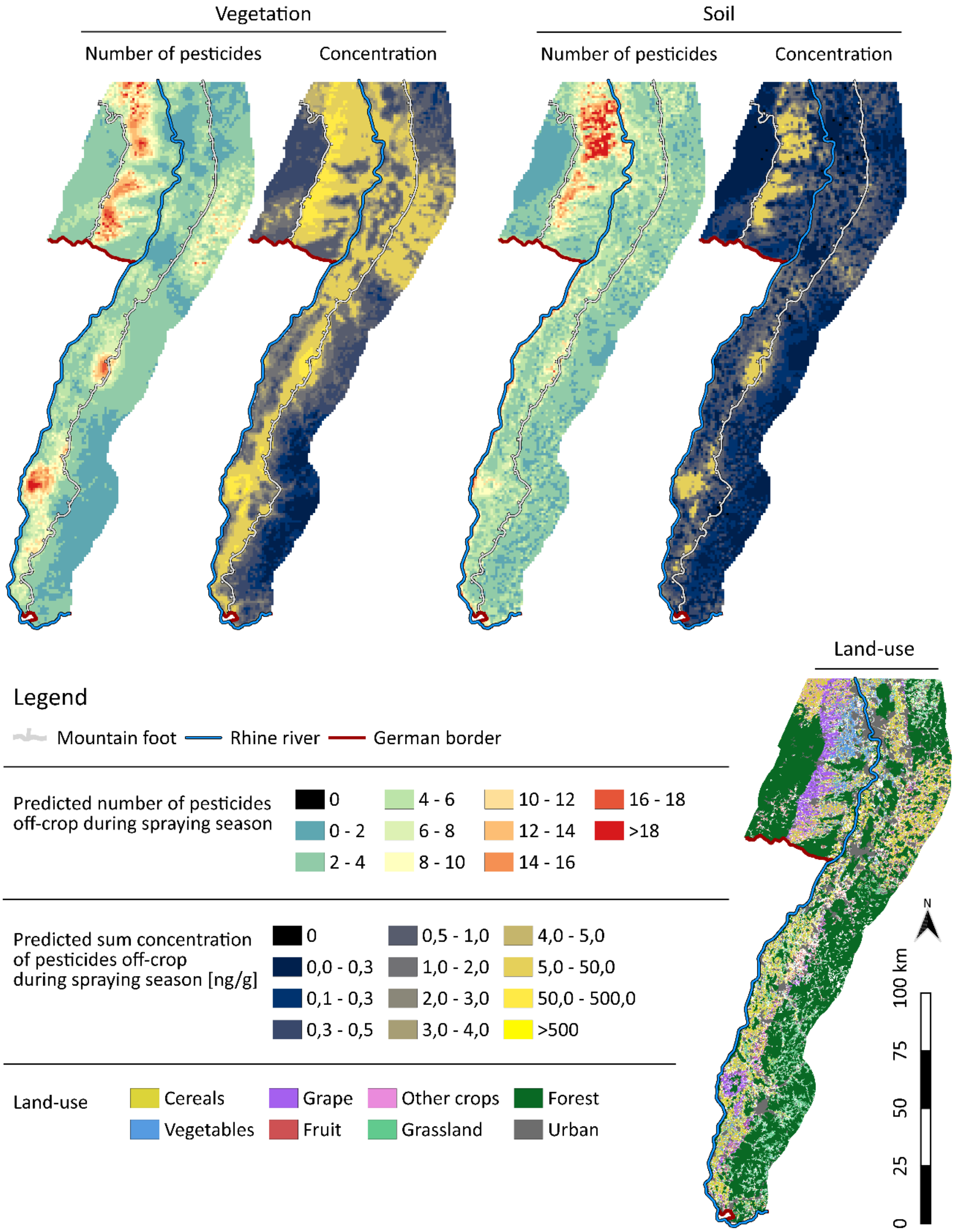

Figure 13. Predicted CUP sum concentrations and number of residues in off-field vegetation and topsoil during spraying season. The number of CUPs and sum concentration ng g-1 in off-field vegetation and topsoil during spraying season were predicted with land use categories in a linear model (CUP sum concentration) or generalised linear model (CUP number). The models explained between 62% and 76% of the variance (Multiple $R^2 = 0.62\text{-}0.76$). Predictions are visualised at a spatial resolution of 1 × 1 km per raster cell. Forest/urban land cover data are from the (European Environment Agency (EEA), 2019); the remaining land use categories are from Blickensdörfer et al. (2021). The prediction map is based on the topsoil and vegetation measurements and their 78 sites only. Figure taken from Paper 1.

In the landscape study, surface waters contained the highest compound diversity (mean 9.6 CUPs), followed by vegetation (6.3) and topsoil (5.4). In the RNBZ study agricultural streams showed the highest diversity (16.9 CUPs), followed by vegetation (10.3, non-target only) and topsoil (8.5, non-target only). This underlines the role of agricultural streams as vectors of pesticide transport throughout landscapes (Wolfram et al., 2023). In addition, the hierarchy of "water > vegetation > soil" underlines differential chemical partitioning characteristics and environmental transport mechanisms operating across matrix boundaries. For example, the commonly detected fungicide boscalid is estimated to have a soil DT_{50} (field) of 254 days and a water-sediment DT_{50} of 545 days but a water DT_{50} of only five days, showing how strong sorption to sediments and reduced degradation under anaerobic conditions may markedly prolong environmental persistence (PPDB, 2024). Moreover, vegetation can take up systemic compounds from soil (Hwang et al., 2017) whereas soil could be less prone to atmospheric deposition simply because of its vegetation cover. However, variable factors such as plant growth, mowing, and grassing complicate concentration measurements and challenge statistical interpretability in field studies. The RNBZ study showed that 42% of detected compounds occurred simultaneously across all three matrices whilst 23% appeared in only two matrices. Landscape contamination study confirmed that also 43% of compounds occurred across all examined matrices. The detection frequencies demonstrate that assumptions of pesticide-free non-target refuge areas within agricultural regions are fundamentally invalid under contemporary application regimes. The documentation of hundreds of unique mixture combinations occurring with low individual frequencies indicates that currently established pesticide regulations systematically underestimate realistic exposure scenarios.

4.2 Entry pathways into non-target areas

The documented pervasive contamination across non-target areas in the agricultural Rhine Valley reveals a fundamental mismatch between current prevention concepts and the spatial reality of pesticide transport when conventional agriculture is covering one third of the landscape (see Paper 1). Agricultural landscapes exhibit complex multi-scale contamination gradients that operate across different spatial dimensions simultaneously (Paper 1, 2 & 3 gradients synthesised in Figure 14).

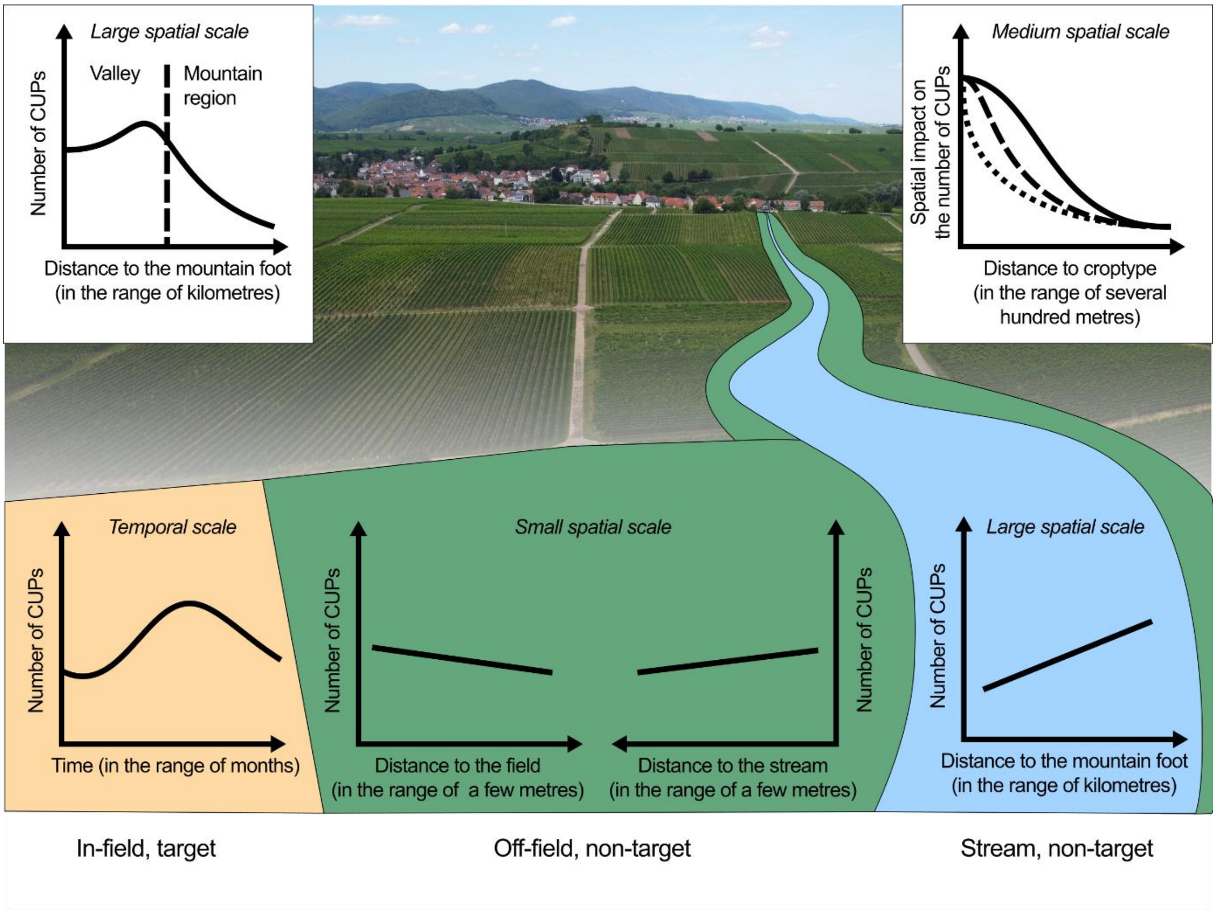

Figure 14. Exemplary gradients responsible for the number of CUPs in vegetation or stream water. Within the temporal scale in-field in viticulture (yellow patch), the number changes over the months with a minimum in March before the spraying season and a maximum in August at the end of the spraying season. Within the small spatial scale (green patch), the number of CUPs decreases slightly over a few metres from the field margin and then increases again with proximity to the stream. Within the medium spatial scale (top right patch), depending on the crop type, the surrounding fields can influence the number of CUPs in a non-target area over several hundred metres. Due to the large spatial scale positioning of the crop types (e.g. viticulture, top left patch), the number of CUPs in non-target areas increases slightly towards the edge of the Palatinate Forest and then decreases within the mountain regions again. On another large spatial scale (blue patch), the number of CUPs detected in stream water increases as it passes through a mosaic of different crop types.

Direct spray drift creates predictable local-scale contamination gradients that form the basis of current regulatory approaches. According to the basic drift values used in pesticide authorisation, 2.77% of the applied amount remains at 1 m distance from the field edge in field crops (Rautmann et al., 2001). A similar drift level is reached at 3 m for early applications in vineyards and only at 20 m for early applications in other fruit (Rautmann et al., 2001). The newer reference values published by the Julius-Kühn-Institut (2022) provide crop-specific drift estimates based on revised empirical data. For conventional arable applications, predicted residual contamination is 0.57% at 5 m, 0.29% at 10 m, and 0.15% at 20 m distance from the field edge. In viticulture, estimated values are higher: 8.02% at 3 m, 1.23% at 10 m, and 0.42% at 20 m. For early-season applications in other fruit crops, predicted drift is substantially higher, with 29.20% at 3 m, 11.81% at 10 m, and 2.77% at 20 m. However, these concentration-based gradients differ from field reality when considering mixture complexity rather than single-compound scenarios. The temporal monitoring provided empirical evidence of persistent multi-compound contamination across distance gradients depending on crop type. In arable systems, mean CUP numbers in topsoil declined from approximately 19 compounds in-field to 13 at 1 m, 3 at 5 m, and 2 at 20 m. Vegetation samples from the same fields showed a different gradient: from 7 compounds in-field to 4 at 1 m, and 3 at both 5 m and 20 m. In contrast, viticulture showed markedly flatter gradients. In topsoil, the number of CUPs declined only slightly from 15 in-field to 12 at both 1 m and 5 m, with 5 compounds still detectable at 20 m. Vegetation samples in vineyards showed 12 CUPs in-field, 10 at 5 m, and 7 at 20 m. In comparison, the RNBZ study revealed different gradients while focusing at more heterogeneous sites and more crop variety (RNBZ: approx. -3.35 CUPs in topsoil and -1.65 CUPs in vegetation vs. temporal monitoring: approx. -3 to -16 CUPs in topsoil and -2 to -4 CUPs in vegetation depending on crop type, over the first 5 m). Overall less decline by distance to the field in viticulture may be explained through the typical three-dimensional spraying that generates airborne particle fractions through upward, downward, and lateral directions designed for canopy coverage. It creates enhanced volatilisation potential through increased surface area exposure and volatilisation from target plants and soils is supported through specific microclimatic conditions including elevated air temperature and wind velocity (Bedos et al., 2002). In conjunction with viticulture shaping CUP complexity in the Upper Rhine Valley, predicted hotspots of more than 18 CUPs in surrounding non-target vegetation are also indicated by the landscape study (Figure 13). Here, the underlying models also showed that the crop type can influence the contamination of a non-target area over several hundred metres.

Atmospheric transport creates medium and long-range gradients extending across entire landscape units, surpassing small “buffer zone scales” entirely. The landscape contamination study revealed CUP transport extending multiple hundred metres into forested mountain regions, with contamination patterns related to distance from agricultural areas. Semi-volatile pesticides may become adsorbed on atmospheric aerosol particles enabling long-range transport (Mayer et al., 2024). In landscape contamination study, a site with maximum distance of 4.4 km from agricultural sources was shown to contain the CUPs fluopyram and spiroxamine. Although not significant, wind direction effects showed trends with downwind regions (Black Forest) experiencing 1.51 more CUPs and 0.699 ng g^{-1} higher sum concentrations on average compared to upwind regions (Palatinate Forest). Contamination on a large scale is also supported by recent findings of rain containing nicotinoids (Putri et al., 2025). Therefore, atmospheric gradients operate through fundamentally different mechanisms than field-edge drift. They are creating contamination patterns that include meteorological and topographic factors rather than simple proximity relationships over a few metres, which makes it difficult to estimate the exact mixtures that may occur at a specific non-target site.

Agricultural streams represent another potential pathway for CUP transport, as they can increase in mixture complexity while passing multiple fields. In addition, the RNBZ study documented counterintuitive gradients close to the streams with the number of CUPs and their sum concentration increasing towards the stream rather than decreasing. Terrestrial RNBZ matrices showed topsoil containing a mean of 9.3 CUPs on the field side versus 7.6 CUPs on the stream side, whilst vegetation contained a mean of 9.8 CUPs on the field side versus 10.7 CUPs on the stream side, uncovering that vegetation near streams can experience greater CUP mixture diversity rather than less compared to field margins. The studied spraying season stream water consistently contained the highest compound diversity with a mean of 16.9 CUPs, supporting the theory of streams acting as an active transport vector. However, the mechanism how streams exchange pollutants with adjacent terrestrial sites is poorly understood, underlining significant research needs at the aquatic-terrestrial interface (Schulz et al., 2015). A mixture composition analyses raised further questions regarding streams as contamination sources: topsoil and vegetation CUP mixtures showed high dissimilarity from their corresponding stream water mixtures at the specific sites (mean Jaccard Indices ≥ 0.7), suggesting that aquatic-terrestrial contamination patterns at the stream side may not be fully explained through simple transfer mechanisms. A nearby study using the same analytical method but searching for 98 pesticides and metabolites showed that flooding can transport pesticides into riparian soil (Fiolka et al., 2024). Although flooding was not directly assessed, on-site observations suggest that flooding likely not explain the increase towards the stream at the studied RNBZ sites. Lateral infiltration may represent another pathway, but CUP mixtures in soil and vegetation differed from stream water and did not change with distance, suggesting limited influence. The absence of site-specific data on soil and weather conditions limits interpretation, especially as pesticide adsorption depends on factors such as organic carbon content, clay content and pH (Kodešová et al., 2011). Moreover, the stream's CUP composition at the time of sampling may not reflect past exposure relevant for terrestrial accumulation.

The analysis of buffer width showed only a small yet significant effect on reducing the number of CUPs in RNBZ topsoil (−2.6 CUPs per 100 m buffer width), with no detectable influence on CUP concentrations or on the number and concentration of CUPs in vegetation. This indicates that the buffering capacity of currently established RNBZs is limited but results in mixture exposure for many adapted aquatic–terrestrial species that rely on these habitats. RNBZs should therefore be regarded not solely as physical barriers for mitigating stream pollution but as ecologically functional habitats that sustain biodiversity and facilitate cross-ecosystem processes. Importantly, prediction models in the landscape contamination study achieved high explanatory power (R^2 = 69–76%) when including land-use effects operating over several hundred metres, underscoring that contamination patterns are shaped by landscape-scale processes rather than solely by field-edge effects.

4.3 Temporal dynamics and chronic exposure

Temporal monitoring across agricultural systems reveals that seasonal pesticide exposure patterns can profoundly affect organism life cycles, creating chronic exposure scenarios that remain unaddressed. In-field topsoil contamination, with 13-20 CUPs depending on the crop type, remained remarkably stable throughout the year, creating persistent background exposure levels that contradict regulatory assumptions of recovery periods between applications. The number of CUP residues in off-crop topsoils (20 metres from the field), with 2-6 CUPs depending on the crop type, remained nearly constant throughout the year, too. In contrast, vegetation exhibited pronounced seasonal fluctuations with contamination peaks coinciding precisely with periods of maximum biological activity. Seasonal fluctuations in CUP numbers were pronounced in vegetation, with in-field maxima (up to 21 CUPs) detected for all crop types in July or August. This temporal pattern may create a critical match with insect life cycles, as the highest contamination levels occur when most adult insects are actively foraging, reproducing, and utilizing vegetation for oviposition. The synchrony between adult emergence and favourable environmental conditions during crop growth, including temperature and host plant availability, is a common adaptive trait in insects (Danks, 2007). Ground-nesting wild bees, which was focused on in the temporal monitoring, exemplify this exposure complexity, with larvae and pupae overwintering in contaminated soils whilst adults are in soil contact for nest burrowing and foraging on vegetation (Willis Chan et al., 2019) during peak contamination periods. In-field, average MHQ indicated a hazard (MHQ > 1) in June and July on arable soils and from September to February on vegetable soils.

Investigating multiple matrices across different spatio-temporal scales, as done in the three studies, has demonstrated that pesticide exposure is shaped by a complex interplay of factors determining when, where, and to what extent an organism comes into contact with pesticides. These patterns are particularly relevant for taxa with complex life cycles, such as dragonflies and damselflies, which may experience cumulative exposure across multiple life stages. At macroecological scales, pesticide effects combined with warming are associated with odonate declines, underlining a key knowledge gap for aquatic-terrestrial taxa responses (Sirois-Delisle & Kerr, 2022). Those responses of odonates are further discussed in objective II & III.

4.4 Implications for regulation and monitoring

The EU pesticide authorisation system follows a two-level structure in which active substances are regulated at EU level, while individual products are authorised by Member States (Storck et al., 2017). Pesticide manufacturers provide ecotoxicological effect data used to derive so-called regulatory acceptable concentrations (RAC) that serve as critical thresholds for predicted environmental concentrations (European Union, 2013, S. 283). However, this structure may be based on a fundamental conflict of interest. In addition, the EU's tiered risk assessment framework allows replacement of conservative Tier 1 regulatory acceptable concentrations (RACs) from laboratory tests with higher-tier RACs. These higher-tier values are derived from mesocosm studies or Species Sensitivity Distributions (SSDs), which use smaller assessment factors and are therefore less conservative. Many insecticides are authorised on the basis of such higher-tier RACs, yet these thresholds may not adequately reflect adverse effects in natural communities, including reduced macroinvertebrate family richness (Stehle & Schulz, 2015). The limitations of the current regulatory approach become apparent when considering the widespread contamination observed in our studies across the Upper Rhine Valley. EU regulations typically assume that acceptable concentrations of individual compounds will not be exceeded in the field. However, this does not consider the reality of mixture exposure. While regulatory assessments focus on individual active substances and specific authorised uses based on individual compound toxicity, we documented 140 unique mixtures with ≥2 components across the Upper Rhine Valley in the first study alone. Current frameworks operate within regulatory silos, assessing each compound in isolation, despite the fact that organisms are simultaneously exposed to multiple pesticides whose combined effects can exceed risk thresholds even when individual levels remain below their respective limits (Kotschik et al., 2024).

The proposed European Soil Monitoring Law represents a crucial opportunity to address these regulatory gaps by establishing legally binding environmental quality standards for chemical pollution in soils (Kotschik et al., 2024). The proposed law aims to improve regulation by targeting substances that are highly toxic to non-target organisms and persistent in soil. (Kotschik et al., 2024). However, current analytical methods capture only a fraction of the more than 450 currently approved active substances in Europe, many with up to 20 metabolites which can effect non-target organisms itself (Kotschik et al., 2024). Our detection of complex CUP mixtures across all three environmental compartments also demonstrates the necessity for comprehensive monitoring that includes vegetation in addition to soil and water, as vegetation serves as a habitat for numerous arthropods and is directly contacted by pollinators as well as sucking and biting insects that may be particularly exposed to those residues. Current EU risk assessment mainly uses a small set of test species, such as honeybees, which may not reflect higher sensitivity of some off-field herbivores. For example, a recent study with the neonicotinoid acetamiprid found that certain plant bugs were far more sensitive than honeybees, in some comparisons with honeybee data from a different formulation exceeding a 10,000-fold difference, and that males in some species were up to twenty times more vulnerable than females, indicating that real-world risks to non-target herbivores may be severely underestimated (Sedlmeier et al., 2025). These gaps in risk assessment and monitoring highlight the importance of aligning upcoming policy targets with measures that ensure reductions in pesticide use and risk translate into tangible decreases in real-world exposure for non-target organisms.

The pesticide reduction targets outlined in EU policies, include the Farm to Fork Strategy's goal which specifies to converts 25% of EU’s agricultural area to organic practices where synthetic pesticides are prohibited (European Commission, 2020a). In addition, the European Commission set non-binding targets to reduce the overall use and risk of chemical pesticides, as well as the use of more hazardous substances, by 50%, based on the quantities of active substances sold per Member State relative to a 2015-2017 baseline (European Commission, 2020b). Even though a 50% “risk” reduction is the goal, repeating the landscape study from 2023 in 2030 could provide evidence of whether the number of active substances in non-target nature has actually been reduced by 50% as well. As proposed, the Soil Monitoring Law calls for the implementation of the "polluter-pays" principle, which is currently applied only in Denmark through pesticide taxation introduced in 1996 and could help steer pesticide use towards less harmful substances (Kotschik et al., 2024). However, more sustainable alternatives to conventional farming, such as pesticide-free cereal production for baking, are already feasible (Finger, 2024) and could influence a substantial share of the EU’s agricultural landscape, given that cereals account for one-third of the total utilisable agricultural area (Eurostat, 2024).

5 AQUATIC STRESSORS SHAPE ORGANISMS AT THE AQUATIC-TERRESTRIAL INTERFACE (OBJECTIVE II)

5.1 Insecticide affects damselfly larvae

Sublethal exposure to chlorantraniliprole, which was the most frequently detected insecticide in water (58.3%) in the RNBZ study, caused distinct, concentration-dependent changes in wing morphology of *C. puella* (Figure 15). While survival, emergence timing, and larval growth remained unaffected (no significant change in days to emergence or weight gain), the highest insecticide concentration of 0.04 mg L^{-1} (40 ng ml^{-1}) significantly altered wing traits of the emerging adults. Front wing perimeter increased by 0.77 mm as well as wing shape FA indicated by a higher FA-score of +24.3%. The insecticide also increased asymmetry in specific wing components. The cell shape asymmetry increased with a 7.0% higher NRMSE of cell circularity and 8.7% higher NRMSE of cell width. These results demonstrate that even without acute toxicity, chlorantraniliprole exposure can impair developmental stability in aquatic stages, leading to more irregular wing architecture in terrestrial adults. This aligns with emerging evidence that many insecticides induce subtle morphological and physiological effects at environmentally relevant concentrations (Gandara et al., 2024). The increase in damselfly wing FA under chemical stress is consistent with prior studies showing that aquatic contaminants can elevate wing asymmetry in Odonata (Hardersen & Frampton, 1999). The detected wing and cell shape shifts may underlie the interference in morphogenetic processes that occur during the final larval stages. In odonates, wings are formed within the larval integument and undergo extensive structural elaboration shortly before emergence. The development of wing cells and veins during this period depends on tightly regulated morphogenetic processes, involving tissue inflation, cuticle synthesis, and coordinated patterning of cellular boundaries (Okude et al., 2021). As chlorantraniliprole acts neurotoxically on ryanodine receptors and disrupts calcium homeostasis in insect muscle (Lahm et al., 2007), it is plausible that such interference affects morphogenesis even without compromising larval viability or growth. The observed shape asymmetries may thus reflect disturbed signalling or localised control failures during tissue differentiation and inflation, particularly in such spatially organised structures as damselfly wings. Importantly, the conducted in-depth spatial analyses of wing indicated that insecticide related cell shape asymmetry was concentrated in specific functional regions of the wing, particularly in wing edge in case of the cell circularity. These regions correspond to complex structural zones in balance of structural integrity and use of resources (Rajabi et al., 2017). Therefore, their shape asymmetry suggests a local loss of developmental control rather than generalised morphological noise. Despite growing recognition of sublethal effects, the observed localised wing asymmetries due to the insecticide underscore how little is still known about developmental disruptions in Odonata, highlighting the need for broader application of high-resolution morphometric tools (see Eshghi et al., 2024). It is important to note, that the concentration that mostly drove effects in this laboratory experiment (40 ng ml^{-1}) was 2,000 times higher than the maximum concentration measured in streams (0.02 ng ml^{-1}) and 4,000 times higher than in puddles (0.01 ng ml^{-1}) during the RNBZ and large landscape study, respectively. Comparability is difficult because two weeks of exposure took place in this study during a specific stage of the larvae's life cycle, whereas environmental exposure may occur over longer periods of time and in combination with other pesticides. Event-driven sampling in small agricultural streams has shown that rainfall events can increase median total pesticide concentrations by a factor of 10 (Halbach et al., 2021). And even though measured in paddy fields, a common habitat of odonates (Huynh et al., 2021), water concentrations of up to 28 ng ml^{-1} have been measured after 2h of application of the double recommended dose (J.-M. Zhang et al., 2012). The findings highlight a critical gap in current regulatory assessments: by not including Odonata species like *C. puella* in standard testing protocols, evaluations overlook important realistic scenarios of non-target insect taxa and their exposure to frequently detected CUPs. Potential terrestrial implications on the detected increase in wing shape asymmetry is discussed in objective III.

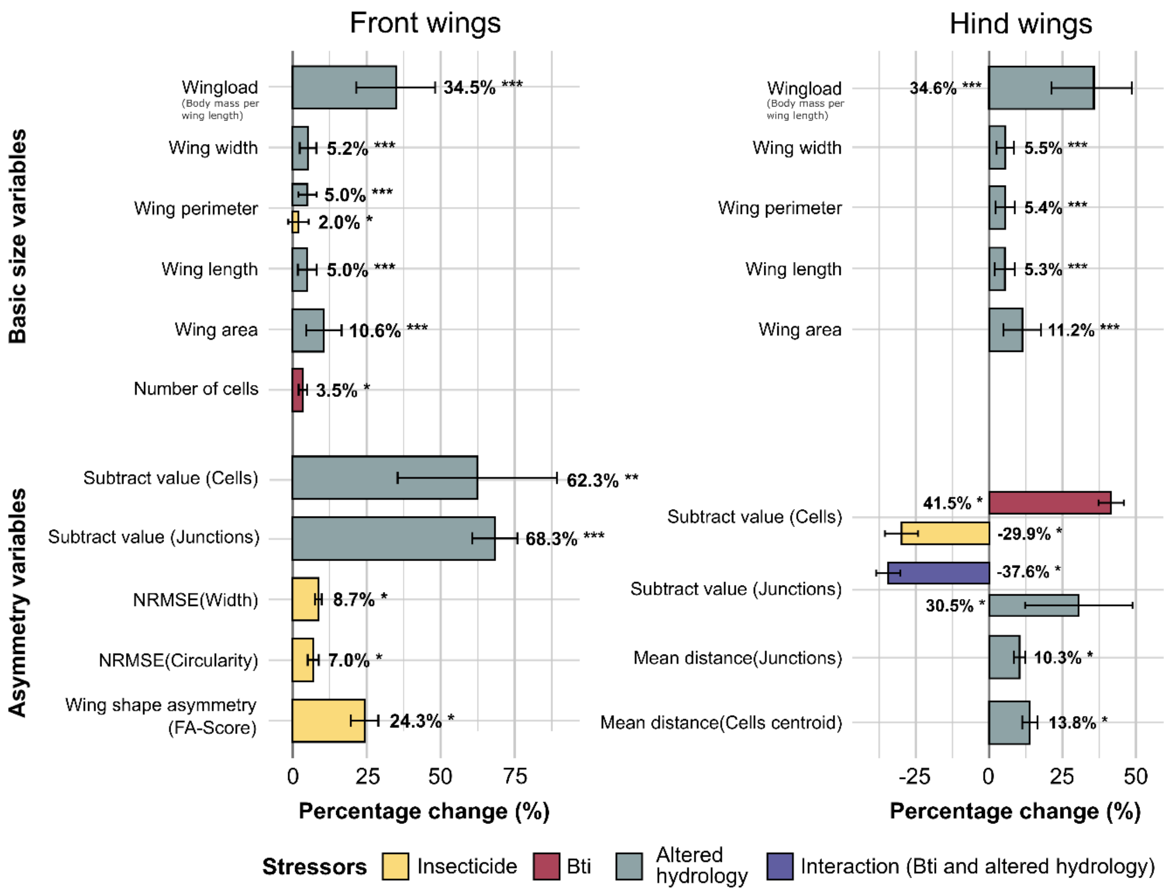

Figure 15. Effects of the three stressors insecticide (yellow), Bti (red), altered hydrology (grey), and their interaction (purple) on wing size (load, width, perimeter, length, area, cell number) and wing asymmetry (NRMSE of width and circularity, mean distance of centroids and junctions, FA-score, and left-right cell number difference). Insecticide effects refer to 0.04 mg L^{-1} vs. control. Generalised linear models were used for insecticide and Likelihood Ratio Chi^2 tests for Bti and hydrology effects, each including sex as covariate. Percentage changes resemble the mean change of females and males.

5.2 Bti affects damselfly larvae

In the mesocosm experiment, Bti altered the wing morphology of *C. puella*, though in more subtle ways than the insecticide. Emerging adults from Bti-treated ponds developed slightly more complex wing structures: on average their forewings had around 5 extra cells compared to controls (Figure 15). This corresponds to about a 3.5% increase in wing cell number, suggesting Bti exposure affected the wing's vein network pattern. This pattern was not accompanied by consistent changes in wing perimeter or area, indicating that additional cells were accommodated within existing wing dimensions, likely reflecting modified developmental dynamics rather than growth promotion. However, Bti-exposed larvae tended to emerge slightly heavier (+1.17 mg body mass) than controls, although this increase was not statistically significant. When comparing the number of cells between the left and right wings (i.e. the subtract value of cells), individuals from Bti-treated ponds showed a 41.5% increase.

However, this corresponds to only about one additional cell difference, as all individuals naturally had around two to three more cells in one wing than in the other. Other asymmetry parameters did not significantly respond to the Bti treatment. The underlying driver of observed shifts in cell number is likely trophic rather than toxicological. Bti acts specifically on dipteran larvae (e.g. mosquitoes, chironomids) and is not expected to affect odonate larvae directly. However, those dipteran larvae are key prey and competitors in aquatic systems, which can cascade to higher taxa (Gerstle et al., 2023, 2024). In our mesocosms, the modest wing alterations in Bti-treated damselflies may reflect indirect food web disturbances. Reported effects in the same system include reduced Chironomidae abundance (benthic kicknet sampling: Gerstle et al. (2023); emergence traps: (Kolbenschlag et al., 2023). At the FPM site, exuviae collections revealed a 54% reduction in Libellulidae and a 27% reduction in Coenagrionidae emergence two years prior to the study Gerstle et al. (2023), suggesting potential food web effects. However, no significant reduction in *C. puella* larvae abundance was observed in the year before our sampling (emergence traps: Schleihauf, not published), nor in chironomid abundance during the sampling year or the year before (emergence traps: Schöndorfer et al., 2025). Bti has been shown to specifically affect the Chironominae subfamily three years before the present study (Stoll et al., 2025), while Tanypodinae and Orthocladinae remained largely unaffected, raising questions about how such selective impacts may alter aquatic prey quality for Odonata.

Although Bti did not massively impair wing patterns of *C. puella*, the increased number of cells and trend in increased body weight suggest that Bti, while reducing target insects, can have side effects on aquatic-terrestrial link organisms. Given the rather few observed effects of Bti on *C. puella* but indications of increased resource availability (increased number of cells and weight), it is plausible that reduced interspecific competition at the same trophic level, and possibly a reduction in predation pressure, contributed to – or even improved – its developmental stability. Therefore, these findings suggest the need to account for indirect trophic pathways when assessing the broader ecological implications of Bti application in aquatic systems. Crucially, disturbances to aquatic insect communities affecting emergence patterns can create temporal mismatches with terrestrial predators that are often unable to compensate through alternative prey (Shipley et al., 2022). Potential terrestrial implications on the detected increase cell number are discussed in objective III.

5.3 Hydrological alterations affect damselfly larvae

Exposure to altered hydrological regimes induced significant changes in wing size and cell position symmetry in *C. puella* (Figure 15), reflecting a trade-off between growth and developmental precision under altered environmental conditions. In contrast to the other stressors, where morphological changes occurred independently of overall body size, individuals emerging from hydrologically altered FPMs showed consistent increases in wing area (+10.6% in front wings), length (+5.0% in front wings), perimeter (+5.0% in front wings) and wing load (+34.5% in front wings). Simultaneously, multiple symmetry metrics indicated reduced left-right consistency in cell structure: The subtract value of cells and junctions increased in front wings by 62.3% and 68.3% respectively and the mean distance of cells and junctions increased by 13.8% and 10.3% respectively. This increase is suggesting that increased investment in growth came at the expense of morphogenetic symmetry. Interestingly, unlike the insecticide, the hydrological stress did not change the overall wing shape outline (no significant effect on the Procrustes FA-score of wing shape). In other words, pond fluctuations mainly affected quantitative size and internal wing architecture rather than altering the basic wing contour. A possible explanation is that extreme water-level changes created periods of resource concentration and scarcity that stimulated faster growth (hence larger size) but also imposed longer stress periods (hence more asymmetries) compared to the short-term neurotoxicological effects of the insecticide. Periodic drying can increase larval density and competition in shrinking pools, followed by pulses of food upon rewetting (Herbst et al., 2019). The mesocosm design may led to such conditions, and the damselflies' trait response suggests a physiological adjustment to intermittent crowding and resource flux. Crucially, this demonstrates that climate-driven hydrological disturbances can directly impact the morphology of aquatic insects. Potential terrestrial implications on the detected increase in wing size and asymmetry is discussed in objective III.

Hydrological changes can also exert effects on aquatic prey availability. In the RSM, we simulated moderate flood events close to a stream instead of ponds and monitored the emergence of aquatic insects. Periodic flooding (4 events of 14 days each, spring to autumn) significantly boosted aquatic insect emergence, especially chironomids: flooded channels produced around 30-40% more chironomid adults than non-flooded controls over the season. This enhanced prey subsidy went together with a measurable effect on spider development. Therefore, the detected increase in wing size of *C. puella* may also underlies a change in aquatic nutrient availability through altered prey dynamics during their larval stage. Further studies are needed to investigate the effect of climate change and its hydrological alterations on specific species, as our results show that even one year of a scenario can cause significant changes at the damselfly species level, the long-term consequences of which are unknown.

5.4 Functional relevance within aquatic stage

Collectively, Paper 4 demonstrates that aquatic stressors can shape the phenotypes of organisms which they bring to the terrestrial ecosystems through emergence. The observed trait shifts then carry potential consequences for individual fitness and terrestrial ecosystem interactions which is discussed in objective III. Fluctuating asymmetry (FA) in particular has here proven to be a metric of aquatic stress response, increasing under detrimental conditions (e.g. +24.3% FA-score due to insecticide exposure, +13.8% cell position asymmetry due to altered hydrology). FA is widely regarded as an indicator of developmental instability, reflecting an organism's inability to buffer its development against stress (Parsons, 1992; Zakharov & Trofimov, 2022). However, in our damselfly experiments, each stressor left a distinct "trait signature" on the emerging adults, with only a few overlapping affected variables. Despite these differences, all point to the same conclusion that anthropogenic changes in aquatic systems significantly influence the development of aquatic insects. Notably, the functional consequences of these trait changes can manifest in the aquatic life stage and at the point of metamorphosis. When larvae face stress, they may prioritize essential processes (like finding food) over symmetry. Or, as noted earlier, pesticide-stressed damselfly larvae likely expended more energy on detox and repair (Monteiro et al., 2019). Such larvae might compensate by reducing activity or altering their active behaviour, for instance, feeding more to meet energy demands or, conversely, becoming less active to conserve energy. This is supported by Stoks (2001), who found that damselfly larvae under predator threat did not eat less but showed signs of lowered assimilation efficiency and higher metabolic rate, implying a stress-induced energy deficit. Thus, the observed change in developmental stability may represent a broader stress response, potentially accompanied by behavioural and other physiological alterations. FA, here as an indicator of developmental stability, is best interpreted within the broader context of stress responses. Rather than reflecting a single endpoint, it captures cumulative sublethal effects that may coincide with or precede changes in growth rates, behaviour, or physiological condition during aquatic development. Behavioural changes may include alterations in activity (general movement and exploration) and boldness (risk-taking or latency to resume activity after disturbance), which have been shown to respond to contaminant exposure in context-dependent ways. In a study, a positive correlation between these traits was already present in urban *C. puella* populations but only emerged in rural populations after pesticide exposure, suggesting that behavioural effects may depend on previous environmental conditions and local population history (Tüzün et al., 2017). Those changes in personalities may affect the viability of populations, and even alter community dynamics (Tüzün et al., 2017).

Crucially, a trait-based approaches may provide an early warning of ecological impact that precede outright population declines. Even when traditional endpoints (survival, emergence rate) show little change, morphology and asymmetry can reveal sublethal aquatic stress as underlined by these results. By the time population numbers drop, significant damage may have already occurred at the individual level. In summary, Objective II demonstrates that anthropogenic stressors in aquatic environments—whether chemical or physical—can significantly alter the development in aquatic insects. Combined with the widespread contamination revealed in Objective I, this highlights the need to either proactively prevent or precautionarily limit pesticide mixture exposure to avoid potential harm to aquatic-terrestrial ecosystems, given their contribution to insect population declines.

6 TRAIT-FITNESS LINKAGE AND CROSS-ECOSYSTEM IMPLICATIONS (OBJECTIVE III)

6.1 Linking morphology to short-term mating success

Male *C. puella* damselflies showed clear links between their morphology and short-term mating outcomes in the early season. Males observed in copula ("mating males") were on average lighter in body mass and had smaller wing size than those captured while actively chasing females without mating ("chasing males"). Specifically, mating males were about 5% lighter in wet body weight (approx. 1.3 mg less) and had around 3% smaller wing area compared to chasing males. This counter-intuitive result challenges the expectation that only the largest, most robust individuals achieve mating success under natural conditions. Instead, in this scrambling (that means actively foraging females for mating, species) smaller body size and wing dimensions appeared to confer a short-term advantage, possibly via enhanced agility or lower wing loading (De Block & Stoks, 2007). Consistent with these findings, a long-term study on the same species reported that male body size correlates negatively with daily mating rate, even though larger males enjoyed greater longevity (Thompson, 1989). In other words, smaller males mated more frequently per day whereas bigger males lived longer but mated less often (Thompson, 1989). Non-territorial damselflies like *C. puella* rely on rapid mate searching rather than prolonged fights, which could explain why speed and manoeuvrability may overplay sheer size in this context (Fincke et al., 1997). Wing morphology beyond simple size also played a role. Computer-vision analysis revealed that mating males from one population had simpler wing venation (fewer cells and junctions) than their rivals. In the Palatinate Forest, for example, mating males had about 6-7 fewer front wing cells (out of around 160 total in front wings) than chasing males. This suggests a potential advantage of less complex wing structure under certain conditions, though the functional significance of this difference remains unclear. In contrast, wing asymmetry did not show a consistent overall effect on mating success in our study. There was no significant difference in wing FA between mating and chasing males alone. The literature on wing symmetry and reproductive success in odonates has reported mixed results. Some studies found that more symmetric males enjoyed higher mating or survival success, while others found no effect. For example, Harvey & Walsh, (1993) reported a positive relation between wing FA and lifetime mating success in a damselfly population, whereas a study on a another coenagrionid species found no effect of wing FA in males actively participating in mate acquisition (Carchini et al., 2000). Such inconsistencies have been attributed to differences in trait measurement reliability and variability as well as variation in environmental context (Simmons et al., 1999). However, significant interactions between short-term mating and location found in Paper 5 underscore that morphological predictors of mating can be context-dependent (which is discussed later in this chapter). In addition, short-term mating success typically refers to immediate reproductive interactions (e.g. copulation events within a limited timeframe), while long-term success reflects lifetime reproductive output, including offspring survival (Hunt et al., 2004). Therefore, short-term mating success may not be related with wing symmetry in *C. puella* alone and long-term success would have to be studied with the recently advancing computer-vision. At the same time, the underlying data variance in the present study showed that extremes of symmetrical and asymmetrical wings can nonetheless be found in successfully mating males in short-term.

6.2 Effect of land-use context on trait-reproduction links

We compared *C. puella* populations from two sites with contrasting land-use surroundings: a fishing pond in the agricultural Rhine Valley and a forested pond at a research station in the Palatinate Forest. Despite similar basic water chemistry (e.g. both sites had low phosphate and copper levels, with the Rhine Valley site showing moderate nitrate 2.4 mg L^{-1}; Paper 6), these habitats may differ in other ways reflecting land-use intensity. We anticipated that the more agricultural site might impose greater developmental stress, potentially leading to higher FA and stronger selection on traits. Contrary to this hypothesis, trait differences between the two populations did not simply mirror an "agricultural stress = poorer traits" pattern. Instead, each population showed a unique trait profile: males from the Palatinate Forest site were significantly smaller on average, weighing 4.6 mg less (-15%) and having markedly reduced wing dimensions (front wing area 7.5 mm^2 (-11%) smaller) than those from the Rhine Valley. Counterintuitively, the Palatinate Forest males also exhibited greater cell shape asymmetry (6-12% higher FA in certain front wing metrics) compared to Rhine valley males. On the other hand, they also showed lower asymmetry in wing cell positioning (12-19% lower FA in certain front hind metrics). These findings indicate that land-use context influenced trait development and variation in different ways, which also showed in detected statistical interactions.

Interaction patterns showed up when the effect of FA on mating was investigated for its dependence on the location: in the Palatinate Forest, mating males tended to have more pronounced cell shape asymmetry in comparison to mating males in the Rhine Valley (+13% higher NRMSE (circularity)), especially with focus on the distal part of the wing (+16% higher NRMSE (circularity)). In other words, the two populations' cell shape asymmetry was similar in terms of the chasing males, but significantly different in terms of the mating males. This hints that more symmetric wings might deliver a benefit under certain environmental conditions, even though a universal effect on mating was not observed. A significant interaction was also found between other wing traits. In the Palatinate Forest, mating males had substantially simpler wings than the chasers (about 10 fewer crossvein junctions and 7 cells on average), whereas in the Rhine Valley this specific trait difference was not significant. Such site-specific selection may reflect differences in ecological factors like density of competitors, sex ratio, or predation pressure (see also discussion Objective II). For instance, the Palatinate Forest had a later, shorter emergence window (earliest male collecting was possible in early June vs. May in the Rhine Valley) and possibly lower adult density compared to the Rhine Valley, which could relax male-male competition but limit mating opportunities. The Rhine Valley, in contrast, likely had an earlier season and different predator communities due to size of the ponds and surrounding open area, potentially favouring slightly different trait optima.

It is notable that anthropogenic factors were present at the research ponds in the Palatinate Forest: as stated in Paper 3, approximately half the forested ponds receive regular Bti treatments to control mosquitoes, and some ponds were experimentally subjected to hydrological manipulation (Bauspiess et al., 2025; Schöndorfer et al., 2025). These stressors likely contributed to the trait differences we observed. The males' smaller size could be partly explained by lower larval food availability, as mosquito control (Bti) reduces chironomid midges which are important damselfly prey (Gerstle et al., 2024). However, as results of Paper 3 show, Bti rather had a promoting effect on *C. puella*'s body weight and wing cell number, which argues against this explanation. It is therefore possible that the more successful males with fewer wing cells originated from ponds without Bti treatment, suggesting that Bti may have had a negative effect on mating despite its seemingly positive influence on cell number and body weight. However, this interpretation remains speculative and requires further investigation.

Synthesized effects can be seen in Table 1, which contrasts how key traits responded to stressors in our studies versus how those traits related to mating success.

Table 1. Terrestrial trait-reproduction links (male) observed or described in literature compared to aquatic stressor effects on C. puella (avg. female/male). Each trait's association with short-term mating success (from field data and other references) is compared to its change under larval stress (from experimental and mesocosm stressor data). All stress effects (Altered hydrology, Bti and insecticides) refer to terrestrial adult females and males.

Morphological Trait	Altered hydrology	Bti	Insecticide	Wings affected	Influence on Reproduction/Fitness	Reference
Adult body weight	↑27%	-	-	-	Mating males were 5% lighter than chasing males (↑ weight ↓ agility) – low weight can reduce energy cost and improves manoeuvrability, a key male trait under sexual selection in the non-territorial damselfly *C. puella*	(Paper 4); (Paper 6); (De Block & Stoks, 2007, low body weight → high flight manoeuvrability advantage)
Wing load (body mass per wing length)	↑35%	-	-	-	↑ wing load ↑ flight speed ↓ flight endurance – high flight endurance is a key male trait under sexual selection in the non-territorial damselfly *C. puella* (endurance > speed)	(Paper 4); (Paper 6); (Gyulavári et al., 2014, flight endurance selected in *C. puella*; high wing load speeds flight but cuts endurance)
Wing length	↑5%	-	-	Front, hind	Mating males showed 1-2% shorter wings than chasing males – consistent with negative body weight/mating correlation (↑ wing length ↓ agility)	(Paper 4); (Paper 6);
Wing width	↑5-6%	-	-	Front, hind	Mating males showed 2% narrower wings than chasing males – consistent with negative body weight/mating correlation (↑ wing width ↓ agility)	(Paper 4); (Paper 6);
Wing area	↑11%	-	-	Front, hind	Mating males showed 3% smaller wing area than chasing males – consistent with negative body weight/mating correlation (↑ wing area ↓ agility). Smaller males have relatively larger wing area per mass, aiding flight endurance	(Paper 4); (Paper 6);(Schmidt-Nielsen, 1984, allometry: smaller organisms → larger wing area relative to mass)
Wing perimeter	↑5%	-	↑2%	Front, hind	Mating males showed 2% less perimeter than chasing males – consistent with negative body weight/mating correlation (↑ wing perimeter ↓ agility)	(Paper 4); (Paper 6)
Number of cells	-	↑3.5%	-	Front	*Site-specific:* In the Palatinate Forest, mating males had 4% fewer wing cells (↑ cell number ↑ "material" cost). No significant mating advantage of cell number in the Rhine Valley found.	(Paper 4); (Paper 6); (Rajabi et al., 2017, wing veins/cells trade-off: more, smaller cells strengthen wing but add mass and cost)
Wing cell shape FA (NRMSE(Width))	-	-	↑9%	Front	Not identified as a mating success predictor	(Paper 4); (Paper 6)

Morphological Trait	Altered hydrology	Bti	Insecticide	Wings affected	Influence on Reproduction/Fitness	Reference
Wing cell shape FA (NRMSE(Circularity))	-	-	↑7%	Front	Site-specific: In the Palatinate Forest, mating males had 7% higher cell circularity FA than chasing males (n.s); Mating males in the Palatinate Forest had 13% higher cell circularity FA than mating males in Rhine Valley (sign.). (context dependent: ↑ cell shape FA ↑ short-term mating)	(Paper 4); (Paper 6); (González-Tokman et al., 2013, infection stress → increased mating effort, potentially linking high asymmetry to "terminal" investment)
L-R wing cell count FA (Subtract value (Cells))	↑62%	↑41.5%	↓30%	Front, hind	Not identified as a mating success predictor	(Paper 4); (Paper 6)
L-R wing junction count FA (Subtract value(Junctions))	↑31-68%	-	-	Front, hind	Not identified as a mating success predictor	(Paper 4); (Paper 6)
Wing cell position FA	↑14%	-	-	Hind	Not identified as a mating success predictor	(Paper 4); (Paper 6)
Wing vein junction position FA	↑10%	-	-	Hind	Not identified as a mating success predictor	(Paper 4); (Paper 6)

6.3 Linking aquatic stressors to short-term mating success

The experiment confirmed that multiple aquatic stressors (insecticide, Bti, altered hydrology) induced significant changes in *C. puella* wing morphology and increased asymmetry. These traits are carried-over into the terrestrial stage, potentially impairing the organism's ecological interactions.

Altered hydrology was implemented in the mesocosm as a climate change scenario. It increased body mass and wing planform dimensions, including length, width, area and perimeter (Table 1). In contrast, successfully mating males at the same ponds were lighter with slightly shorter and narrower wings and a lower perimeter than chasers. If hydrological variability becomes common, *C. puella* may trend towards larger and heavier forms that underperform during scramble search due to reduced agility (Fincke et al., 1997; Kokko & Rankin, 2006). *C. puella* is a generalist and can likely persist because it is behaviourally flexible and tolerant. Persistence does not mean that mating stays unchanged. If wings become larger and heavier, males may mate less often unless they adjust their behaviour to compensate. Long-term consequences for *C. puella* populations remain unresolved, and potential community wide responses are likewise uncertain. Given the observed morphological shifts, in several cases above 20 percent, an effect on the species and on competitors due to climate change is therefore a conservative expectation. Overall, the altered hydrological regime implies that ongoing climate change will redirect morphological distributions and mating success, with effects that are expected to extend to population and community levels, challenging conservation efforts for aquatic-terrestrial habitats.

Insecticide exposure also raised wing perimeter as well as cell shape and wing shape FA (Table 1). In contrast, mating males carried lower perimeter and tended to be lighter with more compact wings, indicating a mismatch with scramble optima that favour agility in non-territorial systems. Counterintuitively, mating males also showed 7% higher cell shape asymmetry in the Palatinate Forest compared to mating males in the Rhine Valley, in the same effect range as caused by the insecticide. This suggests that FA may not represent a universal penalty, and in some contexts, it can even be neutral or even associated with higher mating effort. However, in other ecological or physiological contexts, elevated FA may still be linked to reduced performance or survival, reflecting its role as an indicator of developmental disturbance. FA can covary with behaviour and resource use. High activity during mate search or terminal investment under stress can raise mating effort and co-occur with higher asymmetry rather than directly causing mating failure (De Block & Stoks, 2005; González-Tokman et al., 2013; Purse & Thompson, 2005; Rech et al., 2022). Given that two weeks of exposure produced an FA shift in a range similar to that observed in mating males, the correlation with short-term mating success implies that environmental pesticide concentration in this range may already modulate reproductive dynamics of insects.

Bti increased overall front-wing cell number, whereas in the Palatinate Forest mating males had fewer cells than chasers (Table 1). In the Rhine Valley, higher cell counts showed no clear advantage for short-term mating success. The experimental increase in cell number therefore contrasted with the field pattern, where successful males had fewer cells. This mismatch suggests that resource-driven morphological changes may shift individuals away from the locally favoured phenotype. If Bti alters prey composition and reduces competition for the generalist *C. puella*, extra energy may be channelled into structural elaboration that does not enhance mating performance early in the season. Over time, such trait shifts could disadvantage populations if altered prey communities become the norm, especially for species with limited capacity to adjust morphology or behaviour. Patterns could differ when considering long-term mating success, as traits that are neutral or even disadvantageous in short-term competition may confer benefits over an entire breeding season or lifespan. This represents a limitation of the present study and warrants further investigation to capture the full fitness consequences of Bti-induced trait changes. Patterns could differ when considering long-term mating success, as traits that are neutral or even disadvantageous in short-term competition may confer benefits over an entire lifespan. Nevertheless, Bti still produced an effect size in the range observed in damselfly mating dynamics, highlighting further knowledge gaps in how such indirect stressors influence reproductive processes.

6.4 Terrestrial implications of larval carry-over effects beyond reproduction

Beyond reproductive effects, the same stress-induced morphological changes may influence fitness directly or correlate with other more relevant fitness-traits. Empirical tests on the dragonfly *Sympetrum rubicundulum* have shown that partial wing area loss of 30% in hindwings (approx. 18% of the total wing area) significantly reduced vertical flight acceleration by 29% and flight velocity by 21%, translating to a 22% drop in aerial prey capture success (Combes et al., 2010). In addition to prey capture success, impaired flight performance would likely make adults more vulnerable to their own predators and less effective in dispersal. However, the amount of wing area loss in the mentioned study was rather large, as another study found in most of the collected *Sympetrum striolatum* individuals a maximum of 5% loss of total wing area and up to 45% in forewings and 34% in hindwings only in few cases (Rudolf et al., 2019). A study on lifetime mating success in another coenagrionid species found that individuals with higher wing length FA were recaptured less frequently (Carchini et al., 2005). The authors concluded that the measured FA reflected overall individual quality but likely did not directly affect fitness, given the relatively low FA ratio of approximately 0.3% (Carchini et al., 2005). However, an adjustment of the flapping amplitude due to one wing being smaller than the other has been observed in other insects and discussed for Odonata, too, as an adjustment due to this asymmetry would be needed to avoid spinning their body about the roll axis (Rajabi et al., 2020). Even if such amplitude adjustments allow for some compensation, unintended asymmetries may still affect flight stability, as small differences in wing motion can induce rotational forces and alter body orientation, potentially increasing energetic costs or reducing control precision (Hedrick et al., 2024). For example. it has been shown that flies had more asymmetrical wings than survivors when they were caught by swallows (Møller, 1996). In addition, a study was able to genetically change the shape of *Drosophila* wings to improve their flight performance (Ray et al., 2016), underlining the importance of wing shape or wing shape asymmetries on potential fitness outcomes. However, wing length and shape asymmetries of the magnitude we have found and their direct fitness implications are still insufficiently studied.

The previously mentioned study, where flies had more asymmetrical wings than survivors when they were caught by swallows also found that flies with asymmetric wings were more susceptible to fungus infections-than flies with symmetric wings (Møller, 1996). In short, larval stress that results in morphologically altered adults may show reduced fitness but entangling direct effects and correlations can be challenging. A study directly linking FA at the levels observed in the experiments of Paper 4 to fitness in Odonata (excluding reproductive success and without artificially inducing asymmetry by wing clipping) is currently lacking. Nonetheless, the mentioned findings from other insect taxa suggest that a correlation between observed FA levels in this study and fitness cannot be ruled out. The increasingly precise and comprehensive quantification of wing asymmetry through modern computer-vision could facilitate such investigations in the future.

Crucially, these individual-level effects may scale up to influence population dynamics and community interactions. In addition to changing wing features, exposure to sublethal stressors can reduce survival and impair other flight-related traits such as adult mass, muscle mass, and fat content, thereby limiting the number and quality of dispersing individuals and potentially reducing local population growth (Dinh et al., 2016). If flight ability is compromised, emerging damselflies may become more vulnerable to terrestrial predators, intensifying initial predation pressure. Such changes in prey availability may affect other trophic levels: in riparian food webs, an increased flux of aquatic insects shifted lizard foraging away from terrestrial arthropods, demonstrating that aquatic emergence can actively restructure terrestrial communities (Sabo & Power, 2002). Over longer timescales, not only the quantity but also the physiological quality of emergent prey matters. In spiders, feeding on contaminant-exposed *Chironomus* reduced key fatty acids by around 30% and growth by 40-50% (Pietz et al., 2023). Damselfly asymmetry, as a proxy for developmental disruption, may similarly correlate with nutritional value, including lipid reserves (Dinh et al., 2016), and thus affect their role as prey in terrestrial food webs.

Conversely, boosting aquatic larval conditions can enhance terrestrial consumer populations: the RSM experiment showed that flooding (which increased aquatic insect output by around 45%) improved spider developmental outcomes, as evidenced by 19% higher symmetry in spider prosomes under high prey supply (Paper 6). Improved symmetry indicates reduced developmental stress and is associated with higher fitness in spiders (Hendrickx et al., 2003). This finding underscores that changes in aquatic prey quantity or quality can show in terrestrial predators' morphology and performance, ultimately influencing their reproductive success and densities. In summary, trait-mediated carry-over effects may not only shape the fate of individual damselflies but also the structure of the terrestrial communities that depend on them as both predators and prey.

6.5 Methodological and applied perspective

This thesis demonstrates that combining multi-scale empirical designs with modern digital trait quantification provides a sensitive framework to detect subtle stressor effects in complex ecosystems (Manfrin et al., 2023, Paper 4, 5 & 6). The application of automated morphometric analysis (e.g. WingAnalogy 4.0) facilitated the extraction of cell- and wing-level shape traits with high precision across large sample sizes (Eshghi et al., 2024, Paper 4, Paper 5). Automated image-based tools outperform manual digitisation in reproducibility and allow high-throughput analysis, which is essential for broader ecological applications (Eshghi et al., 2024). Such phenotyping approaches are increasingly vital, as phenomic characterisation also still lags behind genomic resolution, limiting our ability to study the genotype-phenotype map in ecological contexts (Houle et al., 2010). Shape asymmetries and fine-scale distortions in cell geometry can be sometimes more sensitive to developmental instability than traditional size metrics alone (Klingenberg, 2015). In our damselfly model system, the combination of overall wing size, centroid shift and cell circularity asymmetry provided a comprehensive picture of trait disruption under chemical and hydrological stress. Wing traits can be tightly linked to fitness-related performance such as dispersal, mating and predator evasion, and thus act as functional proxies for ecological stress (Hedrick et al., 2024; Outomuro et al., 2016). The detection of directional changes in average body condition or symmetry could serve as early indicators of sublethal stress before population declines occur. However, FA alone should be interpreted with caution, as its direction and magnitude can vary with stressor type, intensity and taxon-specific buffering capacity (Palmer & Strobeck, 1986, and own results). Studies sometimes report inconsistent FA-fitness relationships or non-monotonic patterns under low or moderate stress (Lens et al., 2002). In our work, FA shifts showed up under some but not all stressor-trait combinations, highlighting the importance of combining multiple trait metrics for more robust interpretation but also challenging future simple stress response studies.

For example, visual scoring or automated imaging of wing morphology could be integrated into biomonitoring schemes alongside traditional community indices but never fully replace them because of uncertainties in the dependence on intrinsic invertebrate properties (e.g., life cycle) or extrinsic physical, chemical, and biological factors (e.g., temperature, conductivity, other taxa) (Bonada et al., 2006). However, damselflies offer additional insight through its aquatic-terrestrial life cycle. Current risk assessments rarely consider cross-ecosystem dynamics, although emerging aquatic insects serve as crucial vectors of contaminant transfer into terrestrial food webs and may themselves experience trait-mediated impairments (Schulz et al., 2024).

7 CONCLUSION AND OUTLOOK

This thesis reveals that pesticide contamination has become a defining characteristic of modern agricultural landscapes and demonstrates how pesticides and other micropollutants like Bti, as well as altered hydrological regimes from climate change can affect the developmental stability of organisms at the aquatic-terrestrial interface, with potential of effects propagating across ecosystem boundaries through trait-mediated mechanisms (Figure 16). In simple terms, stress in the water can change the shape and function of organisms in ways that also affect life on land.

The detected large-scale pesticide contamination patterns underpin other increasing evidence that pesticides affect a diverse range of non-target species and may be linked to global biodiversity loss, with recent comprehensive analyses revealing negative responses across multiple taxa and physiological endpoints (Wan et al., 2025). Pesticide use causes substantial non-target effects on soil organisms, thereby contributing to soil biodiversity loss and ecosystem degradation (Beaumelle et al., 2023), while the recovery of European freshwater biodiversity has come to a halt despite decades of conservation efforts (Haase et al., 2023). The complexity observed in this thesis, where chemical stressors and hydrological alterations simulating climate change significantly produced distinct 'trait signatures' in emerging adults, may reflect processes that align with broader ecological patterns reported elsewhere, where the interaction between historical climate warming and intensive agricultural land use has been associated with reductions of almost 50% in insect abundance and species richness (Outhwaite et al., 2022).

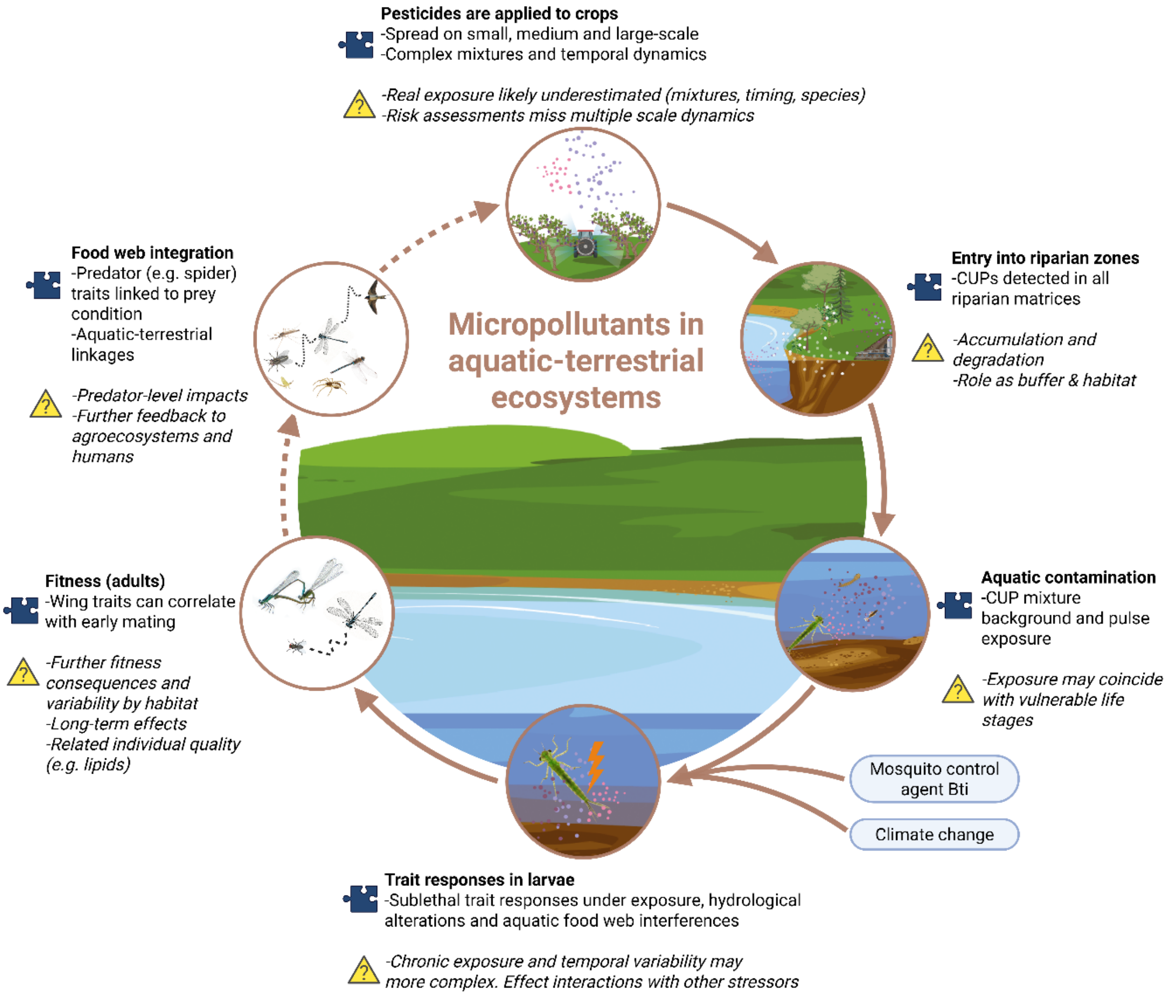

Figure 16. Synthesis of micropollutants in aquatic-terrestrial ecosystems, key findings in this thesis (puzzle pieces) and discussed open questions for further research (yellow triangles). Created with biorender.com

The trait-mediated approach used in this study addresses a critical gap in understanding how sublethal effects may cascade through aquatic–terrestrial ecosystems, a gap that urgently needs to be filled to safeguard biodiversity. In temperate riparian zones, emergent aquatic insects provide substantial prey subsidies to terrestrial consumers, and disturbances that reduce insect emergence demonstrably depress riparian predators such as spiders and birds, underscoring the need to protect land-water linkages (Kowarik et al., 2023; Muehlbauer et al., 2014; Shipley et al., 2022). If these links break, animals on land may simply run out of key food sources. Such interdependencies are exemplified by changes in aquatic subsidies that create temporal mismatches with terrestrial predator life cycles, where birds and other consumers cannot readily compensate for disrupted cross-ecosystem resource flows through alternative prey sources (Shipley et al., 2022). This complexity extends globally, where aquatic emergence is coupled differently to terrestrial systems across latitudinal gradients, ranging from continuous, diffuse emergence in tropical regions to highly concentrated seasonal pulses at higher latitudes, thereby creating diverse vulnerabilities to disruptions in aquatic emergence (Nash et al., 2023).

Several key research priorities emerge from this synthesis (Figure 16). First, the temporal dynamics of exposure require investigation across complete organism life cycles, particularly for species with complex ontogenetic shifts between aquatic and terrestrial phases. This study demonstrates that contamination peaks can coincide with periods of maximum biological activity. It also shows that brief exposure of damselfly larvae during critical developmental windows shortly before emergence can trigger trait responses, suggesting that current risk assessments, which rarely consider seasonal exposure patterns, may overlook critical windows of vulnerability. The timing of exposure may just be as important as the concentration.

Second, the mechanistic basis of trait-mediated effects needs to be studied through integrated physiological, morphological, and behavioural approaches. While FA provides a useful proxy for developmental instability, understanding the energetic costs and performance consequences of altered morphology remains incomplete as seen in the mating success study. Advanced phenotyping technologies used in this thesis revealed subtle indicators related to mating, offering only a glimpse into the complex reproductive processes at play. This serves as a warning that we may still fundamentally misunderstand how environmental stressors influence insect mating behaviours that are critical for maintaining genetic diversity and population viability. In short, we may still underestimate how stress changes insect reproduction.

Third, the disruption of aquatic emergence patterns threatens essential ecosystem services, potentially creating cascading feedback loops that intensify management pressures. The decline in emerging aquatic insects may compromise natural biological control mechanisms, exemplified by how Bti applications reduce dragonfly communities that historically served as effective mosquito predators - potentially necessitating increased Bti applications where natural control once sufficed. Similarly, the loss of emerging insects as prey subsidies may destabilize terrestrial food webs, including those that could support agricultural activities. When these natural regulatory networks are disrupted, agricultural systems might become increasingly dependent on intensive chemical inputs to maintain productivity, potentially further degrading habitats and perpetuating cycles of ecosystem degradation and escalating intervention costs. Understanding and interrupting such potential feedback loops could prove crucial for the future.

Losing natural insect populations may force us into using more chemicals, making the problem worse.

In conclusion, this thesis emphasizes that micropollutants in aquatic-terrestrial ecosystems operate through interconnected pathways that demand integrated management approaches. The hope is that such integrative knowledge will inform more holistic conservation strategies - recognizing that safeguarding aquatic habitats and their emerging insects not only preserves aquatic biodiversity, but also uphold the terrestrial food webs that depend on their cross-ecosystem subsidies. As agricultural landscapes continue expanding under growing food security demands, the challenge lies in developing production systems that maintain the ecological connectivity essential for sustaining both aquatic and terrestrial biodiversity. This means farming in a way that also keeps streams, wetlands, and surrounding wildlife connected and healthy. Ultimately, achieving these conservation goals may require a fundamental shift towards pesticide-free and sustainably managed agricultural systems that prioritize ecosystem health alongside food production.

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9 AUTHOR CONTRIBUTIONS

This is a cumulative dissertation containing three published articles in peer-reviewed international journals and three submitted manuscripts:

Paper 1 (Appendix I):

Ken M. Mauser: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Carolina Honert**: Methodology, Formal analysis, Data curation, Writing – review & editing. **Jakob Wolfram**: Methodology, Formal analysis, Writing – review & editing. Jürg W. Spaak: Methodology, Formal analysis, Writing – review & editing. **Carsten A. Brühl**: Conceptualization, Methodology, Resources, Investigation, Writing – review & editing, Project administration, Supervision.

Paper 2 (Appendix II):

Carolina Honert: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Ken M. Mauser**: Methodology, Formal analysis, Data curation, Writing – review & editing. **Ursel Jäger**: Methodology, Formal analysis, Writing – review & editing. **Carsten A. Brühl**: Conceptualization, Methodology, Resources, Investigation, Writing – review & editing, Project administration, Supervision.

Paper 3 (Appendix III):

Ken M. Mauser: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review & editing. **Tom Dekker**: Conceptualization, Methodology, Investigation, Writing – review & editing. **Jürg W. Spaak**: Conceptualization, Methodology, Writing – review & editing. **Tobias Elsässer**: Methodology, Writing – review & editing. **Carsten A. Brühl**: Conceptualization, Methodology, Resources, Investigation, Writing – review & editing, Project administration, Supervision.

Paper 4 (Appendix IV):

Ken M. Mauser: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review & editing. **Samiksha Paudel:** Methodology, Investigation, Writing – review & editing. **Olivia Sigmund**: Methodology, Writing – review & editing. **Martin H. Entling**: Conceptualization, Writing – review & editing. **Jürgen Ott:** Conceptualization, Writing – review & editing **Carsten A. Brühl**: Conceptualization, Methodology, Resources, Writing – review & editing, Project administration, Supervision.

Paper 5 (Appendix V):

Ken M. Mauser: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Jann Baumeyer**: Writing – review & editing, Methodology, Investigation, Conceptualization. **Shahab Eshghi**: Writing – review & editing, Software, Methodology, Investigation. **Stanislav N. Gorb**: Writing – review & editing, Methodology, Investigation. **Alessandro Manfrin**: Writing review & editing, Investigation. **Carsten A. Brühl**: Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization.

Paper 6 (Appendix VI):

Stephane Mutel: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Project Administration, Resources, Validation, Visualization, Writing – Original, Draft Preparation, Writing – Review & Editing. **Martin H. Entling:** Conceptualization, Funding Acquisition, Supervision, Validation, Writing – Review & Editing. **Ken M. Mauser**: Conceptualization, Formal Analysis, Validation, Writing – Review & Editing. **Federica Spani**: Methodology, Software, Validation, Writing – Review & Editing. **Celia Thomas**: Formal Analysis, Investigation, Writing – Review & Editing. **Collins Ogbeide**: Investigation, Resources, Writing – Review & Editing. **Mirco Bundschuh**: Funding Acquisition, Supervision, Writing – Review & Editing. **Franziska Fiolka**:Investigation, Writing – Review & Editing. **Franziska Middendorf**: Investigation, Writing – Review & Editing. **Ralf Schulz:** Funding Acquisition, Supervision, Writing – Review & Editing. **Alessandro Manfrin:** Conceptualization, Methodology, Supervision, Validation, Writing – Review & Editing.

10 APPENDIX I: PAPER 1

Current-use pesticides in vegetation, topsoil and water reveal contaminated landscapes of the Upper Rhine Valley, Germany

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Current-use pesticides in vegetation, topsoil and water reveal contaminated landscapes of the Upper Rhine Valley, Germany

Ken M. Mauser, **Jakob Wolfram**, **Jürg W. Spaak**, **Carolina Honert** & **Carsten A. Brühl**

Non-target areas in agricultural landscapes serve as invaluable refuges for organisms and safeguard biodiversity. This research aimed to examine the landscape-scale distribution of Current Use Pesticides (CUPs) in the Upper Rhine Valley in Germany, a region characterised by intensive agriculture in the valley and bordered by forested lower mountain regions. We sampled vegetation, topsoil, and surface water at 78 non-target, off-field sites during the pesticide application season in 2022. The sites were located in six 30 km long transects to cover both the valley and mountain regions. Samples were analysed for 93 CUPs. In total, 63 different CUPs (29 fungicides, 19 herbicides and 15 insecticides) were detected in all samples ($n = 186$). CUPs were recorded in 97% of all vegetation and 97% of all topsoil samples (76 of 78 samples each). In total, 140 unique mixtures with ≥2 components were recorded. A prediction map using additional site parameters suggests a widespread presence of CUPs extending multiple hundred meters beyond CUP application areas. Landscape-scale mixture contamination is not addressed in environmental risk assessment for the regulation of pesticides. The study design could serve as a benchmark for evaluating landscape-scale pesticide contamination after implementation of pesticide reduction efforts in agricultural policies and practice.

Pesticides are known to make comprehensive contributions to the decline of several groups of organisms and biodiversity[1–3]. The air in agricultural intense landscapes is particularly loaded with the Current Use Pesticides (CUPs)[4], and CUPs can be transported over long distances[5]. Thus, CUPs have been detected not only in crop fields[6–8] but also in non-target areas[9–13] or even in insects in nature conservation areas[14]. CUPs from agricultural landscapes are able to enter local food webs, potentially magnifying with increasing trophic level[15]. Although a relationship between CUP contamination and short-distance distribution from crop fields has been demonstrated, we just begin to understand CUP contamination at the landscape-scale and the potential exposure of organisms[16].

The complexity of CUP transport processes makes it difficult to predict the exposure of different environmental matrices and non-target organisms, although realistic data on exposure are urgently needed in view of the rapidly advancing biodiversity declines in terrestrial habitats[17,18]. One way to thoroughly evaluate realistic scenarios is to conduct large-scale, long-term monitoring studies to determine the presence of CUPs in the environment. The United Nations Environment Programme calls for additional post-registration monitoring of pesticides and their transformation products in the environment[19], especially in areas where there is high use intensity. Long-term monitoring is already conducted for European surface waters[20], but, in contrast, only a few local and short-term soil and vegetation CUP measurements exist[9,16,21,22], despite the recognized terrestrial biodiversity losses. To assess realistic terrestrial pesticide contamination on a landscape-scale, we conducted extensive sampling across various environmental matrices on a large spatial scale in the Upper Rhine Valley in Central Europe.

The study area is characterized by intensive agriculture, with cropping areas covering 23.4% of the total study region. A diverse range of crops is cultivated, including vegetables (3.4%), cereals (12.2%), vineyards (3.8%) and fruit orchards (2.2%) with some areas exhibiting a high density of cultivated land (Fig. 1, Supplementary Table 1, see methods for details). In the agricultural core of the valley, agriculture and human population are concentrated, where approximately 19.6% of the land is urban and 37.3% is cropping area. The agricultural valley is embraced by the two bordering mountain regions: the Black Forest and Palatinate Forest with low agricultural activity (9.0% cropping area). There are non-target areas of

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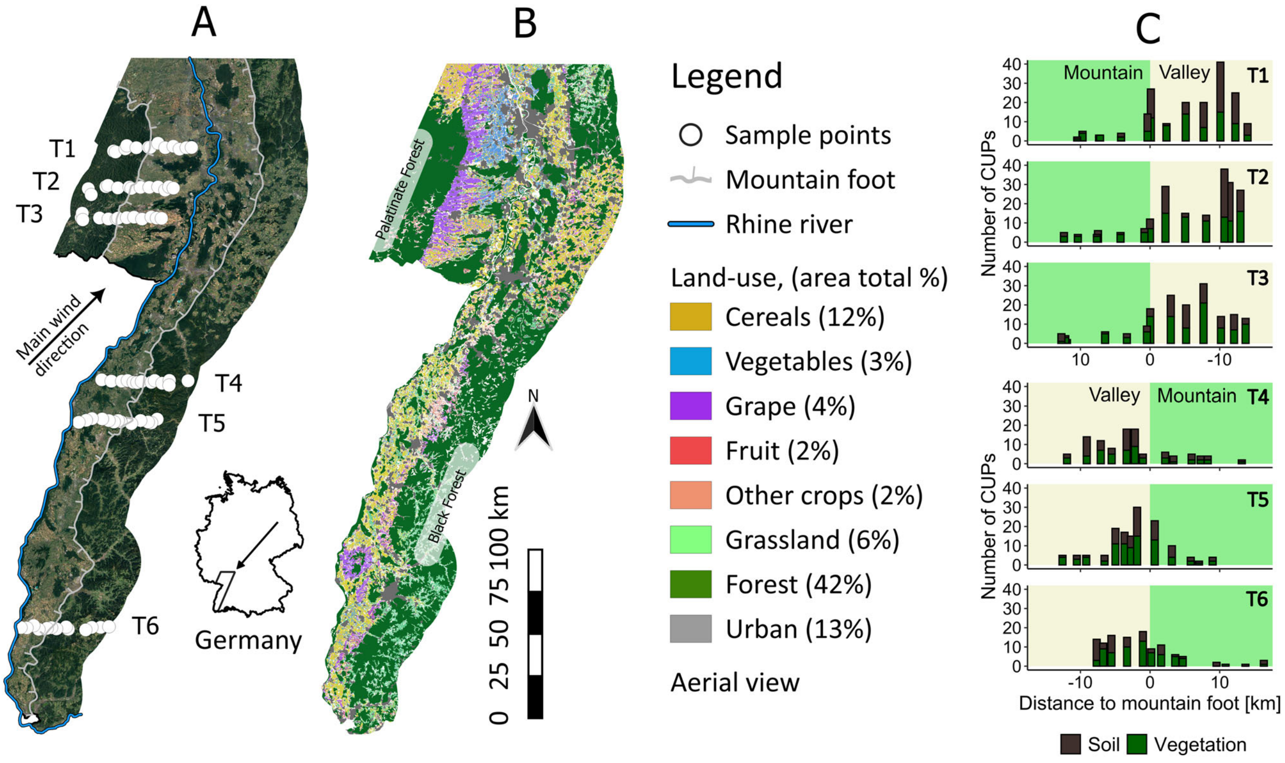

Fig. 1 | Aerial view of the study region Upper Rhine Valley, Germany, its land use and measured cumulative number of Current Use Pesticides residues in topsoil and vegetation per transect. **A** Aerial view with the 78 sample sites (white dots) and six transects (T1-T6). Sample sites were located on line transects T1 - T6 (north to south) with west/east orientation and categorized into mountain and valley depending their location regarding the mountain foot. **B** Land-use (**C**) Measured cumulative number of CUPs in topsoil and vegetation per transect. Water was excluded in (**C**), due to the availability at only 30 sites. Vegetation and topsoil were sampled between June 15th and July 11th 2022. Digital Elevation Model and forest/urban land cover from European Environment Agency (EEA) (2016); remaining land use categories from Blickensdörfer et al. (2021) of the year 2019. Aerial view: © 2025 Google, TerraMetrics.

conservation concern in the mountain regions as well as in the valley, e.g. nature conservation areas with red-listed plant and animal species. In addition, the Upper Rhine Valley has an alluvial plain and is lying on one of Europe's largest groundwater resources which is under profound pressure due to intensive agriculture[23] and source of drinking water for more than seven million people[24]. Non-target areas outside cropping fields were sampled in the agricultural valley and forested mountain regions utilizing a standardized methodology[9,25], and vegetation, topsoil and surface water were analysed for 93 CUPs. Samples were also taken in the Black Forest National Park, the UNESCO Palatinate Forest-North Vosges Biosphere Reserve and Black Forest Biosphere Reserve.

We assumed that due to the prominent SW wind direction, the Palatinate Forest is less contaminated by CUPs than the Black Forest (Fig. 1) and that CUP residues are detected in higher numbers and concentrations next to agricultural fields and only occasionally further away from the application areas. CUP measurements and land-use data were used to create landscape-scale contamination prediction maps with an area of 10,976 km², that can be used to assess the extent of environmental exposure in non-target areas. We specifically addressed the potential contamination of nature conservation areas in the Upper Rhine Valley.

Results & discussion

CUP residues detected

CUP detections in topsoil, vegetation and surface water. In total, 63 of 93 analysed CUPs consisting of 29 fungicides (F), 19 herbicides (H) and 15 insecticides (I) were detected in topsoil, vegetation and surface water samples from the 78 non-target, off-field sites along the six line transects (Fig. 1A). More specifically, 50 CUPs (24 F, 16 H and 10 I) were detected in topsoil and 43 CUPs (23 F, 8 H and 12 I) were detected in vegetation. In surface water samples taken from rivers, small streams, ponds or puddles, depending on the availability at the non-target sample sites, 44 CUPs (21 F, 14 H and 9 I) were detected (Supplementary Table 2). Our total number of 63 CUP detections doubles the recent findings of 27 different CUPs (10 insecticides, 11 fungicides, and 6 herbicides) in the Vinschgau valley in South Tyrol (Italy), which is the largest apple growing area in Europe[16]. One explanation for the higher number of CUPs in the Upper Rhine Valley could be the high crop variability consisting of cereals, vegetables, grape, fruit and other crops (Fig. 1B), requiring different pesticide applications sequences during the season. In addition, the high crop diversity (Supplementary Table 1), as well as the presence of viticulture, could explain the higher number of herbicides and fungicides compared to the Vinschgau Valley. With the Vinschgau Valley's focus on apple growing, pesticide use is both frequent and intensive: on average 38 pesticide applications were conducted from March to September in 2017, with up to 9 different pesticides in one application[26]. The very specific application sequence in apple growing could explain the smaller number of CUPs found in total and different ratios of fungicides, herbicides and insecticides compared to the Rhine Valley. Herbicides have a high sale volume of 48,269 tons (formulation) in Germany, followed by fungicides with 30,072 tonnes (formulation) in 2022[27]. Geographically, Europe is the dominant market for fungicide use in the production of fruits, vegetables, wheat and grape[28], which could explain our frequent detection of fungicides in the Upper Rhine Valley. The most frequently detected CUPs in topsoil in our study were the fungicides fluopyram (94% of all samples), boscalid (42%), spiroxamine (37%), and pyraclostrobin (22%). In vegetation, the most commonly recorded CUPs were the fungicides fluopyram (92%), spiroxamine (55%), cyflufenamid (41%), and boscalid (38%). In surface water, the predominant detections included the fungicide fluopyram (77%), the insecticide pirimicarb (67%), the herbicide metazachlor (63%), and the insecticide tebufenozide (63%). Each of these frequently detected CUPs is categorized as "High alert" or "Moderate alert" by the Pesticides Properties Database[29] in at least one of the three categories "Environmental fate", "Ecotoxicity" and "Human health" (Supplementary Table 3). Of all 63 detected CUPs, 35% are categorized as "High alert" in "Environmental fate", 43% as "High alert" in "Ecotoxicity" and 40% as "High alert" in "Human health".

The majority, 74% of all 1195 detected individual CUP concentrations, were <5 ng g^{-1} (37 CUPs in topsoil, 21 CUPs in vegetation and 42 CUPs in surface water were only detected with concentrations <5 ng g^{-1}). Nonetheless, even individual CUP concentrations can reach comparatively high values in non-target areas as they were detected for fluopyram and boscalid in topsoil (44.25 ng g^{-1} and 51.86 ng g^{-1} both at T1_7) and vegetation (877.61 ng g^{-1} at T2_6 and 380.57 ng g^{-1} at T5_6), due to the distance of <10 m to the nearest agricultural field (Supplementary Table 4) where pesticide exposure is generally higher[30].

Some of the frequent detections could be related to the sales volume in 2022 and their persistence in the environment: metazachlor (H) had a relatively large volume of 517 metric tons of active ingredient (t a.i.), while spiroxamine (F) had 287 t a.i., pyraclostrobin (F) 151 t a.i. fluopyram (F) 113 t a.i. and boscalid (F) 86 t a.i[27]. Moreover, fluopyram and boscalid are highly persistent substances with a typical DT50 (half-life) in soil of 309 days and 484 days, respectively, resulting in a risk to accumulate[29]. Fluopyram and cyflufenamid are categorized as Per- and Polyfluoroalkyl Substances (PFAS), also known as "forever chemicals", and are part of the 37 authorised PFAS pesticides in the European Union[31]. Persistent and widely occurring substances must be considered critically, especially if there is evidence of effects on organisms. Fluopyram, which was detected nearly everywhere (73 of 78 topsoil samples, 72 of 78 vegetation samples 23 of 30 surface water samples), is used as a fungicide but also registered as a nematicide in tropical crops and shows strong effects on target- and non-target nematode communities in topsoils[32]. Fluopyram also poses ecological risks by inducing behavioural alterations in fish and increasing muscle lipid content, with potential involvement of neurotoxic mechanisms[33]. Additionally, growth anomalies in grapevine, caused by a fluopyram metabolite were reported[34]. Fluopyram was detected in 69% of French soils[10] but only on one site in the apple-growing Vinschgau Valley[16], possibly because it is not recommended for integrated apple production there[35]. In addition, Fluopyram and other PFAS degrade into trifluoroacetate (TFA), a highly persistent and mobile contaminant that threatens water resources[36]. With current chemical assessments failing to address its long-term accumulation, mixture toxicity, and persistent environmental risks[37] we underline the risk of widespread fluopyram presence in the Upper Rhine Valley with its groundwater reservoir.

In the case of boscalid, we detected a contamination of 42% of all sampling sites in topsoils (max. 51.86 ng g^{-1}), 38% (max. 380.75 ng g^{-1}) in vegetation and no contamination in surface water. French soils showed a contamination of 85% of their sampling locations with levels up to 1,211 ng g^{-1} in a cereal field[12], whereas in the Vinschgau Valley boscalid was detected in 11% of the soil samples[16]. The fungicide boscalid can decrease the reproductive quality of honey bee queens and is one of the most common pesticides detected in beehives[38]. In addition, boscalid can induce a mitochondrial dysfunction in human cells, suggesting that it is not selective for fungi targets[39]. The fungicide spiroxamine, detected in more than every second vegetation sample and every third topsoil sample, can increase the mortality of the beneficial parasitical insects *Anagyrus vladimiri* and *Trichogramma evanescens* in viticulture[40], indicating the possible impact of CUP contamination on non-target organisms and the reduction of biological pest control potential. Our results show that there can be regional differences (Rhine Valley vs. Vinschgau Valley) but also similarities in terms of pesticide contamination (Rhine Valley vs. France), which results in a need of individual environmental pesticide exposure evaluation. Although the EU National Action Plans ask for the implementation of integrated pest management, which should be adapted to local conditions and crop types, this strategy has not been effective since pesticide use has not decreased in the EU so far[41,42].

CUP residue mixtures in topsoil, vegetation and surface water. CUPs were detected in 97% of all vegetation and 97% of all topsoil samples (76 of 78 samples each). Surface water was available for sampling at 30 of the 78 sample sites, and CUPs were detected in 83% of the samples (25 of 30). Of the total 63 detected CUPs, 27 CUPs (43%) were recorded in all three matrices (vegetation, topsoil and water), 20 CUPs (32%) were detected in at least two of the three matrices and 16 CUPs (25%) were detected in only one of the three matrices (Supplementary Table 5). The detections in almost all combinations of the three matrices and the spatially distributed sample sites with potentially specific habitat conditions, ranging from intensive agriculture in the valley to remote locations in the mountains, show a high probability of pesticide exposure at both patch (site) and landscape (transect) scales (Fig. 2). In total, 140 unique mixtures with ≥2 components were recorded. Of the soil, vegetation and surface water samples, 76%, 87% and 80% contained mixtures, respectively. Samples from non-target, off-field habitats in the agricultural valley showed 90 unique mixtures, whereas 54 unique mixtures were recorded in forested mountain regions. The most often detected mixture contained the fungicides fluopyram + spiroxamine, but this specific mixture occurred only 6 times, indicating the high variability of mixture composition. All other mixtures occurred once (95.7%), twice (2.9%) or threefold (0.7%), demonstrating that there was site-specific contamination. We detected a maximum of 26 CUPs in topsoil, 21 in vegetation and 39 in surface water samples in the agricultural valley (T1_3, T3_4 and T1_3, respectively). Fungicide-intensive viticulture on slopes bordering mixed crops with vegetables, fruit orchards and cereals is typical of this area in Southern Palatinate. Sites without CUP residues in vegetation, soil or surface water were measured only in remote mountain regions (T1, T2, T6 mountain sites 8–13, Fig. 2). However, there was no site where all matrices were uncontaminated. This is in line with a study from the Italian alps, where none of the non-target sites were without CUP detections in soil and vegetation[16]. This CUP exposure could initially have a direct effect on non-target organisms, in case of herbicides directly affecting non-target plants outside of cultivated areas. In addition, herbicides can affect not only plants but various other non-target organisms including humans[43]. Cases of direct acute bee mortality and sub-lethal impairment of their normal biological behaviour have been documented for herbicides, and fungicides and synergistic effects when in mixture with other agricultural chemicals[44]. Traces of insecticides threaten numerous non-target organisms by affecting traits of the individual, including development, physiology, behaviour and communication[45]. In a recent study involving over 1000 pesticides, exposure to sublethal doses (reflecting typical field application and low environmental concentrations) in *Drosophila melanogaster* revealed that 57% of the studied pesticides significantly altered larval behaviour after short-term exposure[46]. Key traits, such as egg-laying rates, reducing reproduction and in the end leading to population decline, were reduced by some pesticides even at concentrations several orders of magnitude below sublethal levels[46]. In addition, nonlinear interactions between many combinations of highly prevalent pesticides suggested widespread synergistic interactions[46]. It can therefore be expected that sublethal effects can also occur at low environmental concentrations, similar to those measured in this study. Our detected high variability of mixtures indicates the difficulty of generalizing CUP exposure and resulting potential effects. The risk of environmentally occurring CUP mixtures is not assessed for the regulation of pesticides in the EU, where only single substances or a few pesticides that are prepared in a single formulation are addressed. CUPs are only tested in the laboratory and in field experiments with a small number of model organisms (Commission Regulation (EU) No 284/2013, 2013) and unexpected consequences due to interactions of different substances (e.g. additive or synergistic effects) or sublethal effects and indirect effects on other organisms are not investigated[47–49]. A realistic assessment of CUP mixture exposure and its associated risks to biodiversity would be extremely challenging, given a large number of different mixtures recorded. The mean number of CUP detections differed between area and

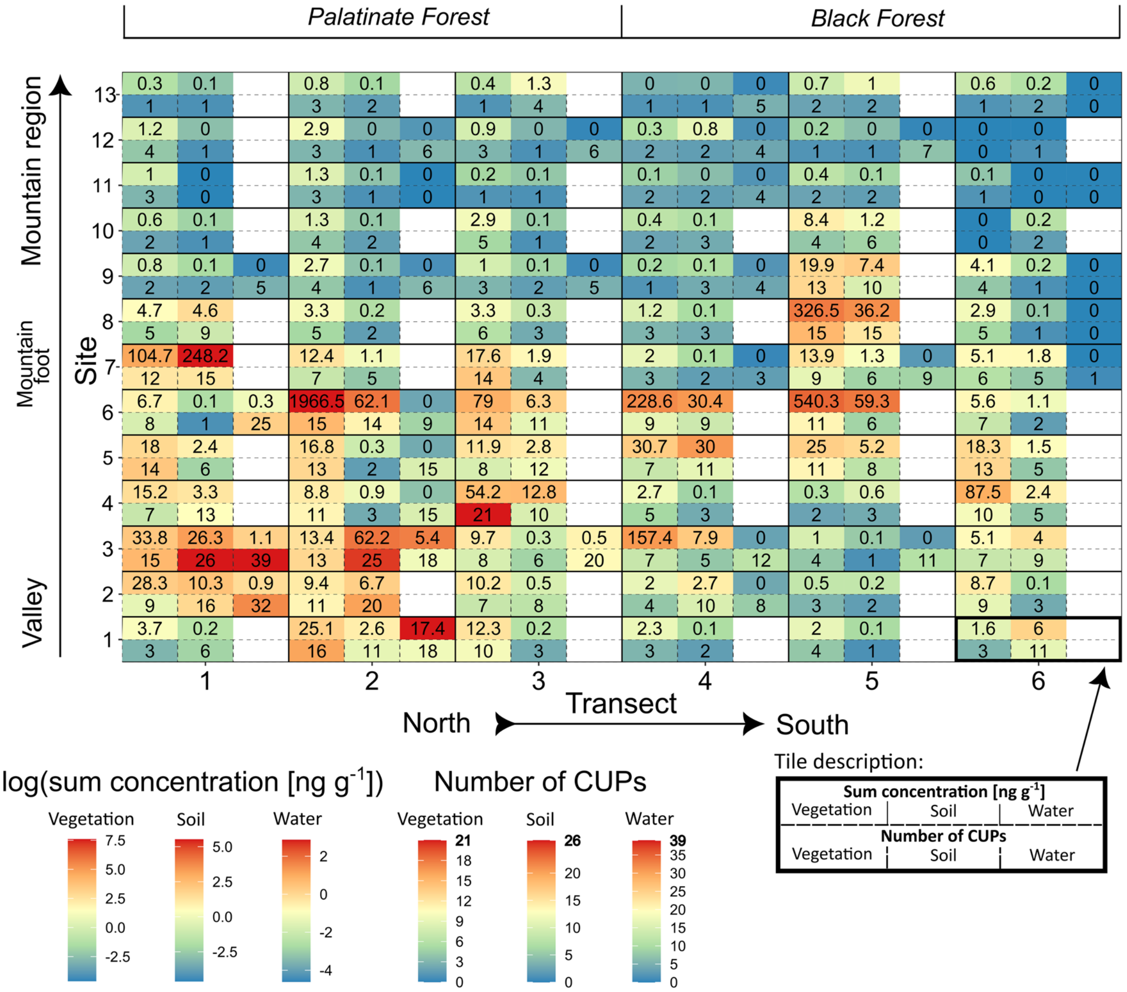

Fig. 2 | Detected Current Use Pesticides sum concentrations and number of residues. Explicit results of the 78 sampling sites for the number and sum concentration of CUPs. Each of the six line transects consisted of 13 sample sites. In each tile, vegetation (bottom left), topsoil (bottom middle) and water (bottom right) and their corresponding sum concentration in ng g^{-1} in the cells above is mentioned (see tile description). Regions defined as valley and mountain region, Palatinate Forest and Black Forest and North and South. Site 1 was always closest to the Rhine in the valley, site 7 at the mountain foot and site 13 most remote in the mountain region. For details on the location of the transects see Fig. 1.

matrices: On average, 6.3 CUPs were found in vegetation (9.2 in the valley and 3.2 in mountain regions), 5.4 CUPs were found in topsoil (8.2 in the valley and 2.3 in mountain regions) and 9.6 CUPs were found in surface water (16.7 in the valley and 3.3 in mountain regions). An analysis of the European soil database, focusing on 76 target CUPs, with an analytical sensitivity of >10 ng g^{-1} (LOQ) for LC-MS/MS compounds and >5 ng g^{-1} (LOQ) for GC-HRMS compounds, showed a mean of 2.7 (median = 3) pesticides in European agricultural soils and 2.4 (median = 1) in Germany[8]. Their mean number of detected CUPs in non-target area topsoils is up to two times lower, compared to our measurements with 93 target CUPs. We included measurements in the number of CUP detections when they reached individual LOD levels and included measurements in site sum concentrations when individual LOQ levels, up to 1000 times lower (topsoil LOQ: 19.11–0.008 ng g^{-1}, vegetation LOQ: 21.5–0.01 ng g^{-1}, water LOQ: 0.01–0.0006 ng g^{-1}, Supplementary Table 6) compared to the previously mentioned study, were reached. Differences in extraction, analytical methods as well as detection limits and number of target CUPs make comparisons difficult, however the analytical sensitivity of our study showed a higher CUP number in non-target areas compared to the target in-field samples of the EU study. Additionally, the EU study was an in-field measurement whereas we sampled in the non-target off-field area. Since 281 different CUPs are currently authorised in Germany[27] and 440 in Europe[50], the number of CUPs and sum concentration is even higher than our results show as only 93 CUPS were included in our analysis. In 2021, a study with the same analytical method was conducted close to the transects T1, T2, T3 but also with samples from cropping areas[22]. The results show that the number of CUPs decreases with distance to the target site but crop type and season play a critical role in contamination prediction[11,22]. It can therefore be concluded that there is an even higher number and sum concentration of CUPs present in fields, vineyards and orchards close to our non-target areas.

We demonstrate a widespread contamination with CUP residues across different environmental matrices and a high variability in mixture compositions. The presence of unique and site-specific mixtures underscores the complexity of CUP exposure in non-target areas, raising concerns about unassessed ecological impacts from diverse, co-occurring residues.

CUP sum concentration in topsoil. Summing up the concentrations of recorded individual CUPs, we detected a mean CUP sum concentration of 8.48 ng g^{-1} in all topsoils, ranging from no detections to a maximum value of 248.23 ng g^{-1} (T1_7, <10 m to vineyards). This is in the same CUP sum concentration range as recently measured in topsoils of the Vinschgau Valley with a mean of 4.15 ng g^{-1}[16]. When split into valley and mountain regions, the valley topsoil showed a 26-fold higher mean concentration (15.97 ng g^{-1}) in comparison to mountain region topsoil

(0.60 ng g^{-1}). A recent study in France revealed 0.1 to 76 ng g^{-1} with a mean value of 30.4 ng g^{-1} in French grassland topsoils and <2 ng g^{-1} in forest topsoils (only >LOQ, 110 target substances)[10] which is comparable to our detections in valley and mountain regions, respectively. A comparison of sum concentrations is difficult due to the lack of comparative studies and the dependence of the method and detection limits (see above). A 15-fold higher mean was detected in French grassland compared to French forest topsoil[10] which is also comparable to our valley (26 times higher) versus mountain region detections. The top ten topsoil CUP sum concentrations (248.23 ng g^{-1} to 10.34 ng g^{-1}) were measured in the valley (Supplementary Table 4).

CUP sum concentration in vegetation. In vegetation, a mean value of 51.21 ng g^{-1} was detected (max: 1966.51 ng g^{-1} at T2_6, <10 m to vineyards), which is slightly lower than the recorded mean CUP sum concentration of 64.5 ng g^{-1} in playground grass and 104.48 ng g^{-1} in vegetation in valleys in South Tyrol[16,51] The study area in these studies is dominated by apple orchards in the valley whereas in our case vineyards are concentrated at the mountain foot and fruit cultivation is more diffuse in the Upper Rhine Valley. One exception is the fruit cultivation area at the mountain foot close to T4_6 and T5_6, possibly explaining the higher sum concentration detected there (Fig. 2). Another explanation for our slightly lower mean sum concentration in vegetation could be the large number of sites with greater distance to the next agricultural activity (mean distance of 650 m). The vegetation of the valley in the whole study area showed a 40-fold higher mean CUP sum concentration (97.55 ng g^{-1}) in comparison to mountain region vegetation (2.43 ng g^{-1}). The top ten vegetation sum concentrations (1,966.51 ng g^{-1} to 33.85 ng g^{-1}) were measured in the valley (Supplementary Table 4). The highest concentration of 1,966.51 ng g^{-1} was mainly driven by the fungicides fluopyram and tebuconazole with 877.61 ng g^{-1} and 777.46 ng g^{-1} respectively, detected together with 13 other CUPs at this sample site (T2_6 distance of <10 m to the nearest vineyard), making contamination by drift from viticulture likely. Three-dimensional application in viticulture results in greater pesticide drift compared to arable applications and greater exposure of near non-target areas[22,30].

CUP sum concentration in surface water. In surface water, a mean CUP sum concentration of 0.86 ng ml^{-1} (max: 17.41 ng ml $^{-1}$ at T 2_1, puddle <10 m to the next field) was detected and the top ten maximum water sum concentrations (17.41 ng ml $^{-1}$ to 0.01 ng ml^{-1}) were all measured in the valley (T1-4). Of the 30 surface water samples (Supplementary Table 7), six were taken from puddles with the highest measured sum concentrations (top six) of 17.41 ng ml^{-1} to 0.30 ng ml^{-1} and 39 to 18 different CUP detections. It can be assumed that the CUPs are concentrated by desiccation, which turns puddles in agricultural areas into toxic soups. Puddles in human modified landscapes are a an important drinking water source for birds[52] and honey bees actively forage in "dirty" water sources for minerals that may be lacking in their floral diet[53]. We emphasize the critical exposure pathway for birds and mammals as well as other vertebrates and invertebrates through water uptake, especially when contaminated puddles are the only source of water in hot seasons or during increasingly frequent droughts. Realistic puddle exposure needs to be addressed as a critical source for affecting biodiversity, and in-field puddle exposure is expected to be even higher. The 19 sampled small streams showed sum concentrations up to 0.03 ng ml^{-1} (Supplementary Table 7) whereby small streams in the valley as well as in the mountain regions were contaminated (15 of total 19 small streams). A study of 124 monitoring stream sections across Germany found that pesticides in agricultural streams were the dominant driver in reducing vulnerable insect populations in aquatic invertebrate communities and event-driven sampling was used to additionally record surface rainfall-induced short-term peak concentrations[54]. In addition to effects in the aquatic environment, terrestrial food webs in the riparian zone may be impacted via reduced insect emergence[55]. The neonicotinoids acetamiprid and thiacloprid, detected in two small streams (T4_3 and T5_7), were the most frequently detected pesticides in 90–100% of emerging insect samples in a study in the same region and were shown to biomagnify in riparian spiders[25]. We just begin to understand the complexity of the aquatic-terrestrial meta-ecosystem[55] and CUP contamination studies which integrate both, aquatic and terrestrial matrices, could support biodiversity conservation efforts by indicating relevant effect pathways. Water sources are quite diverse, and substance transport can vary significantly between small streams and other sources, such as ponds or puddles.

CUP residue gradient and wind effects. Across all matrices, a higher number of CUPs and sum concentration was detected in the valley (mean number of CUPS = 9.6 CUPS, mean sum concentration = 46.7 ng g^{-1}) compared to the mountain region (mean number of CUPS = 2.9 CUPS, mean sum concentration = 1.4 ng g^{-1}. Matrix specific, there was a significant higher number of CUPs in valley topsoil ($z_{76} = 10.51$, $p < 0.001$), vegetation ($z_{76} = 10.08$, $p < 0.001$) and surface water ($z_{28} = 10.64$, $p < 0.001$) as well as a higher sum concentration in valley topsoil ($t_{76} = 6.18$, p < 0.001), vegetation ($t_{76} = 7.03$, p < 0.001) and surface water ($t_{28} = 3.95$, p < 0.001, Supplementary Table 8). The number of CUPs in vegetation and topsoil decreased from the valley to the mountain regions, only increasing by approximately two more CUPs at the mountain foot (Fig. 3A). Despite the decreasing CUP numbers a tenfold increase in the CUP sum concentration in vegetation and topsoil could be observed at the mountain foot compared to the valley (Fig. 3B). This suggests that a detailed representation of landscape contamination can only be obtained with both values. Our results indicate, that CUPs can travel multiple hundred metres from the agricultural valley into forested mountain regions and that the contamination is related to the distance to the mountain foot in both directions. As the mountain foot of the Upper Rhine Valley is widely covered with vineyards and interspersed with fruit cultivation, the observed increase in CUP numbers and especially sum concentrations could be related to the 3-dimensional application required in both crop types and resulting drift. In addition, vineyard site conditions like a high air temperature and wind velocity, enhance pesticide volatilization from target plants and soils[56] which could favour atmospheric transfer.

To test the effect of the main wind direction on the number of CUPs in mountain regions, a Welch Two Sample t-test was applied for all CUP detection numbers in soil and vegetation in mountain areas ($n = 38$). With a main wind direction from southwest to northeast, the mountain areas in T1, T2, T3 would be contaminated upwind and T4, T5 and T6 would be affected downwind, if a connection between wind and CUP transport is assumed. No significant effect could be found for the number of CUPs in vegetation ($t_{32.63} = 0.47$, p-value = 0.640) and the sum concentration in vegetation ($t_{33.86} = -0.411$, p-value = 0.683). However, there was an average trend of 1.51 more CUPs ($t_{30.20} = -1.79$, p-value = 0.084) and 0.699 ng g^{-1} higher sum concentration ($t_{22.14} = -1.72$, p-value = 0.099) in topsoil of the Black Forest mountain regions (T4, T5, T6) which would be contaminated with the main wind direction. Wind can have a direct influence on the direction, transport distance and concentration of pesticides as well as on the transported droplets deposition place[57]. The fact that pesticides are found in ambient air underlines this possible distribution path[4,58,59]. The number of pesticides in air was shown to increase with higher air temperature and decrease with less precipitation[4]. Pesticides have been found in rainwater[60], demonstrating just another aspect in complex meteorological systems accountable for non-target site contamination. Semi-volatile pesticides can get adsorbed on atmospheric aerosol particles which leads to high persistence and long-range transport (>1000 km) in the atmosphere[61]. Nonetheless, it has been shown that long-range CUPs come from different chemical classes with very different physicochemical properties, resulting in the assumption that the assessment of these properties cannot fully explain their distribution[62]. The occurrence of CUPs in remote mountain regions of the Upper Rhine Valley through medium-range transport (1–1000 km)

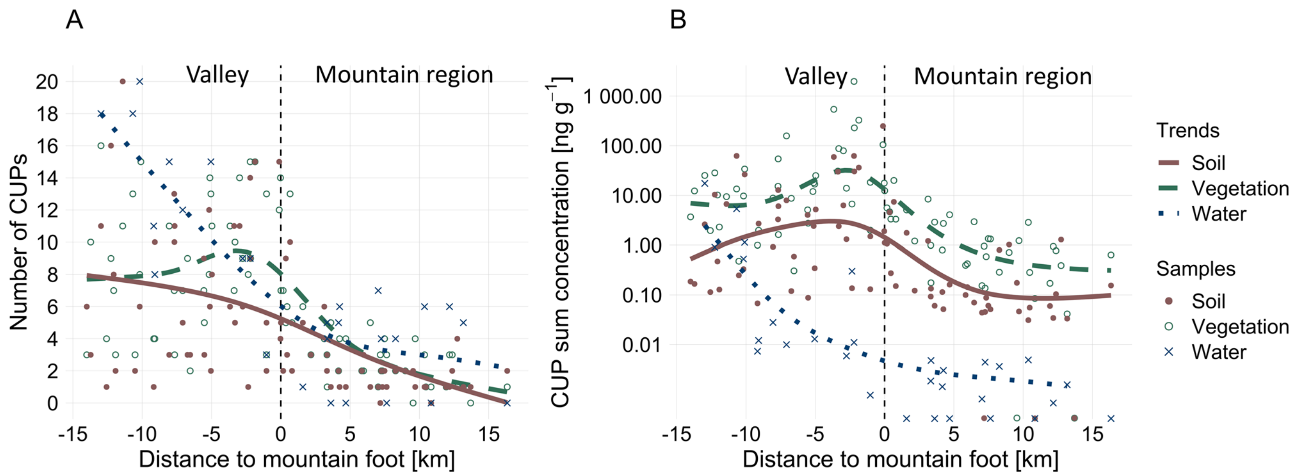

Fig. 3 | Gradient of detected Current Use Pesticides residues from valley to mountain regions. A Number of detected CUPs in vegetation (circles), topsoil (dot) and surface water (cross). **B** CUP sum concentrations in ng g^{-1} in vegetation, topsoil and surface water. Loess smooth trendlines indicate the gradient from the valley (-15 km to 0 km) to the mountain regions (0 km to 15 km). 0 corresponds to the foot of the mountain that defines the Rhine Valley by sudden altitude and land use changes.

questions current pesticide regulation. Medium-range transport, which results in landscape scale CUP contamination, is hardly addressed in current regulation where the main concern lies on short-range transport (drift) and long-range transport, for example to Arctic regions[5].

There is also some agricultural activity in the remote mountain regions which could contribute to CUP occurrence (Supplementary Table 1). The maximum distance from a transect site to agricultural cropping areas (including cereals, fruits, vegetables, grape, other crops) was 4.4 km (T1_10). This site showed contamination of fluopyram and spiroxamine in vegetation and fluopyram in soil. The contamination in the mountain regions with 2–3 pesticides (calculated mean over all matrices = 2.9) can be considered as a ubiquitously present background contamination. As mentioned before, this number will be even higher in reality, as not all approved CUPs were included in our analytical procedure. In summary, pesticides in the Rhine Valley occur not only in the immediate agricultural landscape in the valley itself, but also with a decreasing gradient in the surrounding mountain regions, which are therefore not exempt from CUP contamination, although their sensitive ecosystems could be less adapted to agricultural influence.

Prediction. With the categorized and spatially weighed land use data, the number of CUPs and sum concentrations of off-field topsoil and vegetation during the spraying season was predicted for the area of the Upper Rhine Valley with a resolution of 1 × 1 km (see Supplementary Information for further details). The generalized linear model with a Poisson distribution for the number of CUPs in topsoil and vegetation had multiple R^2 values of 69% and 76%, respectively. The linear model with normal distribution for the sum concentration of CUPs in topsoil and vegetation achieved good explanatory power with multiple R^2 of 62% and 71%, respectively. The model components reveal fruit, grape and vegetables as driving factors for a higher number of CUPs in vegetation and a sum concentration of CUPs in topsoil and vegetation (Supplementary Table 9, 11, 12). A higher number of CUPs in topsoil was driven by vegetables and other (not further categorized) crops (see methods and Supplementary Table 10). Overall, land use types result in the exposure of the surrounding land much further away than expected using drift extrapolations[30], often for multiple hundred metres (See methods section for further details, Supplementary Fig. 1–4). A mean exposure of 4.6 CUPs was predicted for topsoil and 4.3 for vegetation with a maximum of 27 for topsoil and 32 for vegetation (Supplementary Table 14). Contamination in the non-target, off-field areas differ depending on location and neighbouring crops, which can be seen in the varying patterns of the generated maps (Fig. 4).

According to the resulting prediction map, non-target areas located in the agricultural vicinity, are exposed to multiple CUPs in complex mixtures and high sum concentrations, especially in the valley. In the valley and mountain regions of the study area, 37.3% and 9.0% of the land is used for agriculture, respectively (cropping areas without grassland, Supplementary Table 1). A hotspot for the number of CUPs in vegetation was predicted for the slopes at the mountain foot in the southern Palatinate (T1, T2, T3) or the Kaiserstuhl (north of T6), both of which are areas with intense viticulture (Fig. 4, land use). In Europe, the majority of pesticide products for specialty crops are used in viticulture[63]. Approximately 7.2% of the land in the valley (without the mountain region) is used for viticulture but 18.4% for cereals, 6.4% for vegetables, and 2.7% for fruit production[64] (Supplementary Table 1). A slightly different pattern is predicted for topsoil with maxima in a number of CUP detections for the slopes in southern Palatinate (T1-3), too, but also at the valley floor, in the north-western part of the study area (T1). In this region of the valley, vegetables are grown in a large area which contributes to a high predicted number of CUPs. Both topsoil and vegetation show differentiated hotspots within a fruit-growing area close to Achern also called the “Baden fruit paradise”[65] and the wine-growing area Kaiserstuhl. In terms of the amount of pesticide product usage ranks, vegetables and fruit trees follow after viticulture[63]. Predicted sum concentrations reflect an increase in the contamination of vegetation on the wine-growing slopes as shown by the measurement results. This also applies partly to the predicted sum concentration in topsoil, although individual hotspots are more narrowly defined here. The contamination of vegetation appears to be influenced by land use over a greater distance than the contamination of topsoil, as can be seen from the greater distance of explaining land use in the prediction model (Supplementary Table 13).

However, CUP contamination is not limited to agricultural hotspots but is predicted area-wide to occur in the Upper Rhine Valley: more than one CUP is predicted in 98% of topsoil and vegetation samples and contamination-free sites are only expected in remote mountain regions, larger forest blocks or in centres of larger cities that are not closely surrounded by agriculture. We emphasize that this prediction map only shows CUP estimations for non-target areas during the spraying season. A complete contamination map including the target-areas, the cropping areas, is expected to show higher values of CUP numbers and sum concentrations over large areas.

Nature conservation areas. For nature conservation areas (NCA, not including national parks or biosphere reserves) inside the study area, contamination was predicted with a resolution of 200 x 200 m. NCAs are among the most strictly protected areas in Germany[66]. A mean of 4–5

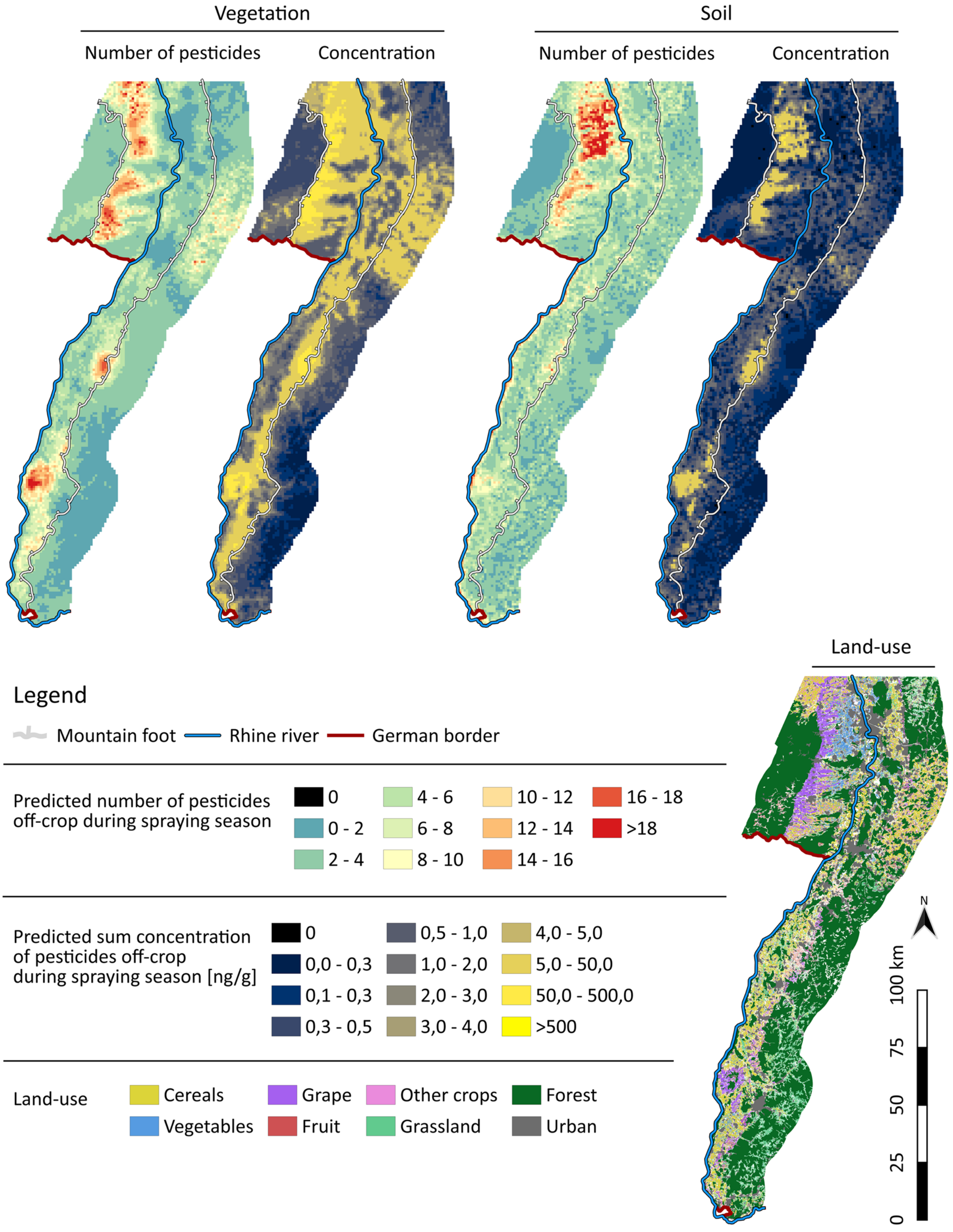

Fig. 4 | Predicted Current Use Pesticides sum concentrations and number of residues in off-field vegetation and topsoil during spraying season. The number of CUPs and sum concentration ng g^{-1} in off-field vegetation and topsoil during spraying season were predicted with land use categories in a linear model (CUP sum concentration) or generalised linear model (CUP number). Multiple R^2 is between 0.62 and 0.76. The raster pixel size is 1 × 1 km. Forest/urban land cover data are from the European Environment Agency (EEA) (2016); the remaining land use categories are from Blickensdörfer et al. (2021). The prediction map is based on the topsoil and vegetation measurements and their 78 sites only.

CUPs was predicted in their vegetation and 5–6 CUPs in topsoil (Supplementary Table 15), which shows that NCAs are not exempt from CUP exposure and are not necessarily refuge areas for protected plants and animal species. Species richness of butterflies decreased in NCAs and other protected areas in Germany[67] as well as insect biomass by more than 75% over 27 years[17]. Insects in NCAs bordering agricultural areas have been shown to be directly exposed to pesticides[14]. In our extrapolation some areas are predicted to be at particular risk due to their location: A maximum value of 19-20 CUPs is predicted for the vegetation of the "Badberg" on the Kaiserstuhl, which could be explained by the intensive surrounding viticulture. The Kaiserstuhl inhabits 16 different NCAs. Non-target and structurally rich margins in agricultural landscapes have been shown to be important for rare and red-listed species[68] and habitats available for non-target organisms in general and terrestrial biodiversity.

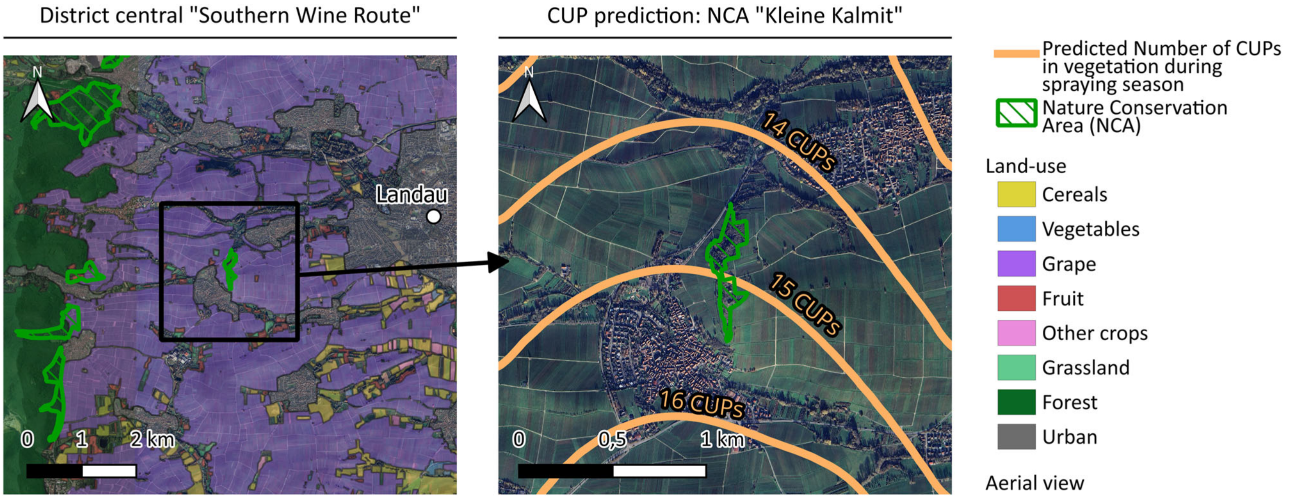

Fig. 5 | Predicted number of Current Use Pesticides residues in the non-target Nature Conservation Area "Kleine Kalmit", Southern Palatinate, Germany. The number of CUPs in off-field vegetation during the spraying season was predicted with a resolution of 200 × 200 m. Multiple R^2 of the glm generated with measurements of the number of CUPs in the overall study area = 0.76. Forest/urban land cover data are from the European Environment Agency (EEA) (2016); the remaining land use categories are from from Blickensdörfer et al. (2021). Aerial view: © 2025 Google, TerraMetrics.

Therefore, one of the main objectives of the Common Agricultural Policy (CAP) of the European Commission is maintaining and increasing landscape features such as hedges, field margins, dry-stone walls and isolated trees to foster biodiversity in agricultural landscapes[69]. The potential of such habitats remains to be critically questioned if they cannot be kept pesticide-free. Our results suggest that the compensation potential of non-target areas established or maintained explicitly for biodiversity in agricultural landscapes strongly correlates with the distance to CUP target areas – the cropping area. This is underlined by the prediction of a maximum of 19–20 CUPs in topsoil for the "Sandgrube bei Schauernheim", a relatively small NCA of 12 hectares surrounded by vast agricultural activities. A nature conservation area in an agricultural neighbourhood with a small size and associated small buffer zone can therefore simply induce high CUP pressure[14]. Another representative example is the "Kleine Kalmit" (5.7 ha), which is situated on a small hill in a large wine-growing area in southern Palatinate (T3). It was predicted to contain a mean of 11–12 different CUPs in topsoil and 14–15 in vegetation during the spraying season (Fig. 5). In another study, 4 CUPs in topsoil and 16 CUPs were detected in vegetation in July 2021 (August: 7 in topsoil and 12 vegetation) in the southern part of the NCA with the same analytical method[22]. Our site T3_6 was closest to the "Kleine Kalmit" with a distance of 1,3 km and 11 different CUPs in topsoil and 14 in vegetation could be detected there. This demonstrates the applicability of the NCA prediction, but particular uncertainties exist due to factors such as the climatic conditions in the sampling year or the soil type at the explicit sampling location.

Our direct measurements in the Black Forest National Park revealed a total of 4 different CUPs. Additionally, at the Feldberg NCA (sampled at 1277 m a.s.l.), 3 different CUPs were detected. Similarly, 3 different CUPs have been shown to reach remote alpine meadows at 2318 m altitude in the Vinschgau Valley[16]. The widespread presence of CUPs in NCAs highlights a critical concern: there is an urgent need to establish larger buffer zones around nature conservation areas[14] and stop the use of substances that can be found in remote areas, such as the fungicides fluopyram, boscalid and spiroxamine. Additionally, pesticide use is not directly prohibited in NCAs in Germany[70] and a general law is needed at the federal level.

Conclusion

Our study reveals that during the spraying season, non-target areas in the agricultural valley and adjacent mountain regions are subject to complex CUP contamination, with varying mixtures depending on the location and surrounding land use. At all study sites there was at least one matrix contaminated, aligning with other studies which find non-target areas contaminated[8,10,16]. Pesticide drift and run-off events from the terrestrial area of landscapes also affects aquatic systems such as small and medium-sized streams, and aquatic contamination again has feedbacks on the riparian terrestrial zone[71]. Natural puddles in non-target areas contained the highest contamination levels and are so far not included in environmental risk assessment procedures. The pervasive spread of CUPs suggests an urgent need to prioritize areas at particular risk of contamination: Our spatial assessments revealed that land use types like fruit orchards, vineyards or vegetable fields affect the surrounding land, often for multiple hundred metres. We underscore the challenges in comparing measurement results, as the number of target CUPs, detection limits and quantification limits still have a significant influence on the detection rate. Nevertheless, we demonstrated the utility of predictive models based on real-world samples to determine background contamination and realistic concentrations, including trace levels of CUPs. It is crucial to quickly anticipate potential environmental stressors rather than relying solely on retrospective data collection, due to the time pressure of the ongoing rapid decline in biodiversity. Our prediction maps might also provide helpful tools in biodiversity management implementation for selecting low contamination areas. CUP measurements and predictions highlight the urgent need to reduce the overall number and amount of substances to (re)establish uncontaminated areas that are crucial for nature conservation and human health. CUP mixtures can reduce the diversity of soil fauna[72] and therefore very likely impact soil fertility, which was recently considered to be directly related to human health[73]. In addition, there are higher levels of pesticide exposure among residents living in close proximity to agricultural lands than among those living at greater distances[74]. Many CUPs are still used preventive[75], but their impact extends beyond fields of application, leading to long-term consequences for biodiversity[1,76]. We show that complex CUP mixtures and their unpredictable components expose entire landscapes and the environmental risk is not assessed correctly. The complex combinations of CUP mixtures and the numerous potential interactions among their components create disregarded environmental pressures across entire landscapes. As this exposure is expected to be chronic as shown for non-target areas in the proximity of 20 m of agricultural fields[22], the current pesticide authorisation and risk management practices do not protect terrestrial biodiversity.

The results of this study highlight the need for changes in agricultural practices in the Upper Rhine Valley, but also for other Central European regions with pesticide-intensive agricultural production to reach global pesticide reduction targets[77]. More sustainable approaches like the production of baking cereals without the use of synthetic pesticides are possible and already in place for many years[78] and would affect a large proportion of the agricultural landscape in the EU as one-third of the total utilisable agricultural area is used for cereal production[79]. Together with the aim of transforming 25% of the agricultural land to organic production by 2030, as formulated originally in the EU Green Deal[80] and integrated in national and international policies[77,81], synthetic pesticide use could be substantially lowered leading to a reduction of pesticide exposure of biodiversity and humans. Our prediction mapping approach could be repeated with measurements in 2030 to evaluate these reduction efforts.

Methods

Study region

The Upper Rhine Valley ranges from Basel in Switzerland to Bingen in Germany (270 km), embraced by the mountain plateaus of the Vosges Mountains and Black Forest in the south and the Palatinate Forest and Odenwald in the north. The Upper Rhine Valley has a mean air temperature of 8.6 °C, mean annual precipitation of 73.5 cm and 15 large cities with more than 100,000 people[82]. Agriculture is a dominant land use in the valley with vegetables and arable crops in the valley lowland and viticulture being mainly present on the mountain foot. The mountain plateaus are separated from the valley by a sudden rise in altitude – slopes (study sites covered 99 to 1378 m) - and are dominated by forest cover. Data were collected close to Ludwigshafen (north, T1) to Staufen im Breisgau (south, T6). The prediction was limited to the area from Worms to Basel (10,976 km^2 prediction area) within Germany, which includes part of the Valley, the Black Forest, and the Palatinate Forest. The study area contained parts of the UNESCO Palatinate Forest-North Vosges Biosphere Reserve and Black Forest Biosphere Reserve as part of the Natura 2000 Network, Nature Conservation Areas and the Black Forest National Park.

Sampling and pesticide analysis

Six line transects with west/east orientation and 30 km length were selected to cover the valley as well as the mountain regions (Fig. 1). Thereby, three transects were set in the northern part at the section of the Palatinate Forest (T1, T2, T3), two transects at the section of the Black Forest National Park (T4, T5) and one transect at the section of the Feldberg (1277 m a.s.l.), the highest mountain in the Black Forest (T6). With a main wind direction from south-west to north-east, mountain areas in T1, T2, T3 would get contaminated against and T4, T5 and T6 with the main wind direction if a connection between wind and long-distance transport is assumed. For each 30 km long line transect, six sampling sites were placed in the valley (TX_1 – TX_6), one close to the mountain foot (TX_7) and six in the mountain area (TX_8–TX_13), resulting in 13 sampling sites per transect. The site selection process yielded an average transect length of 31.5 km and an average distance of 2.6 km between sampling sites along the transects. The sites in the mountain area were selected to capture the sequence of the geographic altitude (sequence of mountain and small valley). For CUP area comparison, the 13 sites of each of the six transects were split into valley and mountain region depending on their position regarding the sudden increase in altitude at the mountain foot (sites close to the mountain foot were categorized, too), resulting in a total of 78 sampling sites. Between June 15th and July 11th 2022, composite topsoil samples consisting of 25 subsamples were taken from a grassland area of 4 x 4 m per sample point with a topsoil corer (diameter: 13 mm, 5 cm deep, Rasengrün, Ingelheim, Germany) in a depth of 0–5 cm. Similarly, vegetation representative of the sample site was manually handpicked in the same area and placed in a 1 L plastic bag. The vegetation samples then included a mixture of different parts (excluding roots) of grass, herbs and leaves of shrubs depending on availability. If a surface water source (puddle, stream or river) was present in the immediate proximity of the sample point, a 250 ml water sample was taken. Transportation to the laboratory and storage in a freezer at −20 °C took place after sampling as soon as possible. Vegetation, topsoil and surface water samples were then further processed in the laboratory.

Vegetation and topsoil samples were analysed[9] for 93 CUPs using a high-performance liquid chromatography coupled with an electrospray ionization tandem mass spectrometry system (HPLC-ESI-MS/MS; HPLC: Agilent Technologies LC 1260 Infinity II series, MS/MS: Agilent Technologies 6495C, Santa Clara CA, USA). Surface water samples were analysed for 75 CUPs with a direct-injection HPLC-ESI-MS/MS method[25]. Details on the extraction procedures and analytical HPLC-MS/MS method and instrument parameters can be found in the Supplementary Information.

CUP sum concentrations were calculated for each sampling site as the sum of all single CUP detections above the limit of quantification (>LOQ) in vegetation, topsoil and water separately. Limits of quantification and detection can be seen in Supplementary Table 6.

Land use and topography data

National-scale crop type maps for Germany from combined time series of Sentinel-1, Sentinel-2 and Landsat 8 data (2017, 2018 and 2019) were used as agricultural land use data[64]. Google Satellite Imagery WMS data was used for satellite images[83]. The land use types were grouped into categories “Grassland”, “Cereals”, “Vegetables”, “Grape” and “Fruit”. Remaining land use types were grouped into “Other crops”, also containing areas specified as “Other agricultural areas”. Forest and urban cover was assessed with the Corine Land Cover (CLC) dataset[84] resulting in a total of 8 different land use categories. Further topographic indices (Altitude, Roughness Index, Topographic Wetness Index, Topographic Position Index, Wind Shelter Index and Wind Exposition Index) and environmental factors (Mean land surface temperature for the years 2018–2020) were evaluated for their correlation with land use data by using them in a separate principal component analysis. Due to a strong correlation with agricultural activities, they were excluded for predictions (Supplementary Information for details).

Optimization and spatial weighing of land use categories

We found CUPs far from the agricultural valley, contrary to the expectation of particularly remote and clean mountain regions. We, therefore, asked how far away the agricultural areas are responsible for the occurrence of CUPs in remote regions. To do so, we grouped the land use into 8 different categories (“Grassland”, “Cereals”, “Vegetables”, “Grape”, “Fruit”, “Other crops”, “Forest” and “Urban”), assuming that similar land use types would lead to similar CUP applications. We dissected the categorized land use area into a raster of 10 x 10 m.

We then computed how much of the land with a certain distance σ to a test-site is from each given land-type use. We assumed that areas closer to the sampling site would contribute more to the CUPs detected at the sampling site and therefore we used a (two-dimensional) Gaussian kernel based on distance to the sampling site as weights for the raster points. Specifically, we computed the mean land coverage of type L around a given sampling site i as

$$W_{iL}(\sigma) = \frac{A}{2\pi\sigma^2}\sum_{l\in L}\exp\left(-\frac{\left(x_i - x_l\right)^2}{2\sigma^2}\right)$$

where A is the area of a pixel, in our case $10m \times 10m = 100m^2$, σ is the kernel width indicating how much of the surrounding land contributes to the land coverage, x_i are the coordinates of the sampling location and x_l are the coordinates of the raster point. The sum is taken over all areas l with land use type L. The factor in front of the Gaussian kernel ensures that $W_{iL} = 1$ if all the surrounding land would be of type L. The area within 1.96σ of the sampling site contributes to 95% of the weighted area. We computed this mean land type coverage for all 78 sampling sites i, each of the 8 land use types L and for σ varying from 50 to 5000 in steps of 50.

Given these mean land coverages for the sampling sites and land use types we searched for the optimal kernel width σ_L for each land type which would lead to the “best” prediction. Specifically, we fit a generalized linear model (glm) for the CUP richness and a linear model (lm) for the sum

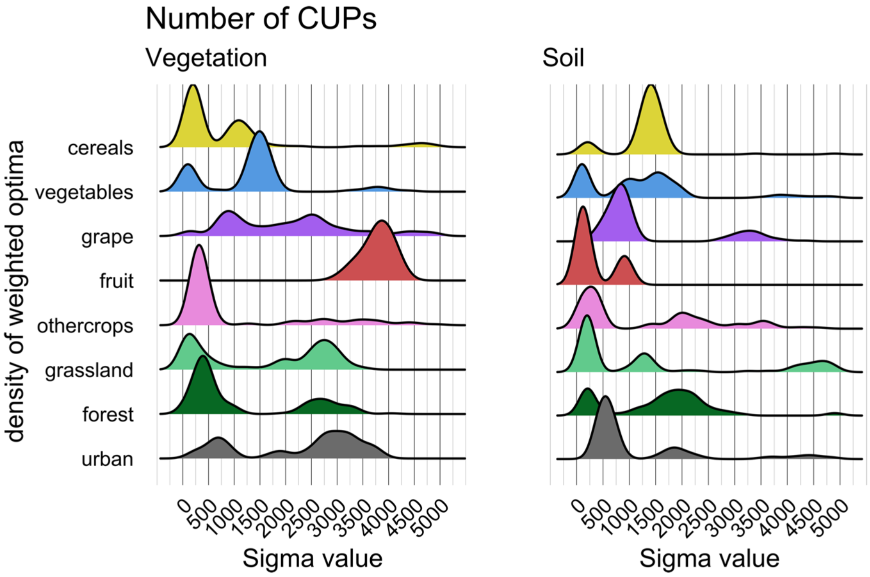

Fig. 6 | Density plot of the weighed optima for the eight land use categories and number of Current Use Pesticides in vegetation and topsoil. Sigma values were the standard deviation of the normal distribution (in metre) which were used for optimization regarding the distance to the prediction site. An optimum was considered as a sigma when the highest multiple R^2 of a model with several sigma compared was reached. Each optimum was additionally weighted with its absolute estimate of the generalized linear model, containing all eight land use categories with a certain sigma combination which resulted in a peak R^2.

concentration and varied the kernel width σ_L for each land type and optimized R^2 for the prediction.

Unfortunately, this results in a nonlinear optimization problem in an 8-dimensional space, which has no simple solution. We therefore used gradient descent methods to find the locally optimal combination of σ_L. We started the optimization algorithm 5000 times with different starting points, e.g. resulting in a total of 176 different local optima for the number of CUPs in vegetation, but we most likely have not found all local optima. For better visual separation we weighted the local optima with their absolute estimate of the resulting model. However, plotting the resulting σ_L reveals that most optima have similar σ_L depending on the land use type (Fig. 6). We caution that we have fit many thousands of linear and generalized linear models during the optimization algorithm. It is therefore not meaningful to compute the *p*-value for the resulting optimal fits, we therefore only report the corresponding R^2 value.

Overall, land use categories affect the surrounding land much further away than tested basic drift values[30], often for multiple hundred metres (Fig. 6). However, some land use categories (e.g. urban or grassland) showed multiple possible σ, indicating that a certain land use type may have different effects on the surrounding land depending on the distance (See SI for all σ histograms). Land use type urban seems to fit a σ at 750 m and 3000 m for the number of CUPs in vegetation. This could be due to the positive relationship of the occurrence of various agricultural practises near smaller villages (σ 750 m), but when there is a lot of urban land in a larger area (σ 3000 m), agricultural land tends to be absent (larger cities). The categories of crop types must be considered differently, which may be due to the specific CUPs used and their application technique. For vegetation and fruit, a maximum σ of 3750 m was determined. Thus, if CUP contamination is predicted for a non-target site for vegetation during spraying season, 68% of information for fruits contribution is between 0 and 3750 m (see Supplementary information for further details).

Prediction

Regularly spaced prediction points with a distance of 1000 m were placed in the study area. Prediction points in mountain regions that were further from the mountain foot than the measured sample sites were removed (>16.5 km).

The measured number of CUPs and their sum concentrations as independent variables and the optimized combinations of the land use categories and their best explaining σ as dependent variables were used inside a generalized linear model (number of CUPs) or linear model (log of (sum concentration + pseudo constant of 0.01)) for prediction. The predicted values were rasterized to generate a map.

Nature conservation areas

Nature conservation areas (NCAs) shape files were provided by the Bundesamt für Naturschutz[85] and considered if they were located inside the study area. Regularly spaced prediction points with a distance of 200 m were generated inside the NCAs. The same generated glm/lms which were used for the large prediction maps were used to predict the number of CUPs in NCAs. To determine the overall mean number of CUPs inside a NCA, all points inside a NCA were averaged. To generate a map of the "Kleine Kalmit", regular spaced prediction points with a distance of 100 m were generated. Previously mentioned glms were used to predict the number of CUPs and rasterized.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data that were calculated in this study are available in the Supplementary Information. Raw Data and calculated tables also available under https://doi.org/10.5281/zenodo.14832057.

Code availability

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. R Code for generating prediction maps is available under https://doi.org/10.5281/zenodo.14832057. Should any code or raw data files be needed in another format they are available from the corresponding author upon reasonable request.

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Author contributions

K.M.M. and C.A.B. planned the project. K.M.M. and C.A.B. arranged the necessary permissions from relevant authorities and sampled the sites.

K.M.M. and C.H. performed the analytical analysis, validated and prepared the data. K.M.M., J.W. and J.W.S performed the statistical analysis and the landscape regression analysis. All authors discussed the results. K.M.M. wrote the original draft. All authors commented and improved the manuscript and agreed on the final version. C.A.B. supervised the entire project.

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11 APPENDIX II: PAPER 2

Exposure of insects to current use pesticide residues in soil and vegetation along spatial and temporal distribution in agricultural sites

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Exposure of insects to current use pesticide residues in soil and vegetation along spatial and temporal distribution in agricultural sites

Carolina Honert✉**, Ken Mauser, Ursel Jäger & Carsten A. Brühl**

Current use pesticides (CUPs) are recognised as the largest deliberate input of bioactive substances into terrestrial ecosystems and one of the main factors responsible for the current decline in insects in agricultural areas. To quantify seasonal insect exposure in the landscape at a regional scale (Rhineland-Palatine in Germany), we analysed the presence of multiple (93) active ingredients in CUPs across three different agricultural cultivation types (with each three fields: arable, vegetable, viticulture) and neighbouring meadows. We collected monthly soil and vegetation samples over a year. A total of 71 CUP residues in different mixtures was detected, with up to 28 CUPs in soil and 25 in vegetation in single samples. The concentrations and numbers of CUPs in vegetation fluctuated over the sampling period, peaking in the summer months in the vegetation but remaining almost constant in topsoil. We calculated in-field additive risks for earthworms, collembola, and soil-living wild bees using the measured soil concentrations of CUPs. Our results call for the need to assess CUP mixture risks at low concentrations, as multiple residues are chronically present in agricultural areas. Since this risk is not addressed in regulation, we emphasise the urgent need to implement global pesticide reduction targets.

Keywords Synthetic pesticide, Non-target organism, Mixture, Chronic

Pesticides are chemicals used to prevent, destroy, repel, or mitigate pests. They can also be used as plant regulators, defoliants, or desiccants[1]. The application of pesticides is recognised as the largest intentional input of biologically active substances into terrestrial ecosystems[2]. Synthetic current use pesticides (CUPs) have been detected not only in cultivated land[2–5] but also in nontarget areas[6–12] and at the landscape level[13–15].

In recent decades, a decline in aerial insect biomass was observed in both, agricultural landscapes[16] and nature reserves[17] in Germany. Many factors, such as climate change, landscape fragmentation and agricultural intensification, including the use of pesticides, are considered stressors of the observed decline[18,19]. However, in a pan-European study that measured the in-field biodiversity of plants, insects and birds, pesticides were identified as stressors with greater explanatory value than structural landscape features or fertiliser use[20].

On cultivated land, a series of CUPs are applied throughout the year. This results in the accumulation of multiple substances. This realistic mixture exposure is not consistently assessed in environmental risk assessment procedures for regulation, as a comprehensive mixture risk assessment is generally not performed. While individual tank mixtures, if listed on the label of the product with a clear name and dose rate and formulations are tested[21], the actual realistic mixtures are not considered[22]. In addition, the current insect exposure assessment does not adress soil or vegetation as sources of contamination[23], despite its acknowledged relevance[24]. Toxicological effect data on pesticide exposure in soil are available for earthworms and collembolans. Recent research has focused on evaluating the potential risks associated with pesticides for pollinators, regarding the presence of CUPs in pollen and nectar and a revised EFSA document was published[25]. Besides the fact, that bumble bees and many solitary bees build their nests in soil[26], exposure in the risk assessment by pesticides in soil is only considered through pollen and nectar contaminations via soil[25,27,28]. Moreover, there is a lack of data on the toxicological endpoints resulting from pesticides on above-ground plant material, like stems and

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leaves, despite various insects using them as nesting material, living on them, or consuming them as larvae (e.g. caterpillars) or adult (e.g. grasshoppers).

Various pesticide mixtures have been measured in recent years in agricultural soils[2–5,8,11,29,30] and non-agricultural soils[5,31] as well as in the vegetation of nontarget areas[7,9,10,32] using multicomponent analytical methods. During a study in Switzerland, agricultural soil samples with 14 years of pesticide history were investigated[33]. Their results indicate that the presence of CUPs goes beyond recent applications, forming a substantial background of various residues in the soil. However, as in most studies, the samples were collected once a year. Thus, it remains unclear how CUP residues change throughout the season, affecting the exposure of non-target organisms.

To investigate the realistic exposure of insects to CUPs in landscapes characterised by agriculture, we sampled and analysed two environmental matrices, soil and vegetation. Samples were taken from nine different agricultural sites (arable management system, vegetable management system and viticulture, each $n=3$) separated by 0.20 to 41.8 km in Rhineland-Palatine, Germany, with an adjacent meadow to each site (for further site details see Supplementary Table S16 and S16b). We sampled every month for an entire year to be able to link exposure to the activity patterns of different insects.

The objectives of this study were (1) to quantify the seasonal variation in synthetic CUP mixtures in soil and vegetation by monthly sampling over one year; (2) to characterise spatial in-field and off-field (adjacent meadows) CUP distributions at different distances (1, 5, and 20 m from the field to the meadow); (3) to calculate the risk of realistic CUP mixture exposure for earthworms, collembolans and a surrogate ground-nesting wild bee. Our research hypotheses were that (a) CUPs fluctuate seasonally in soil and vegetation in numbers and concentration and (b) decrease with distance from the agricultural fields, with (c) far-reaching transportation in 3D (viticulture) compared to 2D (arable and vegetable) applications.

Results

CUP mixtures in soil and vegetation

Of the 93 CUPs analysed, 66 (71%, 30 fungicides (F), 23 herbicides (H) and 13 insecticides (I)) were detected in the topsoil samples (see Supplementary Table S1). Each analysed topsoil sample ($n=468$) contained at least one CUP, 94% contained two or more CUPs and in 53% of all topsoil samples, ten or more CUPs were recorded. On average ten CUPs were recorded in soil samples. A maximum of 28 different CUPs was detected in a single topsoil sample from an arable (winter wheat) field in October (see Supplementary Table S2). The most frequently occurring substances per pesticide group in topsoil samples were the fungicide (F) fluopyram, the herbicides (H) terbuthylazine and pendimethalin) and the insecticide (I) chlorantraniliprole (Table 1). The topsoil samples revealed 349 different CUP mixture combinations with 124 mixtures containing at least one insecticide. The most common mixture of azoxystrobin (F) and fluopyram (F) in all the soil samples occurred 11 times. In the soil, the mean concentration of 29 of the 66 recorded CUPs was < 1 µg/kg. The highest mean concentration for single CUPs in topsoils was detected for metrafenone (F, 26.53 µg/kg see Supplementary Table S1). However, some CUPs, e.g. s-metolachlor (H), azoxystrobin (F) and pendimethalin (H), were detected at concentrations > 500 µg/kg (see Supplementary Table S1). The maximal concentration was recorded for pendimethalin (H, 981.18 µg/kg, see Supplementary Table S1, vegetable field (celery), in-field (−20 m), May 2021)[34]).

Sixty-two of the 93 CUPs (67%, 31 fungicides, 20 herbicides and 11 insecticides; Supplementary Table 1) were detected in the vegetation samples. Almost all vegetation samples (97%, $n=392$) contained at least one CUP, with 89% containing two or more CUPs. The average number of CUPs recorded in the vegetation samples was seven (see Supplementary Table S2). In 30% of the samples, ten or more CUPs were recorded. Up to 25 different CUPs were measured in two vegetation samples (both celery cultivation) from vegetable fields (June and July 2021). Fluopyram (F) and chlorantraniliprole (I) are the most commonly detected fungicides and insecticides (Table 1). Prosulfocarb was the most prevalent herbicide recorded in vegetation (Table 1). In the vegetation samples, 344 CUP combinations were recorded, with 68 mixtures containing at least one insecticide. The most frequent mixture was azoxystrobin (F) and fluopyram (F), which occurred in seven samples. In the vegetation, the mean concentration of 7 CUPs of 68 was < 1 µg/kg and between 1 and 10 µg/kg for 27 CUPs. The highest mean concentration of single CUP in vegetation was recorded for fludioxonil (F, 1,349.55 µg/kg see

	Detection frequency (%)		**Total mean conc. [µg/kg]**		**Maximum conc. [µg/kg]**	
	Soil (*n* = 468)	**Vegetation (*n* = 442)**	**Soil (*n* = 468)**	**Vegetation (*n* = 442)**	**Soil (*n* = 468)**	**Vegetation (*n* = 442)**
Fungicides						
Fluopyram	**93.6**	**70.4**	6.04	28.79	164.62	954.42
Herbicides						
Pendimethalin	**28.2**	16.1	24.22	463.34	981.18	23,161.44
Prosulfocarb	14.1	**35.1**	0.27	4.05	0.78	28.15
Terbuthylazine	**28.2**	12.2	2.34	11.80	205.10	218.52
Insecticides						
Chlorantraniliprole	**14.3**	**10.2**	3.15	102.73	27.09	3,988.48

Table 1. Detection frequency, total mean concentration [µg/kg] and maximum concentration [µg/kg] of the three most commonly detected CUPs in soil and vegetation (in bold).

Supplementary Table S1). The maximal concentration of 67,656.10 µg/kg was measured for dimethomorph (F, viticulture, in-field (−20 m), July 2021).

Temporal CUP distribution

Temporal distribution of CUPs in soil of different crops

The number of CUP residues in topsoils remained nearly constant throughout the year. In arable fields, the CUP numbers were constant until October, when a maximum was detected, afterwards the number of CUPs slightly decreased. In-field at vegetable sites, two maxima were detected (February and October 2021, Fig. 1, see Supplementary Table S3), with a minimum in May. The same was calculated in-field at viticulture sites (Fig. 1, see Supplementary Table S3). The temporal course of the CUP numbers in topsoil samples from target areas (fields) differed only slightly among the three cultures (KL divergence ≤ 0.01, see Supplementary Table S4).

Temporal distribution of CUPs in the vegetation of different crops

Seasonal fluctuations in CUP numbers were pronounced in the vegetation (Fig. 1). In the field, the number of CUPs increased until the middle of summer. Maxima in-field from vegetation samples were detected for all managment systems in July and August (see Supplementary Table S3) and again in October for vegetables. Concerning arable and vegetable crops, similar trends were observed (KL-divergence see Supplementary Table S5) the number of CUPs decreased after the peak and remained high in viticulture (Fig. 1). In the off-field, the number of CUPs remained higher at the viticulture sites compared to the other cultivation types (Fig. 1).

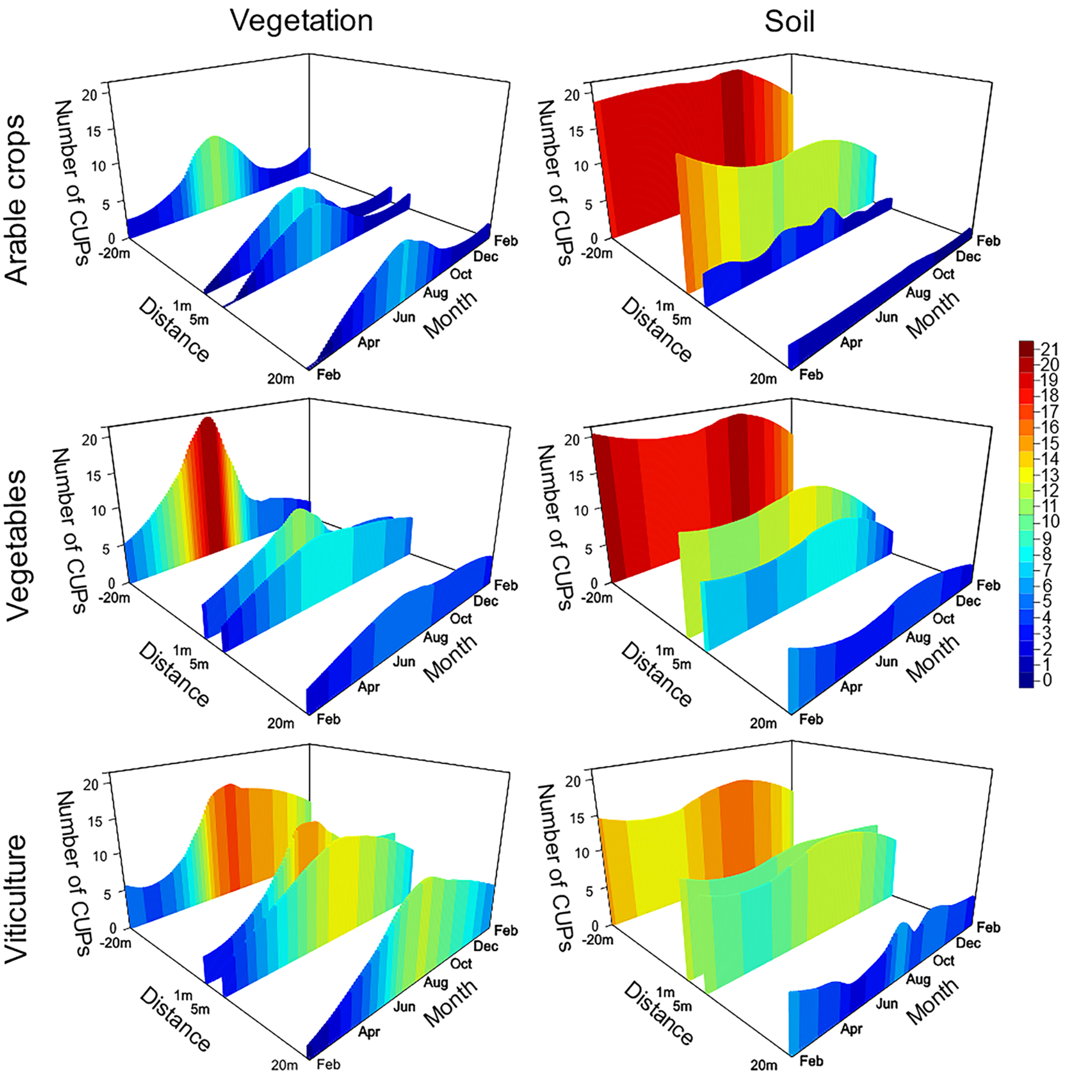

Fig. 1. Number of CUPs throughout the year in soil (left graphs) and vegetation (right graphs) from different agricultural sites (arable crops, vegetables, and viticulture) at four different measurement points (in the target area (−20 m) and off-field in an adjacent meadow (1 m, 5 m and 20 m from the field margin)) over 13 months from February 2021 to February 2022. The colours indicate the number of CUPs. The data were modelled based on the raw data with a loess smooth.

Spatial CUP distribution

An exponential function was used to fit the data and analyse the drift for the different cultivation types. A consistent decrease in CUPs was observed from in-field to meadow for all land use types in terms of soil and vegetation (Fig. 2).

CUPs in soil of different management systems

Arable crops In topsoil from arable fields, an average of 19 CUPs was detected (see Supplementary Table S6). A decrease in the number of CUPs (1 m: 13 CUPs, 5 m: 3 CUPs, 20 m: 2 CUPs, see Supplementary Table S6) was measured at the arable sites with increasing distance (b= −0.47, see Supplementary Table S7, Fig. 2). Insecticides were only recorded in the field and 1 m from the field edge, represented by 6 and 4 substances, respectively (see Supplementary S8).

Vegetables In the field, at the vegetable sites (−20 m), an average of 19 CUPs was recorded (see Supplementary Table S6). A decrease in CUPs with increasing distance was measured off-field (Fig. 2, see Supplementary Table S7: b= −0.52 and see Supplementary Table S6: 1 m: 12 CUPs, 5 m: 8 CUPs, 20 m: 5 CUPs). Over all months, eleven different insecticides were detected in the soils from the vegetable fields. At 1 m off-field, six insecticides were detected, three at 5 m and one at 20 m (see Supplementary Table S8).

Viticulture On average, 15 CUPs were detected in the in-field topsoil at the viticulture sites, decreasing to 12 CUPs at 1 m as well as 5 m and 5 CUPs at 20 m (see Supplementary Table S6). In viticulture, a greater drift into the off-field area was measured compared to arable and vegetable crops (Fig. 2, see Supplementary Table S7: b = − 0.11). The 20 m measurement point at one of the three viticulture sites was situated in a nature conservation area. During the main spraying season, a maximum of 7 CUPs was recorded at this point in August (May: 2 CUPs, June: 2 CUPs, July 4 CUPs). Overall, viticulture sites were dominated by fungicides: residues of 23 different fungicides, and two herbicides and two insecticides were detected in topsoil samples inside the vineyards (see Supplementary Table S8 and Figure S1).

CUPs in vegetation of different management systems

In 14 vegetation samples, no CUP was detected (seven samples at 20 m, six at 5 m and one at 1 m off-field). Twelve of the 14 samples without CUP detection were collected at arable sites (at 5 and 20 m), one at the vegetable site (20 m, February 2022) and one at the viticulture site (1 m, February 2021).

Arable crops On average, 7 CUPs were detected in vegetation in arable crops (see Supplementary Table S6). A decrease in CUP numbers from the cultivated area to the meadow (Fig. 2, see Supplementary Table S7: b = − 1.45) was measured. On average, 4, 4 and 3 CUPs were detected in meadow vegetation samples at 1 m, 5 m and 20 m off-field, respectively (see Supplementary Table S6). Fungicides were detected most predominantly, followed by herbicides and insecticides. Three different insecticides (acetamiprid, thiamethoxam at 5 m, spinosad and thiamethoxam at 20 m; see Supplementary Table S8) were detected in the off-field vegetation.

Vegetables On average, 11 CUPs were extracted from vegetation inside the vegetable fields (mostly crop plants as wild plants were not available in large quantities due to herbicide use; see Supplementary Table S6). At one meter in the meadow, on average, 7 CUPs were detected, 7 at 5 m and 5 at 20 m (see Supplementary Table S6). CUP numbers decreased with the distance from the field (Fig. 2, Supplementary Table 5: b = − 0.22). Fungicides were detected most predominantly. The insecticide chlorantraniliprole was detected at all sampled distances (see Supplementary Table S8). In total, eight insecticides were recorded in-field, five at 1 m, two at 5 m and two at 20 m off-field.

Viticulture On average, 12 CUPs were detected in vegetation from viticulture areas. The number of CUPs decreased slightly with increasing distance, with 10 CUPs recorded at 1 m, 10 at 5 m and 8 at 20 m (Fig. 2, see Supplementary Table S7: b = −0.15, see Supplementary Table S6). At the nature conservation area, a maximum of 16 CUPs was measured in July (May: 2 CUPs, June 7 CUPs, August 12 CUPs). No insecticide residues were detected in any vegetation sample from inside the vineyard or the adjacent meadows.

Realistic mixture exposure risk assessment

The risks of single substances were calculated for the detected CUP mixtures and are summarised in the respective risk quotients.

Collembola

For Collembola, published no-observed effect concentration (NOEC) values were available for 55 of the 66 detected CUPs. In-field, CUP mixtures posed a risk (mixture exposure risk, MRQ) in all topsoil samples for collembola, a high risk was calculated in 13% ($n=5$ of 39) of the samples (mean MRQ > 1), a medium risk (0.1 < MRQ < 1) in 79% ($n=31$), a low risk (0.01 < MRQ < 0.1) in 8% ($n=3$) and no negligible risk (MRQ < 0.01) was calculated ($n=0$) (Table 2). High-risk values (mean MRQ) in-field were calculated in July for arable fields, in October for vegetable sites and from June to August for vineyards. The risk remained at a medium level at arable and vegetable sites but at a low level in February 2021, March 2021 and February 2022 at viticulture sites. In around 20% of the off-field samples, low (22%, $n=26$) and medium (20%, $n=24$) risks were calculated. A high off-field risk was calculated in the meadow soil at 1 m next to the vineyard in June (see Supplementary Table S9). The off-field environment posed a risk to collembola in approximately half of the samples (44%, $n=51$ of 117; see Supplementary Table S9), whereby the occurrence of risks decreased with the increasing distance. On average

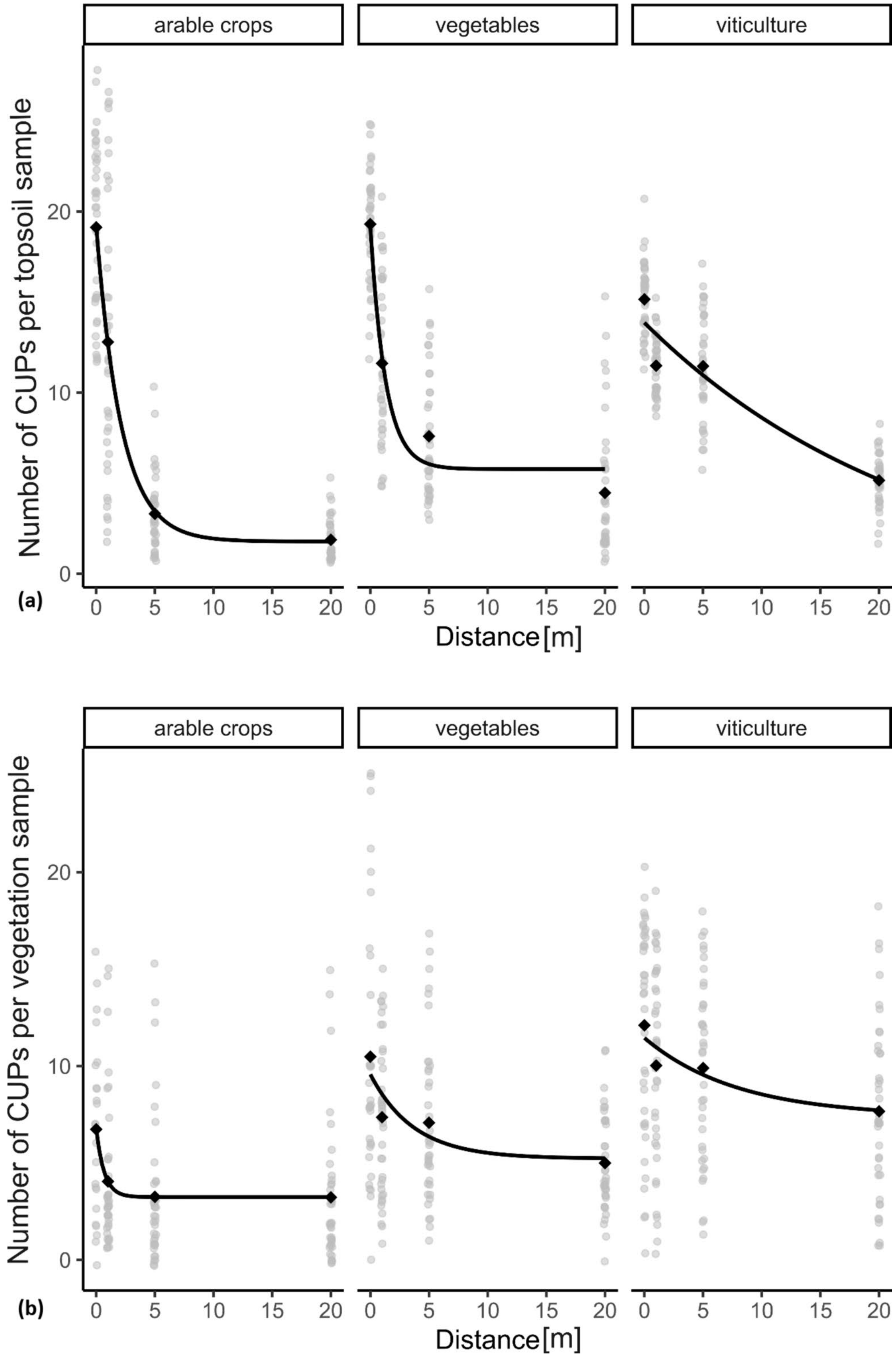

Fig. 2. Number of CUP per sample with increasing distance to the field for all investigated management systems (arable crops, vegetables and viticulture) in soil (**a**) and vegetation (**b**). CUP number per sample and distance. The curves show the exponential fit with the R-function nlsLM[35]. All samples (vegetation: $n = 442$, soil: $n = 468$) collected throughout the year are included. The in-field samples were labelled as distance 0 and the off-field samples were collected at distances of 1, 5 and 20 m in an adjacent meadow.

	Collembola						Earthworms					
	Arable site		Vegetable site		Viticulture site		Arable site		Vegetable site		Viticulture site	
Month	Mean RQT	Max RQT	Mean RQT	Max RQT	Mean RQT	Max RQT	Mean RQT	Max RQT	Mean RQT	Max RQT	Mean RQT	Max RQT
In-field (-20 m)												
Feb’21	**0.34**	**0.51**	**0.18**	**0.43**	*0.04*	*0.05*	**0.28**	**0.43**	**0.24**	**0.31**	**0.36**	**0.39**
Mar’21	**0.38**	**0.82**	**0.11**	**0.30**	*0.04*	*0.09*	**0.30**	**0.44**	**0.30**	**0.60**	***1.65***	***3.83***
Apr’21	**0.53**	**0.83**	**0.41**	***1.17***	**0.13**	**0.25**	**0.40**	**0.73**	**0.53**	**0.70**	**0.61**	***1.44***
May’21	**0.40**	**0.64**	**0.15**	**0.33**	**0.17**	**0.31**	**0.31**	**0.54**	**0.71**	***1.02***	***1.00***	***1.52***
Jun’21	**0.70**	***1.02***	**0.32**	**0.85**	***1.60***	***2.30***	**0.92**	***1.82***	***1.10***	***2.03***	***1.39***	***2.03***
Jul’21	***1.07***	***2.46***	**0.19**	**0.32**	***1.31***	***2.87***	**0.39**	**0.46**	***4.33***	***7.33***	***1.44***	***2.86***
Aug’21	**0.49**	***1.08***	**0.16**	**0.47**	***2.80***	***6.61***	**0.26**	**0.32**	**0.24**	**0.62**	***2.04***	***5.02***
Sep’21	**0.78**	***1.75***	**0.32**	**0.59**	**0.57**	**0.83**	**0.31**	**0.42**	**0.84**	***1.43***	***1.09***	***1.75***
Oct’21	**0.77**	***1.49***	***1.22***	***3.20***	**0.63**	***1.12***	**0.36**	**0.45**	***2.14***	***4.23***	***1.04***	***1.31***
Nov’21	**0.33**	**0.49**	**0.82**	***2.19***	**0.33**	**0.66**	**0.26**	**0.51**	***1.16***	***1.95***	**0.75**	***1.07***
Dec’21	**0.42**	**0.66**	**0.35**	**0.90**	**0.53**	**0.80**	**0.20**	**0.35**	**0.83**	***1.18***	***1.17***	***1.94***
Jan’22	**0.47**	**0.81**	**0.29**	**0.69**	**0.17**	**0.30**	**0.19**	**0.33**	**0.62**	**0.86**	**0.61**	**0.86**
Feb’22	**0.18**	**0.30**	**0.35**	**0.86**	*0.09*	**0.21**	**0.11**	**0.17**	**0.43**	***1.00***	**0.48**	**0.69**

Table 2. Overview of the mixture exposure risks (MRQs) calculated for collembola and earthworms based on the measured environmental concentrations (MECs) of 93 CUPs in topsoils in cultivated areas (in-field) of arable ($n=3$), vegetable ($n=3$) and viticulture sites ($n=3$). The mean and maximum MRQ for both soil organisms at each agricultural site and month are shown. No risk ($MRQ < 0.01$, low risk (italics) $0.01 < MRQ < 0.1$, medium risk (bold) $0.1 < MRQ < 1$, a high ($MRQ = 1$, bold italics) and very high risk (bold italics) $MRQ > 1$).

the MRQ was highest on viticulture sites (mean arable sites ($n=156$): 0.17, mean vegetable sites ($n=156$): 0.11, mean viticulture sites ($n=156$): 0.26).

The maximal Risk Quotient (RQ_{max}), the risk caused by the concentration of a single CUP in the mixture, explained over all months 86% of the risk in arable in-field samples, 69% of the risk in vegetable in-field samples and 79% of the risk in vineyard in-field samples (see Supplementary Table S10). In a few instances also, single substance concentrations alone posed a high risk to collembola ($RQ \geq 1$). The presence of four CUPs, cyflufenamid ($n=8$ of 468 topsoil samples, six in-field and two samples at 1 m), epoxiconazole ($n=4$, in-field), cyantraniliprole ($n=2$, in-field) and imidacloprid ($n=2$, in-field, see Supplementary Table S11), resulted in a high risk for collembola in the topsoil samples.

Earthworms

Toxicological endpoint values (NOEC, or if not available then LC50) for earthworms were available for 64 of the 66 CUPs detected in this study. The mean MRQ ($n=3$) for in-field samples was calculated to indicate a risk for all months and all management systems. A high risk was calculated in 31% of the in-field samples ($n=12$ of 39), a medium risk was calculated in 69% ($n=27$) and no low or negligible risk was calculated (Table 2, see Supplementary Table S9). No high risk (mean MRQ) for earthworms was calculated in-field at arable sites. A high risk was posed by the CUP mixtures present in the topsoils of vegetable fields in June, July, October and November and for March, May to October and December in vineyards. The percentage of off-field mean topsoil samples at risk was 50% ($n=59$ of 117), with 1% ($n=1$) of all off-field samples at high risk, 23% ($n=28$) at medium risk, and 25% ($n=30$) at low risk (see Supplementary Table S9). The number of MRQs indicating a risk decreased with increasing distance. On average the MRQ was highest on viticulture sites (mean arable sites ($n=156$): 0.11, mean vegetable sites ($n=156$): 0.32, mean viticulture sites ($n=156$): 0.34). Medium risks were detected up to 20 m in the meadow of the vegetable sites.

On average ($n=39$), 46% of the MRQ is explained by a single CUP (RQ_{max}) in in-field samples at arable sites and 62% in in-field vegetable and viticulture samples (see Supplementary Table S10). Four single CUPs posed a high risk to earthworms ($RQ \geq 1$): the fungicides azoxystrobin ($n=2$ of 468 samples), boscalid ($n=8$), difenoconazole ($n=12$) and the herbicide terbuthylazine ($n=1$) (see Supplementary Table S12).

Wild bees

LD50 values for honey bees were published for 65 of the 66 CUPs detected in this study. The mean mixture hazard quotient (MHQ) for the acute exposure scenario (2.23 g soil exposure per bee in 48 h[36]) in-field posed a hazard in 20.5% of the topsoil samples (Table 3, see Supplementary Table S13). Based on the average MHQ ($n=3$) a hazard ($MHQ > 1$) was inferred from June to July on arable land and from September to February 2022 on vegetable fields. No risk was identified at vineyards. The maximum MHQ indicated a hazard for wild bees from March to July on arable soils and in February and from August to February on vegetable soils. No maximum MHQ was higher than the threshold of 1 in vineyards (Table 3). Off-field, no hazard was calculated from the measured CUP mixtures (see Supplementary Table S13).

	Arable sites		Vegetable sites		Viticulture sites	
Month	**Mean MHQ**	**Max MHQ**	**Mean MHQ**	**Max MHQ**	**Mean MHQ**	**Max MHQ**
Feb'21	0.37	0.49	0.7	**2.08**	0.05	0.05
Mar'21	0.38	**1.13**	0.01	0.01	0.09	0.18
Apr'21	0.71	**1.8**	0.35	0.52	0.04	0.07
May'21	0.41	**1.19**	0.24	0.37	0.05	0.06
Jun'21	**1.75**	**3.9**	0.08	0.12	0.08	0.12
Jul'21	**1.54**	**4.54**	0.29	0.66	0.1	0.15
Aug'21	0.33	0.94	0.64	**1.91**	0.14	0.29
Sep'21	0.25	0.71	**2.18**	**5.65**	0.09	0.1
Oct'21	0.49	0.74	**7.87**	**22.69**	0.08	0.09
Nov'21	0.29	0.52	**5**	**14.82**	0.06	0.06
Dec'21	0.63	**1.28**	**1.32**	**3.93**	0.08	0.12
Jan'22	0.31	0.68	**1.4**	**3.64**	0.05	0.05
Feb'22	0.01	0.01	**1.55**	**4.09**	0.05	0.05

Table 3. Mean mixture hazard quotient (MHQ) ($n = 3$) and the maximal MHQ were calculated based on the recorded concentrations of 93 CUPSs in in-field topsoil samples of arable, vegetable and viticulture site for solitary wild bees with a surrogate LD50 (honey bee LD50)/10[36], with LD_{50} data taken from PPDB) and an acute contact of 2.23 g of soil over 48 h. The numbers displayed in bold are MHQs > 1, indicating a lethal hazard.

Over all months, 63% of the MHQ was explained by the concentration of a single CUP (HQ_{max}) in arable in-field samples, 69% in vegetable in-field samples and 43% in viticulture in-field samples (see Supplementary Table S10). The concentrations of clothianidin (I, $n = 4$), cyantraniliprole (I, $n = 7$) and thiamethoxam (I, $n = 3$) exceeded the HQ threshold of 1 (see Supplementary Table S14).

Discussion

All topsoil samples in this study, in-field and off-field, contained at least one CUP, with an average of 10 CUPs per sample. This corresponds to other European studies, where at least one CUP was measured in 99%[2] and 98%[3] of the in-field topsoil sample. This suggests that soil in agricultural landscapes is contaminated with multiple CUPs. Methodologically, the number of detected CUPs depends on the number of CUPs that are analysed if targeted methods are used. For instance, in a French study, 111 different CUPs were included in a target analysis of topsoil samples, of which 67 different substances were detected[8]. We analysed 93 different CUPs and detected in total of 71 CUPs, 66 in topsoils and 62 in vegetation. In addition, Glyphosate and AMPA, were among the most frequently detected CUPs in the French study, with 83% and 70% detection rates, respectively[8]. These results correspond to a European-wide study[11], which also detected a mixture containing AMPA and glyphosate (included in 25% of pesticide combinations[11]), to be the most common CUPs in soil samples. Therefore, we can assume that glyphosate and AMPA are very likely also occurring in our soils. In contrast, the most abundant CUP in our soil samples was fluopyram (in 94% of all topsoil samples), which was also detected in 69% of topsoil samples in a French study[8] but was not included in the target substances in other studies[3,11]. Fluopyram is marketed as a fungicide in Europe but is used as a nematicide in tropical crops such as bananas[37], therefore it has negative effects on nematodes and available data indicate a negative effect of a fluopyram metabolite on plants[38] and soil arthropods[39]. Since our analysis covers only a part of the authorised CUPs in Germany, it is fair to assume that even more CUPs are present in in-field soils, resulting in even greater mixture complexity. We detected almost 350 different CUP mixture combinations in topsoil and vegetation by analysing 93 target compounds, with the most common mixture being detected only on eleven occasions in soils and seven in vegetation samples. The amount of combinations makes it impossible to assess the mixture toxicity risk organisms face in reality if this would ever be a regulatory aim. At least one insecticide was detected in 124 mixtures in soils and 68 mixtures in vegetation. In our target method, fewer insecticides than fungicides and herbicides are included, which may explain the lower frequency of insecticides observed in the mixtures. We assume that more mixtures contain insecticides than were observed in this study, as some insecticide groups, such as pyrethroids, can only be detected with different analytical methods. This limitation in detection capacity is an important caveat of our study, as it does not cover all authorised pesticides. To establish pesticide monitoring in the terrestrial environment, all registered molecules, as well as their metabolites or transition products, should be included in a target method and incorporated in the current proposal for an EU Directive on Soil Monitoring[13,40].

A comparison of the detected substances with the application data provided by the farmers would have constituted an interesting research approach. However, as our study was conducted with actual farmers rather than model farms, we were dependent on their willingness to participate, which prevented the collection of such data.

Comparing cumulative concentrations across studies requires caution due to the use of different analytical methods, as underlined in a previous study[5] and discussed above. Individual concentrations can be cautiously compared, considering variations in quantification limits, extraction methods and sampling times. It should also be considered that high variability in CUP concentrations has already been observed in soil samples[41].

Nonetheless, the highest concentration measured in our agricultural topsoil samples was detected for pendimethalin (981.18 µg/kg, vegetable, cultivated area, May 2021). In French arable soils, pendimethalin was detected at a concentration of 923 µg/kg in spring[5] and at a concentration of 1,115 µg/kg in January[8]. In another study, a maximum concentration of pendimethalin (310 ng/g) was recorded after the field season[4]. The comparison shows that our data are quite comparable with those of other European studies when individual concentrations are considered.

The sampling time differed between studies; some were conducted before and others after the field season[42]. It was unclear whether the exposure data recorded in the studies were representative of the entire year or whether they were just displaying a specific moment. Our data show that a single sampling event is not representative of the whole year. Due to the differences in February 2021 and February 2022, there may likely be differences in exposure between years. In light of this, comprehensive, long-term monitoring is essential to gain a full understanding of the situation and its temporal dynamics. In the topsoils, we recorded a high baseline of CUPs throughout the year with various maxima. This result is not consistent with our hypothesis of seasonal variation, which was based on the timing of pesticide application. It might be influenced by multiple factors: CUP residues from deeper soil layers, which have been preserved there, can enter the topsoil through tillage. Crop senescence and harvest residues can also distribute CUP residues into the soil[43]. Moreover, the soil is also treated with CUPs before and after cultivation, mainly with herbicides to prepare the field for cultivation. During the agricultural season, the soil is covered by vegetation and most of the applied substances remain, depending on the development stage, on the crop foliage and only a small fraction reaches the in-field soil[44]. We therefore observed higher concentrations in vegetation than in soil. For example, the highest measured concentration in soils (pendimethalin, herbicide, 981.18 µg/kg) was orders of magnitude lower than the corresponding vegetation sample (23,161.44 µg/kg, vegetable site, in-field, May 2021), although the latter was not the highest concentration measured in vegetation. The highest concentration of an individual CUP in vegetation was recorded for dimethomorph (fungicide, 67,656.10 µg/kg, viticulture sites, in-field, July 2021), as the corresponding concentration in the topsoil was 6.86 µg/kg[34]. The maximal mean concentration for a single CUP was measured at 26.53 µg/kg metrafenone in topsoils and 1,349.55 µg/kg fludioxonil in vegetation, again showing the higher concentrations in vegetation.

To our knowledge, this is the first study investigating the annual variation of terrestrial CUP concentrations. To analyse our data an approach of loess smoothing and a Kullback-Leibler (KL)-divergence was chosen to capture the courses of the year as accurately as possible. By the use of usual complex models like for example GLM/GLMM limitations have quickly been reached with our data set, e.g. due to the unknown distribution of the data or the missing linear relation between the predictor and the explanatory variable. The chosen approach with loess smoothing and KL-divergence was also used in a recent ecology study where time series were tested[45]. In our analysis, we used the data from all three repetitions from one management system to model the general temporal trends, applying both a loess fit and an exponential function to the combined data. The loess fit smoothing over the repetitions, revealing the underlying temporal patterns[45,46], while the exponential model is used in current pesticide emission models to estimate the drift[47,48]. Neither of these methods explicitly accounts for plot-specific variability, as they do not include random factors. CUP contamination in vegetation showed the hypothesised seasonal fluctuations, with an average of 7 CUPs per sample and a maximum of 25 CUPs in two single samples (in-field (−20 m), vegetable: celery, June and July). The observed fluctuations in the loess fit indicate a substantial deviation from the surrounding months. Although the Loess method does not provide p-values or formal statistical significance, the robustness of the fit implies that this peak reflects a meaningful difference, rather than random variation[49]. Grass samples from public areas in the Vinschgau Valley taken four times per year showed statistically significant changes in CUP contamination between seasons[9]. The highest CUP numbers were detected in early summer (May/June), while we measured maxima in late summer (July and August). The Vinschgau Valley is dominated by apple orchards that are treated earlier than the crops that we studied. However, fungicides were the dominant group in both studies.

Vegetation, both in-field and off-field, is more likely than soil to be contaminated by wet deposition and spray drift[50], as soil is covered for a long period by vegetation. Additionally, vegetation takes up systemic CUPs from the soil[51], which may account for some of the CUPs that were measured at lower concentrations. However, dilution effects caused by plant growth, as well as off-field mowing and grassing, can strongly influence the concentrations measured in vegetation samples. These factors present inherent challenges in field experiments, as they are difficult to quantify and cannot be easily accounted for in statistical analyses.

CUP residues were detected in the majority of the samples throughout the year. We show that the soils of agricultural areas, as well as those of the immediate surroundings, are loaded with mixtures of different CUPs throughout the year, indicating potential chronic CUP exposure for insects. Nevertheless, we also detected maxima and minima for the number of CUPs (Supplementary Tables 5, 8). Most maxima were measured in autumn, which can be explained by the accumulation of CUPs throughout the field season.

It is still unclear what effects the presence of up to 28 different CUPs (measured in in-field soil in arable field in October) has on insects and other organisms[52]. Although meadows were less contaminated than cultivated, in-field areas, mixtures of multiple CUPs were detected all year round.

We assumed that the number of detected CUPs decreased with increasing distance to the field. The decrease was tested by exponential functions, commonly used to model pesticide drift (e.g[53]). The hypothesised decrease in CUP numbers was detected for topsoil samples for all three management systems but less so for vegetation samples. In the latter, similar numbers of CUPs were recorded at different distances in the meadow. The number of CUPs was highest in the meadows adjacent to the vineyards and was less different from the in-field values than for the other management systems. This might be explained by the different ways of application; the 3D application, which is used for room cultures like viticulture or in orchards, to cover the canopy, leads to higher drift effects[54]. This finding is consistent with our hypothesis that there are differences between the various application types.

While the study design includes three repetitions per management system, with a total of 18 plots (9 in-field and 9 off-field), we acknowledge that this level of replication may still be considered limited, particularly in the context of the high variability between sites and management systems. The relatively low number of repetitions could make it challenging to fully account for all sources of variation. However, this design was a balance between practical constraints and the need to capture variability across different conditions. Conducting larger-scale studies with more repetitions would have been ideal, but such an approach is often constrained by logistical factors. Future studies could benefit from increasing the number of repetitions or expanding the range of covariates considered in the analysis to better capture the complexity of the system. On the other hand, we collected a large, unique dataset of soil and vegetation samples, comprising approximately 1,000 samples over the course of a year. In this field of research studies so far comprised e.g. 75 soil samples in Hvězdová et al. 2018[2], 317 soils in Silva et al. 2019[11], 180 soils in Pelosi et al. 2021[5], 71 vegetation samples in Linhart et al. 2019[7] or 96 vegetation samples in Linhart et al. 2021[9]. Our comprehensive dataset has the potential to benefit other researchers in a number of ways: It can be used as a benchmark for comparative studies, as a resource for secondary analyses, or as a foundation for model development and long-term monitoring efforts.

In all soil samples ($n=468$), CUPs were detected, while in 14 of 442 vegetation samples, CUPs were not detected. Most uncontaminated samples were recorded at the greatest distance from the field in 2 D crops (20 m off-field: $n=7$ of 14 and 5 m $n=6$). In a study investigating CUP residues in vegetation at similar distances, no CUPs were detected in 5 of 7 vegetation samples at their furthest distance (> 10 m), in 2 of 8 at 5–10 m, and all field edge samples (9 samples) were contaminated[32]. In a landscape characterised by agriculture, CUPs are recorded not only at the edge of the agricultural field but also at further distances, as shown here and also in previous studies[7–10,13,32,55].

Insects that live in an agricultural landscape are therefore potentially exposed to CUP mixtures not only by visiting or living in the fields themselves but also in the surrounding area, e.g. adjacent meadows. We assume that the main source of CUP contamination in vegetation samples from the meadow was drift during applications[10,56] or via volatilisation and/or dust from soil and plants after application, due to the physical-chemical properties of the pesticides, which are known to facilitate such processes (e.g., volatility, adsorption to particles). The pesticide concentrations in vegetation from public playgrounds were already positively correlated with the amount of rainfall and wind speed during application times[7]. However, air deposition[57] and rain events[58] are also inputs of CUPs, with transport from sources several kilometers away.

In our study, we also addressed the risk of CUPs to insects. However, the publicly available toxicology data basis is not sufficient, as there are no toxic endpoints for soil insects. Furthermore, as there is no risk assessment for herbivorous insects (e.g. grasshoppers or caterpillars) required, there are no toxic endpoints for oral insect exposure. Therefore, no risk from the consumption of contaminated vegetation could be calculated. Nevertheless, for approximately half of the CUPs detected in topsoils, the concentrations were below 1 µg/kg, while the concentrations for individual CUPs in vegetation samples were higher (7 of 61 CUPs with concentrations below 1 µg/kg). Concentrations in vegetation samples reached up to 67,656.10 µg/kg (dimethomorph, F, viticulture sites, in-field, July 2021). While insecticides were not detected at all off-field distances in topsoil, they were detected at all distances in vegetation samples from arable and adjacent meadows, indicating that the risk to insects is likely to be even higher here. These findings highlight the need to develop and analyse risk approaches for oral uptake via vegetation.

We showed that, on average, soils from the field posed a high to medium risk throughout the year for earthworms and collembola, except in three months for collembola (mean MRQ, Viticulture, February 2021, March 2021, February 2022, low risk). Furthermore, we showed that samples from meadows also posed a risk to collembola and earthworms, although the risk decreased with increasing distance to the agricultural field for all land use types. However, as nontarget areas also harbour chronic risks for at least earthworms[5,8], potential exposure-free and low-risk habitats for organisms are becoming increasingly scarce. As toxicological endpoints for springtails were not available for all measured CUPs, the risk might be even higher. The bioavailability of residues and the potential for synergistic effects of CUP mixtures need to be further investigated. Recently, a meta-analysis showed that the risk of pesticide exposure, assuming additive effects, underestimates the interaction effect on bee mortality, which in most cases is synergistic[59]. In our study the in-field risk was to some extent explained by single CUP concentrations, explaining on average 43 to 86% of the mixture risks, depending on the management system. Also, some single CUPs represented a high risk. The reduction or even avoidance of CUPs that trigger a high risk might improve the risk situation for non-target soil organisms. We identified eleven fungicides (azoxystrobin, boscalid, cyflufenamid, difenoconazole, dimoxystrobin, epoxiconazole, fluopicolide, fluopyram, metrafenone, myclobutanil, penconazole) five herbicides (clomazone, ethofumesate, pendimethalin, propyzamide, terbuthylazine) and five insecticides (chlorantraniliprole, clothianidin, cyantraniliprole, imidacloprid, indoxacarb) that contribute substantially to the measured risks. However, by changing CUP applications and substances the risk of other substances might increase.

There is currently no approach that addresses pesticide residues in soils as an exposure route for insects. Most wild bees nest in the soil[25,60]. Ground-nesting bees are in direct contact with the soil and larvae are exposed not only to the residues in the soil but also to the pollen. An in-field assessment of the risk to ground-nesting bees from systemic insecticides was recently performed[36]. Due to a lack of information, the authors used toxicological endpoints for honeybees to determine the risk for adult females to experience soil contact during nest building[61]. The ground-nesting honey squash bee (*Peponapis pruinosa*) used in this approach does not exist in Europe, but several other ground-nesting bees are known to build similar nests in agricultural soils in Europe[62]. *Peponapis pruinosa*, it is similar in size to common ground-nesting bees such as *Andrena flavipes*, which have been observed building nests in European agricultural fields[62].

Our wild bee risk evaluation shows that in-field, the CUP residues lead to an MHQ > 1 in arable and vegetable fields but not in viticulture. This may be because insecticides are not or less commonly used in the latter. At the vegetable sites, an MHQ > 1 was calculated from August to February, when most of the ground-nesting adult bees were no longer active. However, larvae and pupae overwinter in soils and are therefore assumed to be continuously exposed to potential pesticide residues, although their dormant phase within cocoons may on the other hand reduce their direct exposure generally uptake of pesticides can currently only be assumed as no data are available. We consider this aspect of the risk assessment as a supplementary evaluation of the potential exposure of soil-living insects and their development stages. The calculated risk may not lead to direct mortality in organisms but sublethal effects that occur at lower concentrations result in population-level effects e.g. due to lower reproduction[63], especially with chronic exposure over longer time spans.

We detected different insecticides in topsoils and vegetation at all sites and in vegetation at arable and vegetable sites. Chlorantraniliprole (I) was the most commonly recorded insecticide in both matrices. It is a newer insecticide used for tree fruits, grapes, vegetables, and maize[64]. Chlorantraniliprole reduced the locomotor activity of honey bees after 72 h of exposure[65] and was already detected in honey bee pollen[66], supporting our results. Pendimethalin, which together with terbuthylazine was the most frequently reported herbicide in topsoil samples, can affect the immune response and reduce the lifespan of honeybees[67]. The herbicide with the most detections in vegetation, prosulfocarb, was also detected with the highest frequency in German urban and agrarian bumblebee samples[68]. Prosulfocarb (H) and terbuthylazine (H), were also detected in all insect samples from 21 nature conservation areas in Germany, as were the fungicides fluopyram and azoxystrobin[52]. These results are consistent with our findings that insects are particularly frequently exposed to these substances.

Current risk assessments are based on the effects of single substances on individual organisms. Furthermore, it is assumed that there is sufficient time after application for populations to recover to previous levels[22]. However, our results show that there are no CUP-free periods. It is therefore improbable that recovery can be achieved in a realistic situation. In particular, in the field soil, high numbers of CUPs are recorded all year round, resulting in chronic exposure of soil insects or life stages of insects, e.g., larvae or pupae. High-resolution sampling over a year enables us to obtain CUP data about the activity of insects. In the vegetation, there is a maximum amount of CUP detected in the summer months, when many insect larvae feed on the plant material (e.g. Lepidoptera larvae) or use the vegetation for oviposition. When exposed to CUP residues as larvae on host plants, effects on longevity and wing length were observed in emerging butterflies[69]. Insecticide-treated plants were found to experience reduced pollination and limited to a single egg-laying event by moths, resulting in a 40% decrease in their offspring[70]. For adult wild bees (*Osmia cornuta*) exposed to sublethal concentrations of a mixture containing only one fungicide and one insecticide, a reduction in reproduction rate was observed, ultimately leading to a decline in population size[71,72]. Furthermore, insecticides have been shown to have carryover effects on wild bees[73] and other nontarget insects[74].

Conclusion

This investigation provides to the best of our knowledge the first temporal resolution of CUP residues in topsoil and vegetation in different management systems and adjacent non-target areas as common structural features in agricultural landscapes. In particular, complex mixtures of multiple CUPs were present throughout the year. They were not only recorded in the cropping area (in-field) but also in neighbouring, non-target meadows at distances of up to 20 m. This constant presence of multiple CUPs in agricultural landscapes leads to a chronic mixture of exposure to insects and other non-target terrestrial organisms.

The documented CUP exposure pattern is particularly worrying considering that most of the adult insects are mainly active in summer when the greatest number of CUPs are detected in vegetation. It has already been shown that flying insects collected in nature conservation areas are exposed to multiple CUPs[52]. In the topsoil, on the other hand, a high number of CUPs are present throughout the year, so adult insects or larvae living in the soil are chronically exposed to several CUPs at low concentrations. It is important to note that the constant presence of CUP mixtures is not part of the regulatory environmental risk assessment procedures for pesticide regulation. The remit of authorities at the EU and national levels lies in the risk characterisation of single substances and only occasionally are mixtures addressed in formulated products with up to four CUPs. We therefore urge authorities to ensure that chronic contamination with complex CUP mixtures occurring in reality is incorporated into authorization procedures and risk assessments, to prevent an unprotected environment. While risk management and mitigation strategies exist, pesticides are nevertheless detected even in areas far removed from agricultural landscapes[55].

Furthermore, together with the demonstrated landscape-scale distribution of CUP mixtures during the application time[13], our analysis demonstrates that this large-scale contamination is to be expected throughout the year. Additionally, the complexity of mixtures and the calculated risks are supposed to be even higher, as our analysis included only a fraction of the CUPs used in Europe (93 of the most commonly used out of 281). In the context of the observed insect declines, it is crucial to recognise, identify and reduce the risks posed by CUPs to insects. As mixture exposure is not addressed in the EU regulation, only reductions in pesticide risk, as included in national targets in the EU (e.g. Germany's National Action Plan aiming to minimize the risk associated with pesticide use[75], or France's commitment to reducing the use and risks of pesticides[76]) or globally by the UN until 2030[77], can change the currently observed declines. Together with conversion targets to reach organic practices in 25% of the agricultural area[78], where no synthetic pesticides are used, the required transformation of agriculture is started and exposure of organisms reduced, allowing the recovery of ecosystem services for a truly sustainable agriculture.

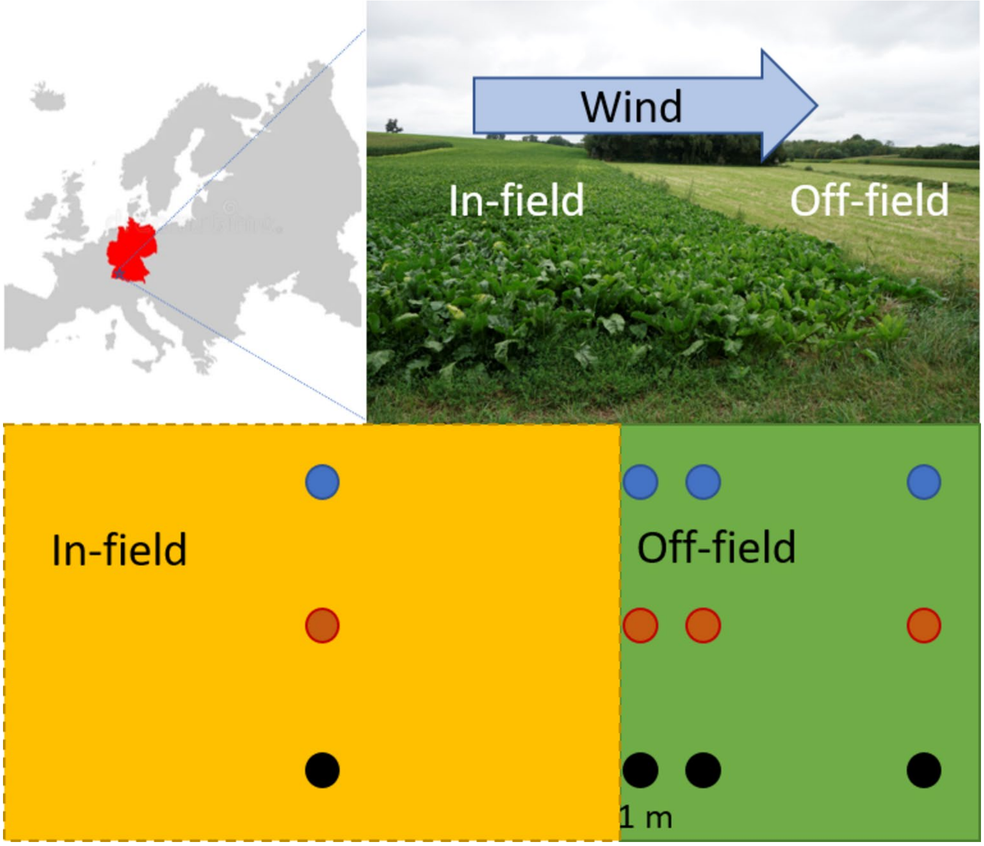

Fig. 3. Study area and layout of the field and meadow: The study was conducted in the southern Rhineland-Palatinate (blue star) in Germany between Bad Bergzabern (district Südliche Weinstraße) and Schwegenheim (district Germersheim). On each of the nine sites, a field and an adjacent meadow were studied. Soil and vegetation samples were taken 20 m from the field border in the cultivated area (field, yellow square) and at three distances (1, 5 and 20 m from the field border into the meadow) in the meadow (green square). For each sample three subsamples (blue, red and black dots) were taken with a 5-meter distance between them, leading to four samples per month and sample site. Samples were taken monthly from February 2021 to February 2022.

Materials and methods

Study sites

This study was conducted on cultivated areas and meadows downwind of the main wind direction (SW) in southern Rhineland-Palatinate in Germany (Fig. 3). Sampling occurred monthly between February 2021 and February 2022 (see Supplementary Table S15), to cover a whole year, including the most important application periods, but also the seasons with less intensive pesticide applications. The study included nine fields with each having an adjacent meadow. Conducting fieldwork across a wider range of sites was not feasible because sampling required visits every four weeks, involving considerable travel time between locations. Ensuring consistent, high-quality sampling within the available resources was prioritized over increasing the number of fields. Furthermore, the process of recruiting participating farmers was challenging, as only a limited number were willing to contribute to the study. Requesting detailed pesticide application records on top of this could have further discouraged participation. Consequently, the study focused on assessing broader residue patterns rather than site-specific application regimes, balancing practical limitations with research objectives. Our farms were also not part of a model farm network and therefore their applications reflect a common and realistic conventional pesticide management.

We chose three fields for each management system (arable farming, vegetable cultivation, vineyards). We investigated three fields for the arable managing system representing crops typical of the Rhenish crop rotation system[79]: one field with a cereal crop (winter wheat) and two with leafy crops (maize, sugar beet). This selection reflects the sequences in the cropping system. While the replication at the field level was limited, the fields were chosen to represent different cropping practices commonly observed in the region. The vegetable fields followed a succession planting pattern (e.g., fennel followed by celery, then radish, Supplementary Table 16). Although multiple fields of the same crop per management system, such as wheat, would have been preferable to reduce variability, the selection was constrained by the fields that were available for inclusion in the study. Given the limited number of fields accessible, which included a mix of crops for arable farming and vegetable cultivation, the study aimed to reflect the diversity of agricultural practices encountered in real-world conditions and to capture variations in agricultural practices and pesticide application methods. Arable farming typically involves long-term crops that remain in the field for extended periods, with pesticides applied in a two-dimensional (2D) spraying. In contrast, vegetable cultivation is characterised by quicker crop rotations, also involving 2D pesticide applications. Vineyards, on the other hand, involve a permanent crop, with pesticide applications occurring in three dimensions (3D).

We included an adjacent meadow to each field as a non-target area for comparison. All the farms followed conventional management practices involving the use of synthetic pesticides. Pesticides were applied to the fields according to good agricultural practices based on the specific crop. Six of the nine meadows were used for long-term hay production, two others were privately owned or municipal long-term green spaces and one was a nature conservation area. All meadows were only mowed once or twice during the year and consisted mainly of grasses. The sampling was conducted with the explicit permission of the landowners. All landowners were informed, that the information they provided is used for publication. In order to ensure that landowners remain anonymous, no further details of the exact locations are given.

Sampling and sample preparation

Sampling was carried out at each of the nine sites, at four sampling points each (20 m in the cultivated field (in-field, target area), 1 m, 5 m and 20 m from the field edge into the meadow (off-field, non-target area), Fig. 3). Samples were taken at all sites ($n = 9$) and distances ($n = 4$) each month ($n = 13$). At all distances and months, it was possible to take soil samples, resulting in 468 topsoil samples, while not at all in-field samples vegetation was growing at all months, resulting in a total of 442 vegetation samples. For each sample, a composite sample was obtained from three subsamples collected at a distance of 5 m from each other (Fig. 3) to get representative samples.

For the soil samples, after removing the vegetation and root layer, 0–5 cm topsoil was taken with a flower onion planter tool (Gardena, Ø 12.5 cm) at three locations per distance and stored in a sample bag combining the three subsamples. The topsoil samples were weighed in the field, transported to the laboratory and stored in a freezer (−20 °C). The samples were then freeze-dried for 48 h (Alpha 1–4 LSCbasic, Christ, Osterode, Germany), sieved (2 mm, standard-compliant test sieve, ISO 3310-1, Haver & Boecker OHG, Oelde, Germany) and homogenised. For the extraction of CUPs from topsoils, 5.00 g ± 0.01 g of soil material was weighed (ME802, Mettler Toledo, Ohio, USA; d = 0.01 g).

For the vegetation samples, cultivated plants and “weeds” (if available) were collected in the field. For the off-field samples, differing grasses and herbaceous plants were collected in the meadow. The samples were hand collected at three locations (see above) using laboratory gloves, filling half of a 1 L freezer bag with the three subsamples. The above-ground vegetation (approximately upper 20 centimetres) was taken as vegetation samples. The combined vegetation samples were first air-dried overnight in the laboratory at room temperature, frozen (−20 °C) and subsequently freeze-dried for 24–48 h, depending on the moisture of the sample. The vegetation samples were ground in a knife mill (Grindomix GM 300, Retsch, Haan, Germany) for 2 × 1 min at 3000 rpm to avoid heating. The ground vegetation was stored in small freezer bags at −20 °C until extraction. For extraction, 1 g ± 0.01 g of vegetation powder was weighed into 50 ml tubes. The reason for using less matrix for vegetation samples compared to soil samples is primarily due to the availability and volume of material collected during the sampling process. Nonetheless, the precision is the same with also a low error margin for vegetation samples. The relative error of about 1% for vegetation would not significantly influence the CUP concentrations and can be neglected.

All tools used in the field were cleaned with ethanol (≥ 98%, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and tap water e.g. the onion planter tool and in the lab with acetone (≥ 99.9%, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and tap water. All other materials e.g. sieves were also cleaned with acetone and tap water.

Analytics for CUP residues

Validated multicomponent analytical methods[80] were used to extract 93 CUPs (36 fungicides, 36 herbicides, 21 insecticides) from soil and vegetation. Details on the CUP selection can be found in[80], the most frequently-used herbicides and fungicides as well as all insecticides from 2016 to 2017 recorded by the Julius-Kühn Institute were selected[81]. By 2021, three fungicides, five herbicides and eight insecticides had been withdrawn from the market, in total 77 of the 93 substances were approved in 2021. In brief, 5.00 g of soil or 2.00 g of vegetation was extracted with 10 ml of acetonitrile (MeCN, HPLC gradient grade ≥ 99.9%, Honeywell, Charlotte, USA) containing 2.5% formic acid (CH_2O_2, HiPerSolv Chromanorm ≥ 99.0%, VWR, Radnor, USA) and either 5.00 g–2.00 g of salt (ammonium formate, NH_4HCO_2, reagent grade ≥ 99.0%, Sigma-Aldrich, St. Louis, USA) for soil and vegetation, respectively. The samples were placed in an overhead shaker for 1 h (Stuart drive Rotator drive STR4, Cole-Parmer, Vernon Hills, USA); afterwards, the samples were centrifuged (MegaStar 1.6R, VWR, Radnor, USA, 6 min at 3000 rpm) and filtered (17 mm HPLC syringe filter, PTFE, pore size 0.2 µm; La-Pha-Pack, Langerwehe, Germany). The vegetation extracts were further cleaned with graphitised carbon black (GCB, Carbon SPE Bulk Sorbent, Agilent Technologies, Santa Clara, USA). For the clean-up, 1 ml of vegetation extract was added to 7.5 mg of GCB in a 2 ml Eppendorf tube. The tubes were vortexed vigorously for 60 s and the supernatant was filtered through 2 µm filters directly into the measurement vials.

The extracts were analysed by high-performance liquid chromatography-tandem to triple quadrupole mass spectrometry via electrospray ionisation (HPLC)-ESI-MS/MS (HPLC: Agilent Technologies LC 1260 Infinity II series, MS/MS: Agilent Technologies 6495 C, Santa Clara CA, USA). The instrumental performance and potential carry-over effects were controlled by injecting quality control and analytical blank samples, respectively. For quality control, all the samples were spiked with 50 µL of the deuterated standard imidacloprid-D4 (98.9%, Dr. Ehrenstorfer GmbH, Augsburg, Germany) in acetonitrile (MeCN, HPLC gradient grade ≥ 99.9%, Honeywell, Charlotte, USA) (concentration: 10 mg/L). The solvent was allowed to evaporate for 30 min under a fume hood. A blank sample was spiked with imidacloprid-D4 after extraction (concentration: 10 mg/L). The peak areas of imidacloprid-D4 in the samples and of the respective blank samples were compared to check for extraction quality.

HPLC-ESI-MS/MS data were processed with Agilent Mass Hunter Quantitative Analysis software (Version 10.0., Build 10.0.707.0, Agilent Technologies, 2006–2018, Santa Clara CA, USA). CUP residues were considered positive if the MRM-transition products were present and if the retention time, response and quantifier/qualifier ratio corresponded to the standard sample at a comparable concentration (range: 70–130%). The quantification of CUP concentrations in all samples was performed using external matrix-matched calibrations. Detailed information on the limit of quantification (LOQ) and limit of detection (LOD) is available at[80]. For the total number of detected CUPs in the samples, residues < LOQ were also included. However, for the calculation of total CUP concentrations, only residues > LOQ were used.

Risk assessment

We used the measured topsoil CUP concentrations to calculate a conservative cumulative chronic risk for earthworms and collembola, as well as a hazard quotient for wild bees. For these calculations, only soil contact exposure was considered, as no other exposure routes were included. To date, there are no recommendations for approaches that include potential synergistic effects[5], so additive approaches were chosen. For HQ calculations, we used an assessment factor of 10 for comparability with other recent studies[5,8,12,82]. The Hazard Quotient (HQ) approach was used to assess the risk of ground-nesting solitary bees[36]. There is limited availability of toxicological endpoints for insects and approved risk assessment approaches. We chose the proposed approach to determine the risk posed to ground-nesting insects from soil based on the concentrations detected. The honey bee lethal effect endpoints (median lethal dose, LD_{50}) were used because honey bees are the current regulatory standard and are considered to be an appropriate proxy for all bee species[83]. We used the contact acute LD_{50} values reported in the "Pesticides Properties DataBase" (PPDB[84]). A surrogate solitary bee contact LD_{50} (honey bee $LD_{50}/10$) was also used. The matrix of a ground-nesting bee (*Peponapis pruinosa*) was exposed as described previously[36]. This wild bee does not occur in Europa. However, other wild bees such as *Andrena flavipes*, *Halictus scabiosae* or *Systropha planidens* also nest in soil, are similar in size and occur in or near agricultural areas in Europe[85,86]. We estimate a similar exposure to *Andrena flavipes* whose nests are similar to *Peponapos pruinosa* which is common in Europe and was detected in European agriculture fields[62].

It was estimated that female *Peponapis pruinosa* bees have contact with 2.23 g of soil during their burrowing activity for nest construction during one day[36]. Only soil contact exposure was addressed in the calculation.

The HQ was then calculated for each CUP residue in each topsoil sample as follows:

$$HQ = \frac{(Matrix\,exposure\ [g])\left(MEC\ \left[\frac{ng}{g}\right]\right)}{\frac{honey\,bee\,LD_{50}}{10}\left[\mu\frac{g}{bee}\right]}) = \frac{(2.23\,g)\left(MEC\ \left[\frac{ng}{g}\right]\right)}{\frac{honey\,beeLD_{50}}{10}\left[\mu\frac{g}{bee}\right]}) \tag{1}$$

Individual HQ values from one topsoil sample were further summed to obtain the mixture hazard quotient (MHQ, Eq. 2) in the topsoil sample.

$$MHQ = \sum HQ \tag{2}$$

An HQ > 1 indicates a potential lethal hazard occurring from the CUP residues. The concentration of residues was given in µg of active ingredient (a.i.) per kg of soil matrix, which equals ng (a.i.)/g (matrix); the amount of exposure to the matrix was reported in g of matrix per bee and the LD50 values were reported in ng a.i. per bee.

Additionally, the risk posed by cumulative concentrations of CUPs for earthworms was calculated following the methodology proposed by[12] and applied in recent studies[5,8,12,82,87]. The chronic no-observed-effect concentration (NOEC) for earthworms (*Eisenia fetida*) and collembola (*Folsomia candida*) from the PPDB were utilised. If a no-observed effect concentration (NOEC) was not available, reports to the European Commission and reports of the European Food and Safety Authority were used. If no NOEC was available, then the lethal concentration where 50% are killed (LC50) was used. The toxicity assessment for earthworms was based on 55 NOECs and 9 LC50 values, while for collembola, all were based on the NOEC ($n = 55$; see Supplementary Material Table S18). The NOEC was divided by an assessment factor of 10[5,8], or the LC50 by an assessment factor of 1000[5], to compute the predicted no-effect concentration (PNEC)[88]. The risk quotient (RQ) was then individually calculated for each CUP as the difference between the PNEC and the measured environmental concentration (MEC) in each soil sample (Eq. 3, SI, Eq. (1) to (7)).

$$RQ = MEC/PNEC = MEC/(NOEC/AF) \tag{3}$$

Subsequently, the mixture exposure risk (MRQ, Eq. 4) was calculated for each topsoil sample.

$$MRQ = \sum RQ \tag{4}$$

The MRQs were further categorised into four risk groups: negligible risk, MRQ < 0.01; low risk, 0.01 < MRQ < 0.1; medium risk, 0.1 < MRQ < 1; high risk, MRQ = 1, very high risk ≥ 1[89]. The same procedure was used for the data from the springtail *Folsomia candida*.

Statistical analysis

For the statistical analysis, Rx64 4.0.1 was used[90]. For samples with values below the LOQ, the concentration was set to 0 for statistical analyses. To avoid multicollinearity issues, principal component analyses (PCAs) (package: stats, function: princomp[90]) were constructed. To identify local CUP minima and maxima during the year, a linear model was built to subtract the trend for the number of CUPs of the year from the number of shorter-term fluctuations; furthermore, a peak detection algorithm was used[91,92]. This enables us to compare CUP residues and insect development stages. The algorithm used a threshold delta value to determine when a peak (maximum or minimum) was reached. The algorithm iterates through a given vector, updating the maximum or minimum candidate based on the current value and the threshold. If a current value deviated by more than a delta from a previous extremum, a new extremum was detected. When a peak was identified, its position and value were recorded. To increase the overall detection rate, the delta was set to 0.3. A delta of 0.3 was considered appropriate as it allows for the detection of a broader range of relevant signals without significantly compromising the reliability of the results. We acknowledge that it is somewhat subjective. In general, delta values are often set based on the dataset, to increase the overall peak detection rate (e.g. in[93]). The peak finder is only used to standardize the interpretation of the peaks and does not influence the measured and fitted peaks themselves. We constructed a generalised linear model (GLM) to see, whether the predictors have a significant influence on the number of CUPs (see SI GLM section). For the analyses of the courses over the year, the following approach was used, which has recently been applied to quantify differences in time series patterns across ecological systems[45]: A loess smooth was used to obtain a coherent progression over the year and distance to the field margin[49]. The loess smoothing was further cross-validated, for further details on the loess smoothing, see the SI Loess smoothing section. Individual loess smooths were compared with the Kullback-Leibler (KL) divergence. KL-divergence is used to measure differences in distributions or patterns[45,94]. It can be considered as a measurement of information[95].

The KL divergence was calculated as follows:

$$KL\left(P\|Q\right)=\sum P\left(P\right)*\log2\left(P\left(P\right)/P\left(Q\right)\right)=H\left(P,Q\right)-H\left(P\right) \tag{5}$$

where $H(P,Q)$ denotes the joint entropy of the probability distributions P and Q and $H\left(P\right)$ denotes the entropy of probability distribution P[96]. KL divergence, while using direct yearly progressions, allows a comparison of curve patterns (for the SI, see the KL divergence section).

The distance to the field margin was fitted with an exponential function with nlsLM from the minpack.lm package[35]. It was assumed that the measurements in cultivated areas (−20 m) also represented the concentration at the field margin (0 m). This assumption was made because the −20 m measurement is considered representative of the general in-field concentration. Measurements at the field border (0 m) would likely not be fully representative, as the field edges are often subject to lower pesticide rates due to drift reduction techniques, which aim to minimize off-target drift. Rather than interpreting the 0 m measurement as the CUPs at the field edge, it is more accurate to consider it as indicative of the in-field measurement.

The exponential function was set up as:

$$y=a*e^{(b*x)}+c \tag{6}$$

where a is the amplitude, b is the base, c is the constant term, y is the response variable and x is the explanatory variable. In this study, the value a served as a vertical scaling factor, describing the amount of additional CUP detected at the margin in comparison to higher distances. The value b indicates the decay speed (if negative). Lower b values represent faster decay. The mean of the respective subset of the − 20 m distance was used for c, describing the background concentration that the function should have reached.

Data availability

Data is provided within the manuscript, the supplementary information and under https://doi.org/10.5281/zenodo.11517560.

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Author contributions

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Declarations

Competing interests

The authors declare no competing interests.

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12 APPENDIX III: PAPER 3

Current-use pesticide exposure of riparian ecosystems – stream buffer or non-target habitat?

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Current-use pesticide exposure of riparian ecosystems – stream buffer or non-target habitat?

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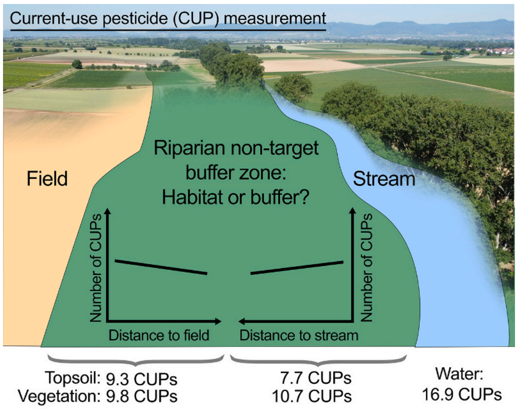
HIGHLIGHTS

- Riparian buffers show pervasive CUP residues across matrices.
- Wider buffers cut CUP numbers in topsoil, not in vegetation
- Viticulture drives higher upstream CUP loads in riparian zones.
- Soil and vegetation CUP mixtures differ from stream water compositions.

GRAPHICAL ABSTRACT



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Keywords:
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ABSTRACT

Riparian non-target buffer zones (RNBZ) are widely implemented to reduce pesticide pollution of surface waters, yet their dual role as protective barriers and semi-natural habitats remains contested, particularly where management targets mitigation rather than conservation. We quantified gradients of pesticide contamination from fields across RNBZ to small streams by sampling vegetation, topsoil, and water at 24 sites spanning RNBZ widths of 10–260 m. At each site we collected samples 1, 2, and 5 m into the RNBZ from both field and stream sides. 93 current-use pesticides (CUPs) were analysed, and residues were detected in all matrices and at every site, demonstrating widespread contamination across riparian zones. On average, topsoil and vegetation contained complex mixtures, with 9.3 and 9.8 CUPs on the field side and 7.7 and 10.7 CUPs on the stream side. Greater RNBZ width significantly reduced CUP numbers in topsoil but not in vegetation. In wine-growing catchments, upstream RNBZ topsoil and vegetation showed higher CUP contamination than downstream, indicating viticulture as a key driver. Contamination increased toward the stream within RNBZ topsoil and vegetation, consistent with accumulation or entry via flooding or infiltration. Jaccard dissimilarities indicated distinct CUP mixture patterns between stream water and riparian soil and vegetation. These findings highlight that RNBZ width alone cannot explain pesticide dynamics and that riparian contamination may reflect a widespread, landscape-scale phenomenon rather than a local anomaly. Effective mitigation should integrate vegetation

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structure, land-use context, and contamination pathways to strengthen both the protective and habitat roles of riparian zones.

1. Introduction

Riparian zones are defined as transition zones between terrestrial and aquatic environments, typically along rivers and streams. These transition zones facilitate water-land interactions (Schulz et al., 2015), support high biodiversity and provide essential ecosystem functions such as water quality regulation, erosion control, carbon sequestration, and habitat provisioning (Prado et al., 2022). Riparian zones also support a high diversity of specialized terrestrial organisms including ants, spiders, and birds (Larsen et al., 2010; Ramey and Richardson, 2017). Maintaining intact riparian zones is central to riverscape conservation, and recent syntheses advocate variable width, multi-tier buffers tailored to geomorphology and the requirements of riparian biota rather than uniform strips (Graziano et al., 2022). At the watershed scale, such buffers restore lateral, longitudinal and vertical connectivity, facilitate species movements, and strengthen resilience to climate change and other stressors (Graziano et al., 2022). Therefore, the EU Biodiversity Strategy for 2030 addresses riparian zones in a specific target to restore at least 25,000 km of free-flowing rivers by removing barriers and restoring floodplains and wetlands (European Union, 2020).

Buffer zones, vegetated buffer strips, riparian buffers, forested buffers, filter strips, or ecological buffers are among the many names which describe a space designed to separate an anthropogenic source of contamination, i.e., agricultural fields, from ecologically important non-target ecosystems such as freshwater streams (Prosser et al., 2020). Importantly, in the context of pollution and stream management, riparian ecosystems represent the buffer. While the Common Agricultural Policy (2023–2027) requires a minimum riparian buffer width of 3 m along watercourses, no uniform EU-wide standard for broader buffer widths exists, and decisions regarding wider riparian strips are delegated to individual Member States through their national CAP Strategic Plans (European Commission, 2022). In Germany, pesticide applications are generally prohibited within 10 m of surface waters, or 5 m if a vegetated buffer strip is in place, (Bundesministerium der Justiz und Verbraucherschutz, 2025). This legal buffer does not necessarily correspond to an ecologically functional zone and does not specify mandatory structural requirements.

Current-Use Pesticides (CUPs) are a source of surface water pollution that are frequently applied in modern agriculture. The negative consequences of realistic CUP exposure are still insufficiently understood and regulated (Brühl and Zaller, 2019). Recent findings suggest that, e.g., insect populations are highly vulnerable to CUP contamination, with documented cases of behavioural and reproductive impairments caused by pesticide mixture exposure at low concentrations in the range of our detections (Gandara et al., 2024). Furthermore, nonlinear interactions among many combinations of widely prevalent pesticides indicate the presence of widespread synergistic effects (Gandara et al., 2024). CUPs reach riparian zones via drift, overspray, run-off events, soil infiltration and atmospheric transport (Reichenberger et al., 2007), contributing to widespread surface-water contamination across Europe (Stehle and Schulz, 2015). Nonetheless, erosion rills connecting agricultural fields with surface waters can undermine the protective function of buffer zones (Stehle et al., 2016), illustrating that their effectiveness is not solely determined by width but also by factors such as the source-area-to-buffer ratio, run-off intensity, soil texture, and vegetation structure (Prosser et al., 2020).

In addition, buffer zones can effectively reduce pesticide inputs into streams only if the contamination originates directly from adjacent agricultural fields. However, the pesticides in the buffer strips may stem from three conceptually different pathways: first, directly from adjacent fields; second, from fields further upstream that were transported in the stream and were deposited on the bank during flooding events; or third, from non-adjacent fields on a much larger geographical scale via atmospheric transport resulting in wet and dry deposition. A study in the same area revealed that crop type composition up to 4 km away may influence the pesticide load at terrestrial non-target sites (Mauser et al., 2025).

Given the highly context-dependent effectiveness of riparian non-target buffer zones (RNBZ) and the urgent need to safeguard aquatic–terrestrial systems in agricultural landscapes, RNBZs are increasingly recognized as multifunctional elements. They function not only as pollution barriers that reduce pesticide inputs to adjacent streams, but also as valuable terrestrial habitats that sustain high levels of biodiversity. We assessed CUP exposure in an intensively used agricultural landscape in Southwestern Germany. This region reflects structural land-use patterns typical of intensively cultivated temperate landscapes across Europe, providing insights that may be relevant beyond the local scale. During the spraying season, we sampled the RNBZ along three streams between the nearest agricultural field and the stream. At a total of 24 RNBZs, crop types in the adjacent field varied from viticulture to cereals to vegetables, with RNBZ widths ranging from 10 to 260 m (Supplementary Table 2). At each site, we systematically collected topsoil and vegetation samples both from the agricultural field side and from the stream side while also collecting a water sample from the stream.

We hypothesized that (H1) most CUPs originate from adjacent agricultural fields and decrease in number and concentration with every meter into the RNBZ. Similarly, (H2) CUPs may enter from the stream and decrease in number and concentration into the RNBZ. Furthermore, we anticipated that (H3) riparian CUP contamination is not solely determined by RNBZ width but also influenced by surrounding crop types, particularly by viticulture with intensive 3D application techniques. We further expected that downstream sites exhibit higher CUP contamination and mixture complexity.

2. Methods

2.1. Study area

The study area was located in the Southern Palatinate in Southwest Germany, a region with extensive agricultural land use and a distinct separation between the lowland Upper Rhine valley and the low mountain range of the Palatinate Forest. Although agriculture is the dominant land use in the valley, with vegetables and arable crops primarily cultivated in the lowlands and viticulture concentrated on the slopes of the low mountain range, the landscape also includes remnants of riparian woodland and smaller forest patches along the lowland tributaries of the Upper Rhine.

2.2. Study streams

We sampled RNBZs along the streams Speyerbach (49°21′01.0″N 8°11′02.8″E), Queich (49°12′27.7″N 8°03′43.9″E) and Otterbach (49°04′09.0″N 7°59′21.4″E), which are tributary streams of the Rhine River and originate within the Palatinate Forest. The three streams pass through the foothills of the sparsely cultivated Palatinate Forest and then flow through the same agriculturally intense landscape (upstream viticulture and further downstream mixed crops, including vegetables), enabling good comparability. Sections of the streams partly flood the adjacent riparian zone, although the stream banks are generally steep, and only the lower parts are flooded.

The three study streams are designated as heavily modified water bodies (Landesamt für Umwelt Rheinland-Pfalz, 2024). Ecological status is rated as poor in Speyerbach and moderate in both Queich and Otterbach. The chemical status, based on measurements of selected priority substances (including pesticides, metals and metalloids, industrial chemicals and aromatic compounds, and persistent organic pollutants), is rated “not good” in Speyerbach and Queich, whereas Otterbach meets “good”. Biological monitoring (macroinvertebrates, diatoms, macrophytes, and fish) further indicates agricultural pollution stress in Speyerbach and Queich but not Otterbach.

2.3. *Study sites and sampling scheme*

Between June 15th and 29th’ 2024, we sampled 24 sites along the three streams (Fig. 1). The sampling sites were selected starting from the foot of the low mountain range, following up to lowlands (for site locations and crop type refer to Fig. 6). Sites covered different RNBZ width and surrounding land-use conditions (Supplementary Table 2). We assessed pesticide contamination in water and along seven positions for topsoil and vegetation along a transect perpendicular to the stream. The RNBZs typically consisted of grasslands, shrubs or larger riparian trees between the nearest field and the stream.

At each site, a single water sample (S0) was collected directly from the stream to determine the presence of aquatic CUP contamination as a source of contamination. Topsoil and vegetation samples were taken within the RNBZ at three distances from the stream: 1 m (S1), 2 m (S2), and 5 m (S5). Additionally, topsoil and vegetation samples were collected from adjacent agricultural fields, with a CUP source reference sample inside the field (F0) and additional samples at 1 m (F1), 2 m (F2), and 5 m (F5) into the RNBZ (Fig. 1).

This paired sampling design allowed us to assess CUP contamination gradients extending into the RNBZ from two potential sources: the field and the stream. Sites with a minimum RNBZ width of 10 m were selected with the RNBZ on the opposite stream side being at least equally wide, ensuring a reliable spatial linkage to the adjacent agricultural field. At two sites where the RNBZ width was exactly 10 m, the centre sample was assigned to the stream side for descriptive contamination assessments (S5, then F5 missing). For statistical analysis of the gradient from both directions, the centre sample was taken for both fits (S5 and F5).

2.4. *Sampling*

Composite topsoil samples consisting of five subsamples were taken at each RNBZ position (F1, F2, F5, S5, S2, S1) along a transect of 4 m with a distance of one meter between subsamples with a bulb planter (diameter: 12.5 cm, Gardena GmbH, Ulm, Germany) at a depth of 0–5 cm. Similarly, vegetation representative of the sampling site was handpicked along the same transect and placed in a 1 L plastic bag. The vegetation samples included a mixture of different parts (excluding roots) of grasses and herbaceous plants. One topsoil field source reference sample (F0) was taken inside the field similarly at a depth of 0–5 cm in addition to a source vegetation sample including a mixture of different parts (excluding roots) of crops and weeds present inside the field. The present in-field crop types were categorized into “Cereals” (n = 14), “Viticulture” (n = 4), “Vegetables” (n = 4) and “Other” (n = 2, rapeseed or just recently planted). From the stream, a 15-ml water sample was taken as a source (S0). Samples were transported to the laboratory and stored in a freezer at −20 °C. All samples were then further processed in the laboratory.

2.5. *Pesticide analysis*

Vegetation and topsoil samples were analysed for 93 CUPs using high-performance liquid chromatography coupled with an electrospray ionization tandem mass spectrometry system (HPLC-ESI-MS/MS; HPLC: Agilent Technologies LC 1260 Infinity II series, MS/MS: Agilent Technologies 6495C, Santa Clara CA, USA) (method: Bakanov et al., 2023). Water samples were analysed for 74 CUPs by direct-injection HPLC–ESI–MS/MS (method: Roodt et al., 2023). The difference in analyte number reflects matrix-specific method validation, with 93 CUPs validated for solid matrices and 74 for aqueous samples. All CUPs analysed in water were also included in the broader set analysed in solid matrices. Comprehensive details on the extraction protocols, analytical methods, performance and quality control (e.g., recoveries, blanks, and instrument stability) are provided in the Supplementary Information. CUP sum concentrations were determined for each sample as the total of all individual CUP compounds quantified above the limit of quantification (LOQ) in vegetation, topsoil, and water respectively. The specific LOQs and limits of detection (LODs), including their calculation procedures, are provided in Supplementary Table 1.

2.6. *Data analysis*

To test H1, which predicts that pesticide contamination declines with increasing distance from adjacent agricultural fields, and H2, which assumes a similar decline from the stream margin into the RNBZ, we fitted generalized linear models (stats package core, version 4.3.2, R

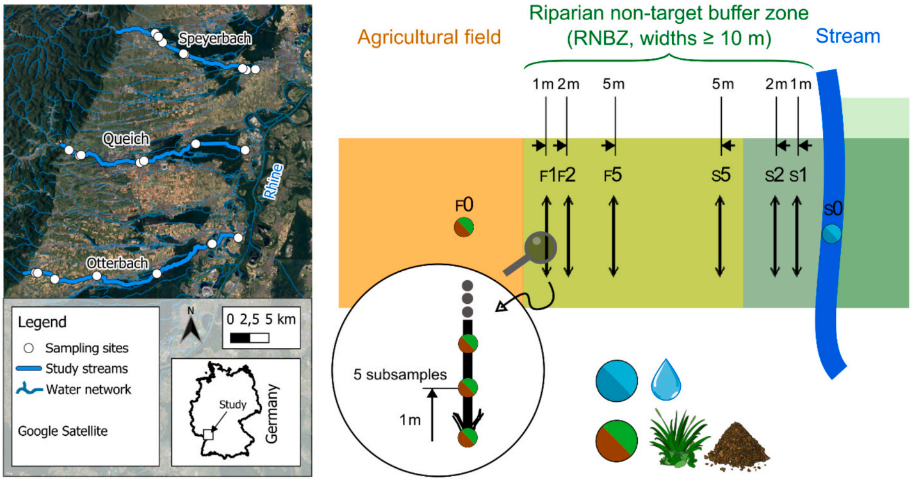

Fig. 1. Study map (left) and sampling design (right) at each of the 24 sampling sites. Topsoil (0–5 cm depth) and vegetation samples were taken within the RNBZ at three distances from the stream: 1 m (S1), 2 m (S2), and 5 m (S5). Additionally, topsoil and vegetation samples were taken from the adjacent agricultural field, with one source reference sample collected inside the field (F0) and additional samples (out of 5 subsamples) at 1 m (F1), 2 m (F2), and 5 m (F5) into the RNBZ. A water sample was also collected as a source reference (S0). Aerial images from Google & TerraMetrics, (2025). Details of crop distribution can be found in Fig. 6.

Core Team, 2023) for each of the 24 sampling sites and each side (F and S). Separate models were calculated for the response variables number of CUPs and CUP sum concentrations using Distance from the field [m] as the explanatory variable. The estimate, i.e. slope of each of the sites was then used for further comparisons.

To test H3, which states that pesticide contamination in RNBZs is not solely determined by buffer width but also influenced by the surrounding crop type, we used generalized linear models on the subset of topsoil and vegetation samples (F0–F5, S5–S1). The explanatory variables used were RNBZ width [m] and Distance downstream [km] as a proxy for crop type, including their interaction.

To further examine our spatial assumption that downstream accumulation of pesticides results in more complex contamination in lower stream sections, we applied generalized linear models to the water data. The response variables were again the number of CUPs and CUP sum concentrations, with Distance downstream [km] and Stream (Speyerbach, Queich, Otterbach) as explanatory variables, including their interaction.

For all generalized linear models, the number of CUPs was used with a Poisson family and identity link. The CUP sum concentrations were natural-log-transformed (with a pseudoconstant of +0.01) and used with a Gaussian family and identity link. Significance was tested with a *t*-test for Gaussian family models and the Wald test for Poisson family models (base package core, version 4.3.2, R Core Team, 2023). The normal distribution of the residuals was visually checked for the Gaussian models via Q–Q plots. The homoscedasticity and linearity were checked using residuals vs fitted plots where no major irregularities were observed (stats package core, version 4.3.2, R Core Team, 2023). To investigate the compositional similarity of CUP communities (binary presence–absence patterns of CUPs) between our different matrices (topsoil, vegetation, water), pairwise Jaccard dissimilarity indices were calculated for each sampling location with the vegdist function (version 2.6-4, Oksanen et al., 2022). CUP detections above the limit of detection (>LOD) were considered as present.

3. Results

3.1. CUP detections

We detected 65 of 93 analysed CUPs, consisting of 30 fungicides (F), 23 herbicides (H) and 12 insecticides (I) from a total of 356 samples (24 sites with 7 positions (including in-field) with two overlapping (see above) and 24 water samples). More specifically, 56 CUPs (25 F, 20 H and 11 I) were detected in the topsoil samples, 47 CUPs (25 F, 14 H, 8 I) were detected in the vegetation samples, and 34 CUPs (17 F, 9 H, 8 I) were detected in the water samples. The most frequently detected CUPs in topsoil in our study were the fungicides fluopyram (100% of all topsoil samples), boscalid (71.7%), spiroxamine (66.9%), azoxystrobin (57.2%) and fluopicolide (54.8%). Diflufenican was the most frequently detected herbicide in topsoil samples (37.4%). The neonicotinoid clothianidin was the most frequently detected insecticide (10.2%). The most frequently detected CUPs in vegetation were the fungicides fluopyram (99.4%), mandipropamid (90.4%), spiroxamine (86.75%) and cyflufenamid (72.9%) as well as the herbicide terbuthylazine (92.1%). The systemic insecticide chlorantraniliprole was most frequently detected in our vegetation samples (6.0%). The most frequently detected CUPs from the water samples were the fungicides fluopyram (100%), azoxystrobin (100%), metalaxyl (87.5%) and dimetomorph (83.3%) as well as the herbicide napropamide (91.7%). The most frequently detected insecticide was again chlorantraniliprole (58.3%). A mixture of 14 CUPs was recorded in at least 20% of all samples (topsoil, vegetation, and water). Among the 65 total CUPs, 27 CUPs (42%) were detected in all of our matrices, 15 CUPs (23%) were detected in at least two matrices, and 23 CUPs (35%) were detected in only one matrix (Supplementary Table 4).

When focusing on the RNBZ (284 samples: F1–F5 and S1–S5), we detected 54 different CUPs (24 F, 19 H and 11 I) in riparian topsoil and 40 CUPs (22 F, 12 H and 6 I) in riparian vegetation. We detected CUPs in all RNBZ topsoil and vegetation samples. The majority (84%) of all 2663 individual CUP concentrations in the RNBZ topsoil and vegetation were <5 μg/kg. We recorded a total of 258 mixtures with >2 components in the RNBZ (topsoil, vegetation). The most frequently detected mixtures contained the fungicides fluopyram + azoxystrobin and boscalid + fluopicolide + fluopyram + metrafenone + spiroxamine, although these two specific mixtures occurred only five and four times respectively. RNBZ topsoil contained a mean of 9.3 CUPs on the field side (min = 1 CUP, max = 21 CUPs, F1–F5) and 7.6 CUPs on the stream side (min = 1 CUP, max = 26 CUPs, S1–S5). The RNBZ vegetation contained a mean of 9.8 CUPs on the field side (min = 3 CUPs, max = 17 CUPs) and 10.7 CUPs on the stream side (min = 2 CUPs, max = 16 CUPs). Regarding detected concentrations, the RNBZ topsoil contained a mean CUP sum concentration of 43.9 μg/kg on the field side (min = 0.1 μg/kg, max = 438.9 μg/kg) and 6.3 μg/kg on the stream side (min = 0.2 μg/kg, max = 112.4 μg/kg). The RNBZ vegetation contained a mean CUP sum concentration of 272.4 μg/kg on the field side (min = 1.7 μg/kg, max = 3219.0 μg/kg) and 34.0 μg/kg on the stream side (min = 0.7 μg/kg, max = 132.8 μg/kg). In RNBZ topsoil, the highest individual maximum concentrations (>100 μg/kg) were found for difenoconazole ((F), 226.3 μg/kg; F1 sample), boscalid ((F), 187.3 μg/kg; F1), azoxystrobin ((F), 175.8 μg/kg; F1) and dimethenamid-P ((H), 111.5 μg/kg; F1). In RNBZ vegetation, the highest individual maximum concentrations (>1000 μg/kg) were found for cyazofamid ((F), 1254.4 μg/kg, F5), dimethenamid-P ((H), 1101.5 μg/kg, F1), metalaxyl ((F), 1029.3 μg/kg, F5) and azoxystrobin ((F), 1021.2 μg/kg, F1).

3.2. Contamination from field and stream sources

The in-field source (F0) topsoil contained a mean number of 11.6 CUPs (min = 4, max = 20) and a mean sum concentration of 115.9 μg/kg (min = 0.4 μg/kg, max = 522.8 μg/kg). In-field vegetation contained a mean number of 10.8 CUPs (min = 3, max = 17) and a mean sum concentration of 2371.1 μg/kg (min = 1.4 μg/kg, max = 43,823.1 μg/kg). Source stream water (S0) contained a mean number of 16.9 CUPs (min = 2, max = 25) and a mean sum concentration of 0.8 μg/L (min <0.01 μg/L, max = 9.3 μg/L). Contamination decreased from both sources, the field and the stream, into the RNBZ (Fig. 2).

The decrease in log(sum) concentration in the topsoil (Fig. 3) was steeper on the field side (−0.43 ln(μg/kg)/m) compared to the stream side (−0.04 ln(μg/kg)/m). Similarly, the decrease in the number of CUPs was steeper on the field side (−0.67 CUPs/m) compared to the stream side (−0.33 CUPs/m). A similar pattern of decreasing CUPs from both sources was observed for vegetation, with a steeper decrease in log(sum) concentration on the field side (−0.21 ln(μg/kg)/m) compared to the stream side (−0.09 ln(μg/kg)/m). In contrast, the decrease in the number of CUPs was slightly smaller on the field side (−0.33 CUPs/m) compared to the stream side (−0.35 CUPs/m). Concentration trends of the individual commonly detected pesticides are presented in Supplementary Fig. 1.

3.3. Effect of RNBZ width and crop type

When vineyards were present at F0, the mean number of CUPs and sum concentration of topsoil and vegetation at the field side were greater compared to vegetables and cereals (Fig. 4). In addition, vineyard sites contained an overall higher number of CUPs in vegetation and sum concentration in topsoil and vegetation on the stream side compared to other crop types. Vineyard sites were the only sites where topsoil at the stream side showed a reverse trend, with a decreasing number of CUPs and sum concentration toward the stream.

For the number of CUPs in topsoil, RNBZ width had a significant negative effect (Est = −0.026 CUPs/m, z = −3.62, $p < 0.001$, Table 1), which remained negative both at the mountain foot (Distance = 0 km)

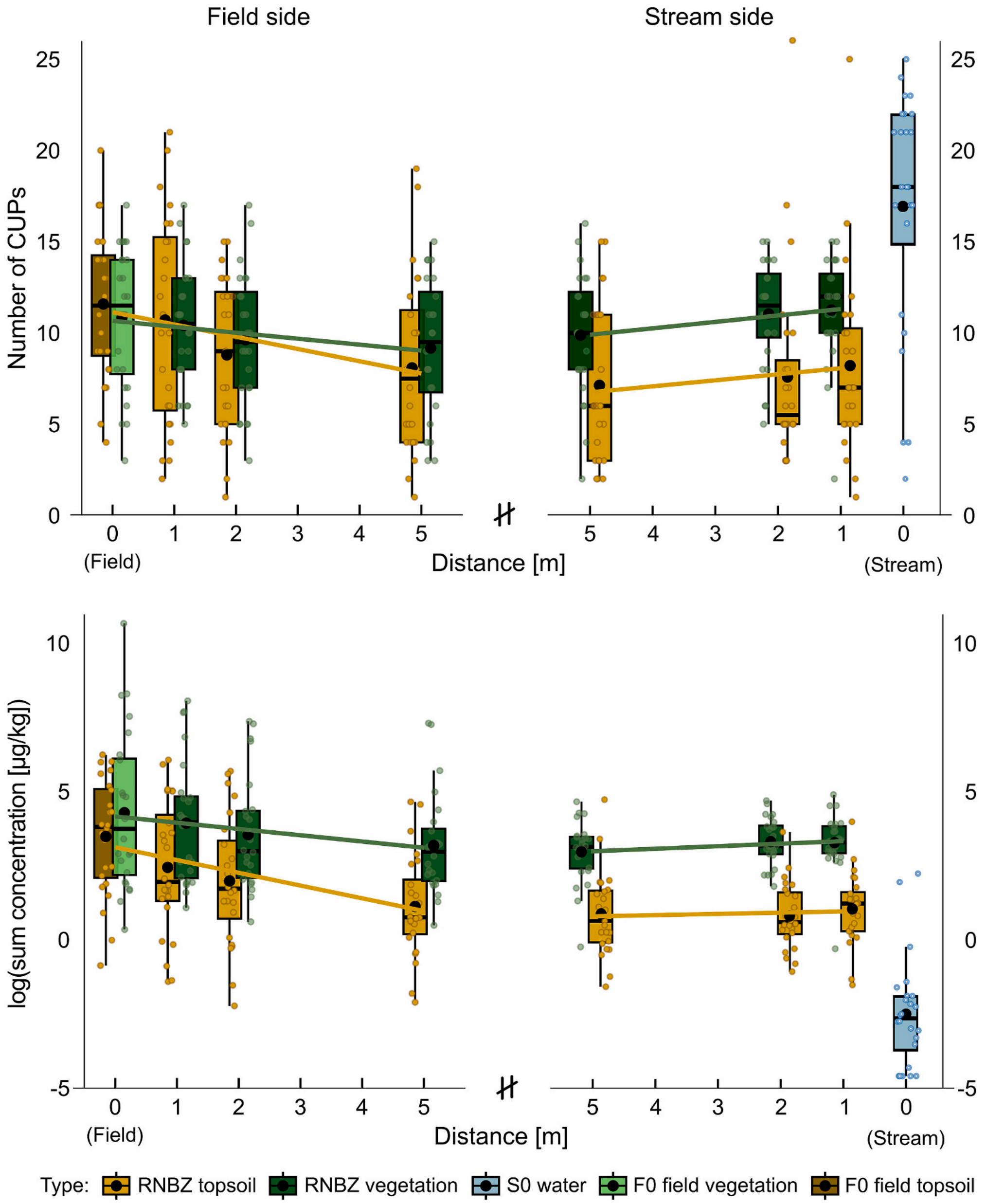

Fig. 2. Combined plot of CUP detections from the field side and the stream side. Each boxplot represents the average number of detections or their log(sum concentration) at a given sample point (from F0 to S0), with field 0 being taken in the field and stream 0 being taken in the stream. In yellow are topsoil samples in green are vegetation samples and in blue are water samples. Each bold yellow or green line represents the mean estimate over all site estimates (mean of all individual fits of all sample sites).

and the downstream locations. Distance downstream as a proxy for crop type also had a significant negative effect (Est = −0.097 CUPs/km, z = −2.78, p = 0.006), with no meaningful change across different widths (interaction Est = 0.001, z = 1.69, p = 0.092). For log(sum) concentration, neither the RNBZ width (Est = −0.002 ln(µg/kg)/m, t = −0.55, p = 0.581) nor the distance downstream (Est = −0.024 ln(µg/kg)/km, t = −1.26, p = 0.209) nor their interaction (Est < 0.001, t = −0.14, p = 0.887) were significant.

For the number of CUPs in vegetation, RNBZ width had no significant effect (Est = −0.002 CUPs/m, z = −0.18, p = 0.855), whereas distance downstream as a crop type proxy had a significant negative effect (Est = −0.139 CUPs/km, z = −3.83, p < 0.001) that was consistent across different widths (interaction Est < 0.001, z = 0.57, p = 0.572). For log (sum) concentration, the RNBZ width (Est = 0.005 ln(µg/kg)/m, t = 1.30, p = 0.197), distance downstream (Est = −0.022 ln(µg/kg)/km, t = −1.13, p = 0.262) and their interaction (Est < −0.001, t = −1.92, p = 0.057) had no significant effects.

3.4. CUP mixture composition analysis

An analysis of Jaccard Dissimilarity Indices (JDI) of CUP mixture composition (Fig. 5) to further analyse the source of RNBZ contamination revealed that both the topsoil and vegetation mixture compositions at the stream side (S) and field side (F) were clearly dissimilar from the stream water mixture composition (S0), with mean JDIs of ≥0.7. Nevertheless, all stream-sided RNBZ topsoil and vegetation mixtures at the specific sampling locations shared at least one CUP occurrence with its stream water sample (S1–S5 vs S0: topsoil JDI_{S0} min = 0.55, mean = 0.81, max = 0.95; vegetation JDI_{S0} min = 0.48, mean = 0.71, max = 0.93). Notably, the greater mean JDI_{S0} for stream-sided RNBZ topsoil (0.81) compared to stream-sided RNBZ vegetation (0.71) indicates that the topsoil CUP mixtures were compositionally more dissimilar to the

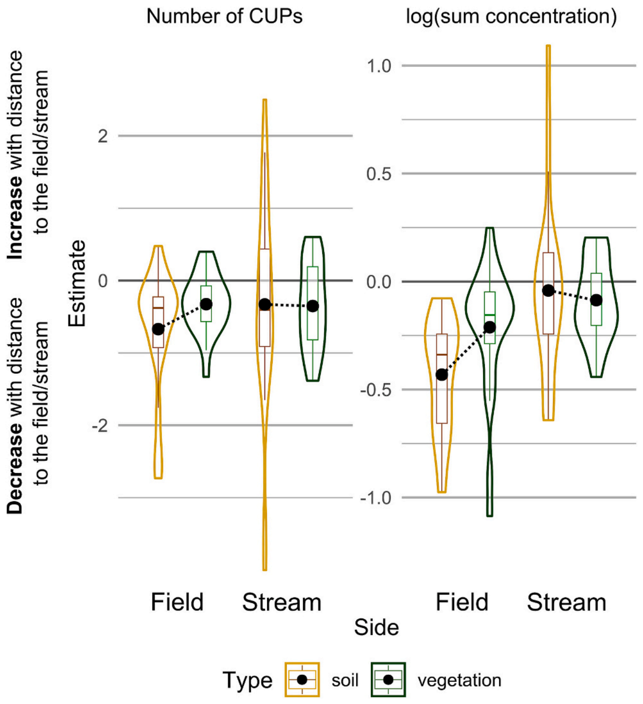

Fig. 3. Violin plots representing the estimates for the number of CUPs and log (sum concentrations) for topsoil (yellow) and vegetation (green) samples from the field side and the stream side. Estimates above 0 represent positive slopes, whereas estimates below 0 represent negative slopes. Negative slopes indicate that pesticide contamination was highest in the field or stream and decreased moving into the RNBZ, whereas positive slopes indicate that greater contamination within the RNBZ decreased toward the field or stream. Estimate means (black dots) indicate negative slopes, implying that the RNBZ grows less contaminated from both the field side and the stream side.

stream water CUP mixtures. There was no consistent pattern of stronger dissimilarity for either the stream side or the field side mixtures in relation to the stream water (Fig. 5). The compositional dissimilarity between stream water (S0) and adjacent matrices (topsoil or vegetation) remained relatively constant across lateral distances on both sides, indicating that, in our case, proximity to the stream does not explain spatial CUP mixture patterns in riparian zones.

The RNBZ topsoil and vegetation CUP mixtures matched more closely with in-field mixtures (F1–F5 and S1–S5 vs F0: topsoil JDI_{F0} min $= 0.06$, mean $= 0.54$, max $= 0.95$; vegetation JDI_{F0} min $= 0.00$, mean $= 0.43$, max $= 0.92$). This pattern was also evident when only the stream side of the RNBZ was considered: S1–S5 vs F0 showed JDI_{F0} values of 0.25–0.95 (mean 0.68) for topsoil and 0.15–0.92 (mean 0.49) for vegetation.

3.5. CUP contamination in stream water

With respect to the number of CUPs in stream water, the Queich showed a significant increase in detections of 0.62 CUPs/km downstream ($z = 3.89$, $p < 0.001$, Table 2, Fig. 6). The Speyerbach started with substantially lower CUP detections compared to the Queich (estimate $= -13.88$, $z = -4.72$, $p < 0.001$) but increased more steeply downstream by 1.52 CUPs/km (sum of main effect and interaction; interaction $z = 3.29$, $p = 0.001$). In contrast, the Otterbach started with significantly greater CUP detections than the Queich (estimate $= 11.82$, $z = 3.62$, $p < 0.001$) but showed no clear linear downstream trend, with a near-zero slope of −0.01 CUPs/km (sum of main effect and interaction; interaction $z = -3.23$, $p = 0.001$). In terms of the log(sum) concentration in stream water, the Queich showed no significant downstream trend (0.003 ln(μg/L)/km, $p = 0.838$). The Speyerbach had a similar starting value to that of Queich (estimate $= -0.056$, $p = 0.888$) and a comparable downstream slope of 0.010 ln(μg/L)/km (interaction $p = 0.802$). In contrast, the Otterbach started with substantially higher concentrations than the Queich (estimate $= 1.515$, $p < 0.001$) and exhibited a significant decrease downstream of −0.046 ln(μg/L)/km (interaction $p = 0.016$).

4. Discussion

In their buffer function RNBZs are intended to mitigate agricultural pollution and protect adjacent aquatic ecosystems (Prosser et al., 2020) by retaining CUPs in vegetation and soil in the riparian zone, but this inevitably results in the exposure of organisms within this habitat to CUP mixtures. Although our data are regionally collected and cover only a single time point during one spraying season, the consistently high number of CUP mixtures across all sites and matrices suggest recurring input pathways. These mechanisms may not be restricted to our study area and could also affect riparian zones in other intensively farmed regions.

4.1. Contamination in the RNBZ habitat

Pesticide contamination in terrestrial non-target areas has been extensively demonstrated beyond RNBZs, even in conservation areas (Brühl et al., 2024; Honert et al., 2025; Mauser et al., 2025). This contamination also exposes the special aquatic–terrestrial RNBZ biodiversity, the extent and consequences of which remain unknown. Our detections of 54 different CUPs in RNBZ topsoil and 40 CUPs in RNBZ vegetation during the spraying season exceeded recent detections of 39 CUPs in regularly flooded topsoil and 25 CUPs in rarely flooded topsoil, 33 CUPs in regularly flooded vegetation and 27 in rarely flooded vegetation in the same study area in October 2022 (max. 98 CUPs and metabolites, Fiolka et al., 2024). These differences in vegetation may be explained by its temporal variability and relatively high CUP abundances at non-target sites during the spraying season (Honert et al., 2025). In the case of soil, it was demonstrated that contamination is much more constant over time (Honert et al., 2025), leading to the conclusion that our samples may have been taken from generally more contaminated sites.

Our detected mean stream-side concentrations were 6.3 μg/kg in topsoil and 34.0 μg/kg in vegetation. These values are approximately 50–150 times and 300–850 times higher than the 0.12 μg/kg and 0.04 μg/kg in regularly and rarely flooded topsoil and the 0.10 μg/kg and 0.04 μg/kg in regularly and rarely flooded vegetation, respectively, reported in the mentioned study (Fiolka et al., 2024). Another recent study covering the same region reported a mean CUP sum concentration of 8.48 μg/kg in topsoil across the Upper Rhine Valley and adjacent low mountain regions, with a maximum of 248.23 μg/kg in vineyard-adjacent valley sites (Mauser et al., 2025). Our detected topsoil concentrations of 6.3 μg/kg at the stream side are thus comparable to their average across the entire region but clearly below their highest valley-specific values. Similarly, the stream-side vegetation samples in our study contained a mean CUP concentration of 34.0 μg/kg, which is within the range of the overall mean of 51.2 μg/kg reported in the Upper Rhine Valley study and below the maximum of 1966.5 μg/kg found near vineyards (Mauser et al., 2025). Compared with French non-target topsoil, where a mean CUP sum concentration of 30.4 μg/kg in grassland and <2 μg/kg in forest soils was detected (n = 110 CUPs, Froger et al., 2023), our stream-side topsoil values fall in-between those reported here, highlighting the transitional character of RNBZs between agricultural and more natural systems. Atmospheric and hydrological transport facilitates widespread CUP movement across landscapes (Mayer et al., 2024). Even rainwater contains pesticides (Putri et al., 2025), contributing to ubiquitous exposure. While the ubiquity of CUP residues across Europe is increasingly recognized, research on RNBZs has mostly focused on model or reference systems (Stutter et al., 2021).

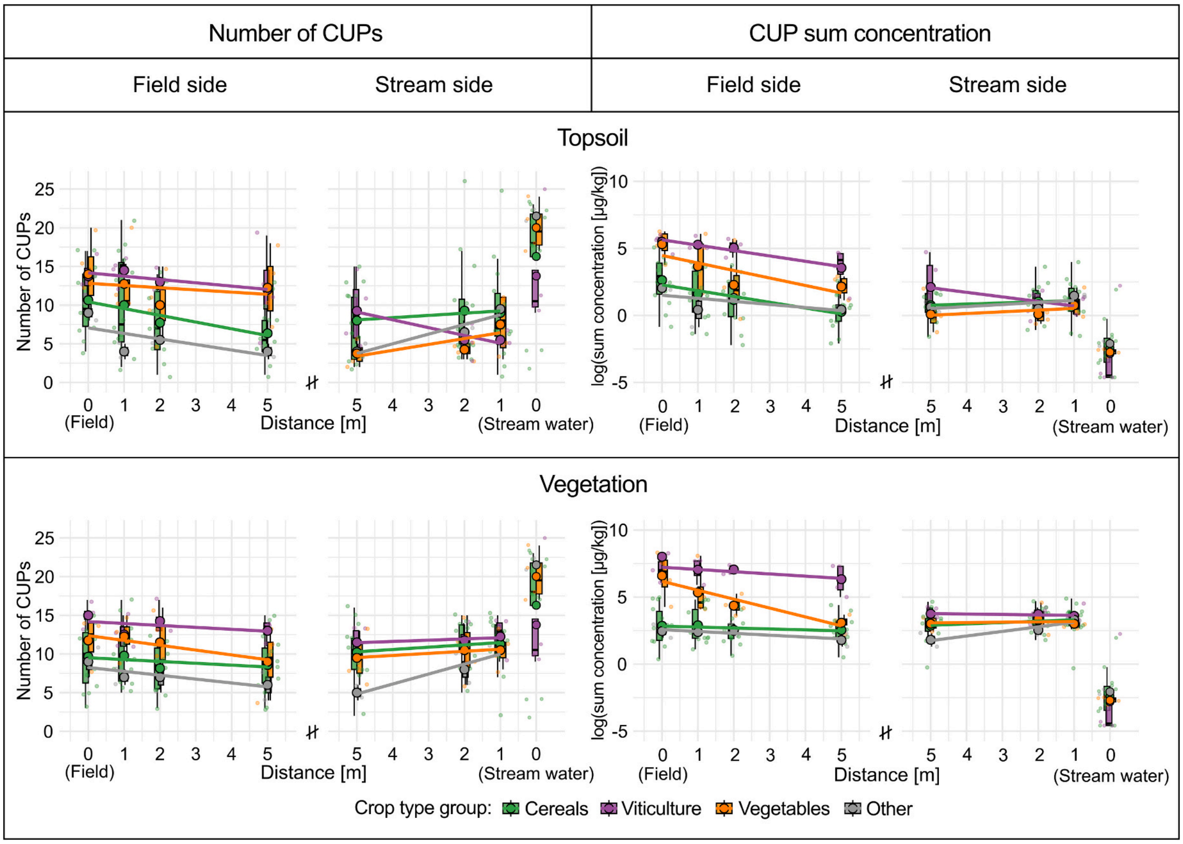

Fig. 4. Combined plot of CUP detections by crop type. Each boxplot represents the average number of detections or their log(sum concentration) at a given sample point (from F0 to S0) with distance [m] from the field or stream. Colour represents the crop type at the time of sampling at F0 with cereals (green, n = 14), viticulture (purple, n = 4), vegetables (orange, n = 4) and other (gray; recently planted, n = 2). Each line represents the mean estimate over all site estimates of a given crop type.

Table 1

Results of Generalized Linear Models (GLMs) analyzing the effects of RNBZ width and distance downstream (as a crop type proxy) on contamination levels in topsoil and vegetation. The number of CUPs was modelled using Poisson GLMs with an identity link, while log(sum) concentration was analysed using Gaussian GLMs. Estimates, standard errors, test statistics (z values for Poisson models and t values for Gaussian models), and p values are reported for fixed effects, including RNBZ width, distance downstream, and their interaction. Significant effects ($p < 0.05$) are shown in bold.

Type	Variable	Term	Estimate	StdError	testValue	pValue	
Topsoil	Number of CUPs	(Intercept)	11.335	0.62	18.15	**<0.001**	(***)
		RNBZ width [m]	−0.026	0.01	−3.62	**<0.001**	(***)
		Distance downstream (crop type proxy) [km]	−0.097	0.03	−2.78	**0.006**	(**)
		RNBZ width: Distance downstream	0.001	<0.01	1.69	0.092	
	log(sum) concentration [ln(µg/kg)]	(Intercept)	2.623	0.32	8.11	**<0.001**	(***)
		RNBZ width [m]	−0.002	<0.01	−0.55	0.581	
		Distance downstream [km] (crop type proxy)	−0.024	0.02	−1.26	0.209	
		RNBZ width: Distance downstream	<0.001	<0.01	−0.14	0.887	
Vegetation	Number of CUPs	(Intercept)	12.126	0.67	18.16	**<0.001**	(***)
		RNBZ width [m]	−0.002	0.01	−0.18	0.855	
		Distance downstream (crop type proxy) [km]	−0.139	0.04	−3.83	**<0.001**	(***)
		RNBZ width: Distance downstream	<0.001	<0.01	0.57	0.572	
	log(sum) concentration [ln(µg/kg)]	(Intercept)	3.981	0.34	11.82	**<0.001**	(***)
		RNBZ width [m]	0.005	<0.01	1.30	0.197	
		Distance downstream [km] (crop type proxy)	−0.022	0.02	−1.13	0.262	
		RNBZ width: Distance downstream	<−0.001	<0.01	−1.92	0.057	

Yet, their role at the interface to agricultural sources makes them essential for understanding pesticide dynamics at the landscape scale.

In our study, the fungicides fluopyram and spiroxamine were among the most frequently detected CUPs in riparian habitats, similar to earlier findings from the Upper Rhine Valley (Mauser et al., 2025). Several substances are present in a large proportion of environmental samples, as has also been demonstrated in France (e.g., the herbicide glyphosate or the fungicide fluopyram, Froger et al., 2023) or Italian alps (e.g., the insecticide methoxyfenozide or the fungicide fluazinam, Brühl et al., 2024), with fluopyram showing the highest monthly detection frequency, leading to chronic year-round exposure (Honert et al., 2025). Previous studies have demonstrated that pesticide exposure in non-target areas contributes to declines in biodiversity (Dicks et al., 2021; Geiger et al., 2010; Gunstone et al., 2021). CUPs can enter food webs and accumulate moving upward through trophic levels, leading to sublethal effects that impact species survival and ecosystem functioning

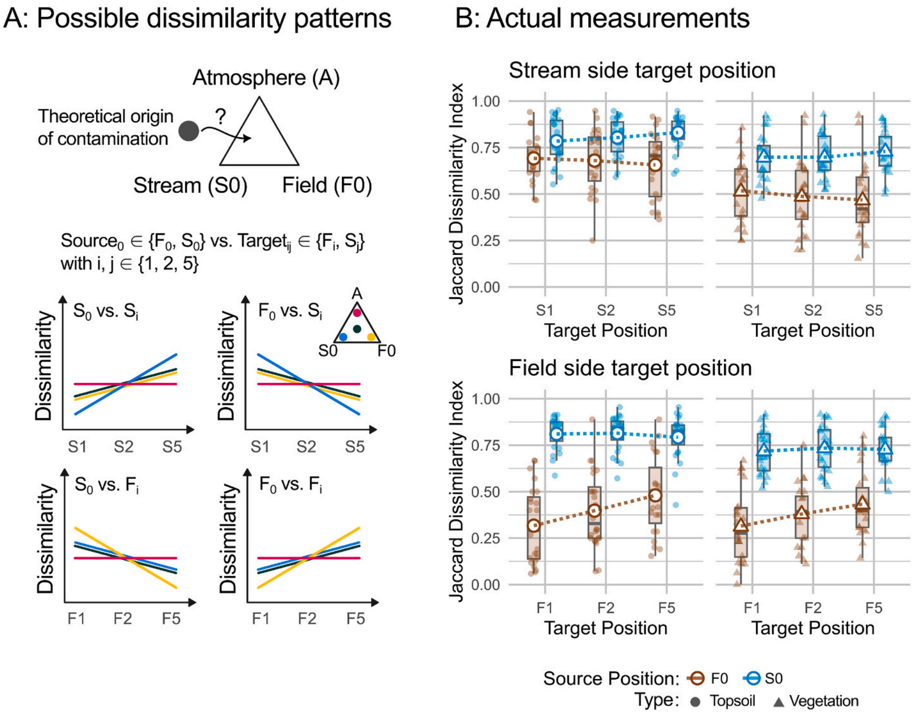

Fig. 5. Possible dissimilarity patterns with the origin of contamination from the atmosphere, stream or field (A). Examples: S_0 vs. S_i (top left) – Strong increase = stream source; weak increase = partly field, partly atmospheric input. F_0 vs. S_i (top right) – Strong decrease = stream source; weak decrease = field or atmosphere. S_0 vs. F_i (bottom left) – Strong decrease = field source; weak decrease = partly stream, partly atmosphere. F_0 vs. F_i (bottom right) – Strong increase = field source; weak increase = partly stream, partly atmosphere. Boxplots of Jaccard dissimilarities between measured CUP mixtures in stream water and adjacent terrestrial samples (B). Top panels compare stream water (S0, blue dots) or in-field samples (F0, brown dots) to stream-side samples at 1-, 2- and 5-meters distances to the stream (S1, S2, S5). The bottom panels compare stream water (S0, blue dots) or in-field samples (F0, brown dots) to field-side samples at 1-, 2- and 5-meters distance to the field (F1, F2, F5). The dots represent topsoil; the triangles represent the vegetation dissimilarities of one pairwise comparison at one specific site. White-filled circles or triangles denote group means. Higher values represent greater dissimilarity between pesticide mixtures; lower values indicate more similar CUP compositions.

Table 2
Results of Generalized Linear Models (GLMs) analyzing the effects of distance downstream and stream on contamination levels in water. The number of CUPs was tested using Poisson GLMs with an identity link, while log(sum) concentration was analysed using Gaussian GLMs. Estimates, standard errors, test statistics (z values for Poisson models and t values for Gaussian models), and p values are reported for fixed effects, distance downstream, stream (Queich/Speyerbach/Otterbach) and their interaction (differences in the effect of distance downstream between the three streams). Significant effects ($p < 0.05$) are shown in bold.

Variable	Term	Estimate	StdError	testValue	pValue	
Number of CUPs	(Intercept Queich mountain foot)	8.869	2.029	4.371	**<0.001**	(***)
	Distance downstream [km]	0.620	0.159	3.889	**<0.001**	(***)
	Stream Speyerbach	−13.875	2.938	−4.722	**<0.001**	(***)
	Stream Otterbach	11.822	3.266	3.619	**<0.001**	(***)
	Distance downstream: Speyerbach	0.902	0.274	3.289	**0.001**	(**)
	Distance downstream: Otterbach	−0.630	0.195	−3.229	**0.001**	(**)
log(sum) concentration	(Intercept Queich mountain foot)	−0.010	0.24	−0.042	0.967	
	Distance downstream [km]	0.003	0.026	0.207	0.838	
	Stream Speyerbach	−0.056	0.4	−0.143	0.888	
	Stream Otterbach	1.515	0.33	4.614	**<0.001**	(***)
	Distance downstream: Speyerbach	0.007	0.03	0.255	0.802	
	Distance downstream: Otterbach	−0.049	0.018	−2.674	**0.016**	(*)

(Michalko et al., 2024). In our study, most detections in topsoil, vegetation and water were below known acute or chronic effect thresholds. However, some field-side samples exceeded invertebrate NOEC thresholds (PPDB, 2024): in vegetation, cyflufenamid reached a maximum of 10.9 times the collembola NOEC threshold (0.195 mg/kg F2 sample, NOEC Collembola 0.018 mg/kg). In topsoil, the NOEC of difenoconazole reached a maximum of 1.1 times greater than that of earthworms (0.23 mg/kg F1 sample, NOEC Earthworm 0.200 mg/kg). These findings indicate that exceedances may occur locally under field conditions. While our dataset does not include biological or effect-based endpoints, the frequent detection of diverse CUP mixtures in riparian habitats raises concern about potential exposure risks. The documented decline of over 75% in flying insect biomass in German nature conservation areas (Hallmann et al., 2017) and that local insect communities showing higher CUP exposure with increasing proportion of surrounding agricultural area (Brühl et al., 2021) place our findings in a broader context of growing concern over non-target contamination and biodiversity loss. Since realistic mixture exposures occurring in non-target habitats are not assessed at all (Brühl and Zaller, 2019), there is a gap between regulatory evaluations and actual environmental conditions.

In support of our first hypothesis that most of the CUPs originate from the adjacent agricultural fields, the number of CUPs and the CUP sum concentrations decreased with increasing distance from the field. In this study, vegetation showed a smaller decrease (−0.33 CUPs/m) than

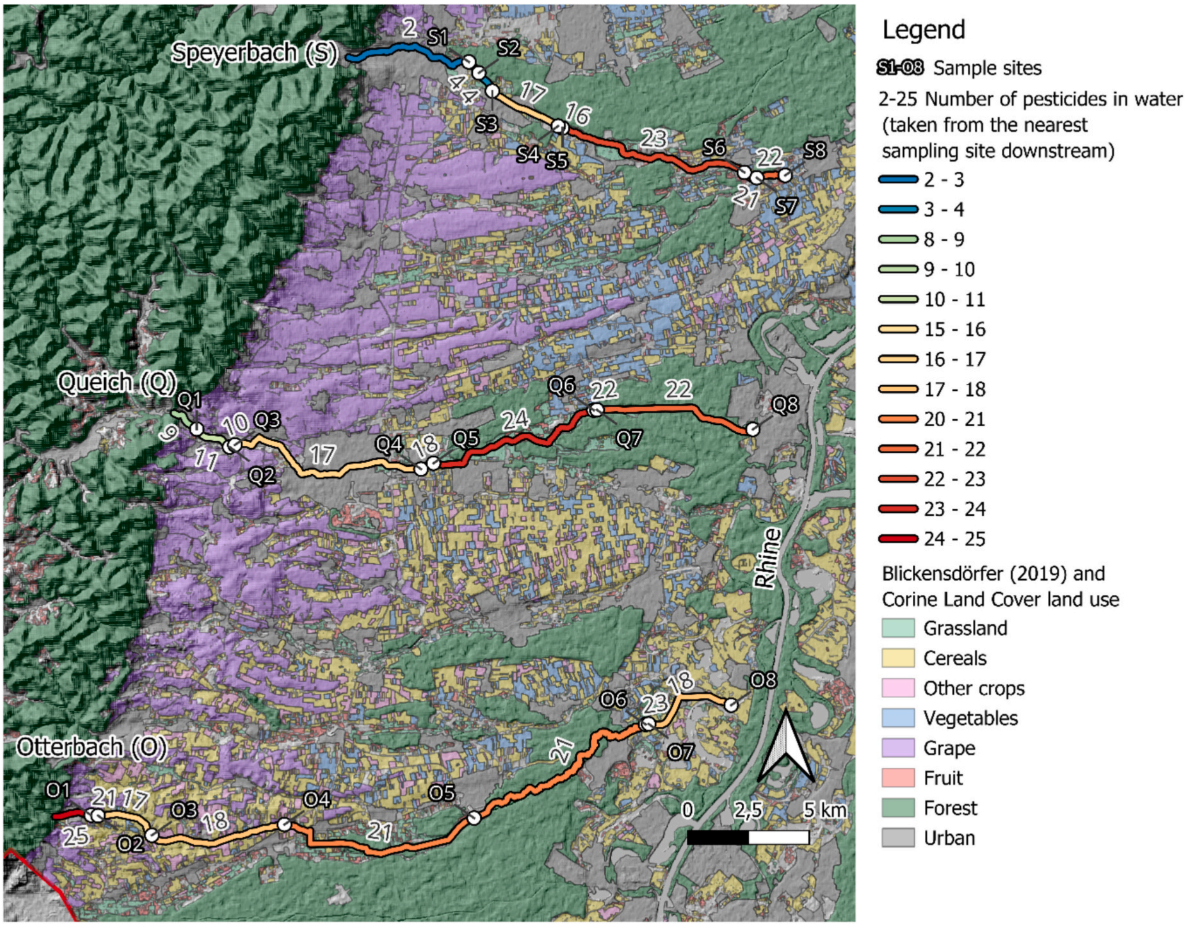

Fig. 6. Map of the Speyerbach (top), Queich (middle), and Otterbach (bottom) and their number of CUP detections in water as well as surrounding land use types and conservation areas. The number of CUP detections in water are coloured from dark blue to dark red, representing low to high CUP detections. Agricultural Land use data from Blickensdörfer et al. (2021). Altitudinal data, forest and urban cover from European Environment Agency (EEA).

topsoil did (−0.67 CUPs/m), resulting in reductions of 1.65 and 3.35 CUPs at 5 m distance of 5 m, respectively. Although our measurements of non-target area contamination fall within the range of CUP numbers recorded in a year-round study (Honert et al., 2025), we found a less steep decline in CUP numbers over a 5-meter distance from the field margin (riparian assessment: approx. −3.35 CUPs in topsoil and −1.65 CUPs in vegetation vs. temporal monitoring: approx. −3 to −16 CUPs in topsoil and −2 to −4 CUPs in vegetation depending on crop type, over the first 5 m). This may reflect the fact that measurements were taken at a single point during the spraying season, without accounting for temporal variability across the entire year, as in the mentioned study. However, the relatively small decrease in the number of CUPs in vegetation with distance from the field also follows our other findings of an insignificant effect of RNBZ width on contamination in vegetation. Therefore, narrow RNBZs may greatly differ in CUP mixture complexity of the surrounding fields but may share similar CUP dynamics throughout the year.

Following our second hypothesis, that CUPs may also enter from the stream into the RNBZ, the number of CUPs and CUP sum concentrations at the stream side were shown to increase toward the stream (S5–S1). Although we did not collect hydrological or meteorological data, field observations suggest that only a few of the sites, mainly mid- and downstream flood regularly (e.g., S8, Q4, Q5, O5, Fig. 6). Some of these sites were situated close to a previous study, whereby it was determined that flooding events can transport pesticides to riparian soil and expose riparian vegetation, likely through uptake from contaminated soil (Fiolka et al., 2024). The sampling in the mentioned study was specifically performed on the lower part of the bank, close to the stream surface, whereas we sampled in the upper part. Flooding therefore might only occur at sampling position S1, although not regularly, depending on heavy rain events. Therefore, flooding of the banks with contaminated water from the streams may have not produced the overall pattern in our data. Another possible entry route for pesticides from the stream into the RNBZ soil could be lateral infiltration, i.e., the horizontal movement of stream water into adjacent soils. The rate of this process varies with soil texture and initial moisture content (Malik et al., 1987), which were not assessed in our study. Moreover, as both the topsoil and vegetation CUP mixtures at the stream side and field side were clearly dissimilar from the respective stream water mixtures and no change in dissimilarity between the stream (S0) and S1, S2 and S5 was recorded, the specific composition of CUPs may not simply be related to the adjacent stream water. In this study, we sampled 24 sites over a two-week period without accompanying information on precipitation, runoff or soil conditions. Given the dynamic nature of the stream system, relevant contamination events may have occurred outside the sampling window, limiting our ability to link terrestrial and aquatic mixtures directly. Moreover, local differences in environmental factors such as soil organic matter content, clay content, pH, or recent meteorological events (e.g., rainfall or drought) could influence pesticide entry and persistence in riparian soils (Kodešová et al., 2011). A subsequent study including streambank microtopography, soil characteristics and time-resolved water chemistry could help to clarify these pathways. Taken together, in line with hypothesis two, the observed increase in CUP numbers and concentrations toward the stream suggest that pesticide input from the stream into adjacent riparian areas is plausible, even though the exact mechanisms remain uncertain. The clear compositional differences between stream water and adjacent soil and vegetation point to complex, site-specific processes. Based on current data, the CUP mixtures at the stream side tend to resemble the adjacent fields rather than the stream.

4.2. Crop type

Our RNBZ CUP detections in relation to their adjacent field (F0) emphasize that non-target contamination is shaped by adjacent crop types. The RNBZ vegetation and topsoil along vineyards at F0 exhibited overall greater CUP contamination compared to sites with other crop types at F0, whereas at vegetable sites, field-side vegetation and topsoil also showed greater contamination than did cereal sites (Fig. 4). As vineyards are located primarily along slopes close to the low mountain foot (Fig. 6), these upstream sites showed greater contamination compared to downstream RNBZs. In addition, vineyard sites at F0 were the only sites where contamination on the stream side decreased on average toward the stream, suggesting a reduced influence from the stream itself but an increased contribution of drift from the surrounding vineyards toward the stream. Vineyards showed exceptionally high fungicide treatment frequencies (index: 19.2), exceeding winter wheat by 4 times and corn by 8 times, with only apples showing relatively high rates (29.3; Julius Kühn-Institut, 2023). Therefore, the number of fungicides applied in viticulture may explain our increased detections at the RNBZ at viticultural sites and therefore at sites closer to the mountain foot due to their geographic position. The elevated number of CUPs and their sum concentration in the RNBZ (Fig. 4) may also align with crop-specific application patterns: unlike conventional arable farming, where pesticides are applied two-dimensionally on the ground or crop, three-dimensional vineyard spraying results in a significant proportion of airborne particles that can be transported over short to medium distances, increasing pesticide exposure in non-target areas (Julius Kühn-Insitut, 2022). Additionally, vineyard site conditions, such as increased microclimatic air temperature and wind velocity, increase pesticide volatilization (Bedos et al., 2002).

4.3. RNBZs and their role as buffers

The role of the RNBZ in effectively reducing pesticide inputs into aquatic ecosystems has already been questioned (Prosser et al., 2020). This raises concerns about the reliability of the RNBZ in safeguarding aquatic biodiversity in intensively managed landscapes. Pesticide retention can vary based on vegetation type, soil properties, and hydrological conditions, with some studies indicating that riparian zones may even act as secondary sources of contamination under certain conditions (Stutter et al., 2021). The presence of CUPs in the topsoil and vegetation of the RNBZ, particularly close to streams (S1–5), demonstrates that CUPs are transported over the entire width and reach areas near the stream, finally ending in stream water. Widespread CUP occurrence in riparian waters indicates buffer strip failure through structural shortcomings: erosion rills bypassing buffers (Stehle et al., 2016), subsurface drainage circumventing surface interception (Reichenberger et al., 2007), and site-specific factors such as slope (Dabrowski et al., 2002).

Our results partially support the third hypothesis that riparian CUP contamination is not solely determined by RNBZ width. Specifically, RNBZ width had a significant negative effect on the number of CUPs detected in RNBZ topsoil (−2.6 CUPs per 100 m), but no significant influence on the number of CUPs in RNBZ vegetation or on the total CUP concentrations in both RNBZ topsoil and vegetation. Our observation that vegetation retains high numbers of CUPs while topsoil contamination decreases only marginally with increasing RNBZ width might be a result of plant surfaces acting as primary interception zones for pesticide deposition. This suggests that in wider RNBZs, much of the CUP load may be intercepted by aboveground biomass before it reaches the soil, possibly explaining the lack of a strong decreasing trend in total CUP sum concentrations in soil.

Sampling took place during peak vegetation growth, whereas other studies also observed only a minor decline in CUP numbers with increasing distance from arable fields or vineyards (Honert et al., 2025). Therefore, seasonal effects such as plant growth and mowing may affect concentrations in RNBZ vegetation. In addition, there is a large-scale input that most likely does not come from directly neighbouring fields but might be a result of atmospheric transport (Mauser et al., 2025). Continuous contamination in stream water and at the stream side (S5–S1) across sites with buffer widths ranging from 10 to 260 m indicates that Germany's mandatory 5 m no-spray zones provide limited protection. RNBZ buffer strategies should therefore be tailored to local land use and pesticide application practices. In cropping regions with high-drift potential such as vineyards, riparian buffers alone may provide insufficient to protect vulnerable aquatic species in the stream, especially when buffers are narrow or lack structural complexity.

Consistent with our expectation that more complex CUP mixtures occur downstream, we observed an increase in the overall number of CUPs in water samples along the stream, except in the Otterbach. At the Otterbach, complex mixtures of up to 25 CUPs were detected as early as the mountain foot (Fig. 6), indicating an early entry point of viticultural CUPs. These sites also showed the highest total CUP concentrations (9.27, 6.92, and 0.77 μg/L), primarily driven by dimethomorph (9.16–0.76 μg/L). Excluding dimethomorph, individual CUP concentrations remained low (≤0.02 μg/L).

In contrast, the upper Queich flows through areas dominated by small gardens, shrubs, and riparian trees, suggesting lower early CUP input. Nonetheless, nine CUPs were detected at the uppermost Queich site (Q1), consistent with previous findings of up to six CUPs near the source ~20 km upstream (Mauser et al., 2025). While low-level contamination here may result from atmospheric deposition, other sources such as private gardens, drainage pipes, and subsurface flows from distant vineyards likely contribute. Notably, over 80% of small lowland agricultural streams in Germany fail to meet pesticide-related ecological targets (Liess et al., 2021).

We collected stream water samples only once at each of the 24 sites, likely missing contamination peaks experienced by organisms after rainfall (Liess et al., 2021). Event-driven sampling in small German agricultural streams showed that rainfall can increase median total pesticide concentrations tenfold and detected pesticides from 5 to 14 per sample, with up to 41 in some cases (Halbach et al., 2021). Regulatory acceptable concentrations (RACs) were exceeded in 60% of event-driven samples versus 23% of grab samples. This indicates our single sampling likely underestimates both the magnitude and frequency of peak exposures.

5. Conclusion

Our findings illustrate a potential conflict in RNBZ function, as they are designed to prevent contamination but are themselves exposed to substantial CUP inputs. The contamination pattern suggests multiple input pathways, including agricultural drift—confirmed as the dominant source by mixture analyses—and large-scale atmospheric deposition. Our field data, together with consistent findings from other studies, indicate that current buffer widths are often insufficient to prevent CUP contamination of riparian habitats, particularly in intensively managed landscapes such as viticultural areas.

RNBZs should be recontextualised not merely as physical buffers to reduce stream pollution but as valuable, ecologically functional habitats that support biodiversity and cross-ecosystem processes. The meaningful protection of aquatic ecosystems and riparian biodiversity requires fundamental reconceptualization beyond mere increases in buffer width. Integrated approaches must combine substantial pesticide use reductions, structurally complex buffer designs, and landscape-scale contamination mitigation. After all, riparian zones are also named the green arteries of our landscapes, connecting habitats and allowing species exchange (Weissteiner et al., 2016). The measured contamination of stream water and riparian habitats suggests that conservation goals may not be met without a significant reduction in overall pesticide use, as included in the Montreal Biodiversity Framework, to halt and reverse biodiversity loss, with a target of reducing the overall risk from pesticides by at least half to be reached by 2030 (Convention on Biological Diversity, 2022).

CRediT authorship contribution statement

Ken M. Mauser: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tom Dekker:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Jurg W. Spaak:** Writing – review & editing, Methodology, Conceptualization. **Tobias Elsässer:** Writing – review & editing, Methodology. **Carsten A. Brühl:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2026.181503.

Data availability

Data will be made available on request.

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13 APPENDIX IV: PAPER 4

Effects of chemical and hydrological stress on the wing morphology of a damselfly

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Physiological Ecology

Effects of chemical and hydrological stress on the wing morphology of a damselfly

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Dragonflies and damselflies are exposed to various anthropogenic stressors in the aquatic-terrestrial ecosystem, which can affect their development and fitness. The symmetry of their wings, shaped during the aquatic larval stage, can serve as an indicator of environmental stress during development. Recent advances in computer-vision now provide the opportunity to standardize and enhance the precision of 2D assessments of entire wings, including many structural parameters, enabling a more reliable comparison of the effects of multiple anthropogenic stressors. We investigated the effect of 3 anthropogenic stressors on the fluctuating wing asymmetry of the damselfly *Coenagrion puella*: (i) Exposure to the agricultural insecticide chlorantraniliprole in a climate chamber experiment, (ii) alteration of the aquatic community with the mosquito control agent Bti (*Bacillus thuringiensis israelensis*), and (iii) altered hydrological regimes, both of which were applied in a floodplain mesocosm experiment in a full 2-factorial design. We found changes in wing size and several asymmetry parameters in response to the insecticide and altered hydrological regimes, whereas Bti treatment increased the number of cells in front wings. Our results show that damselflies' wing morphology and symmetry can be affected by anthropogenically induced stress in aquatic ecosystems. The intensity of stressor effects varied across treatments, with altered hydrology causing the strongest changes in wing size and asymmetry.

Keywords: aquatic-terrestrial linkage, odonata, emergence, chemical pollution, drought

Introduction

Dragonflies and damselflies link freshwater and terrestrial ecosystems. Due to their amphibiotic biphasic life cycle, disturbances during the aquatic larval stage can carry over to affect adult behavior, reproduction and lifespan in the terrestrial phase (Tüzün and Stoks 2018, Samways et al. 2025). During the aquatic larval stage, damselflies are exposed to several anthropogenic stressors. In an agricultural landscape, micropollutants (eg pesticides) reach streams and ponds by artificial drainage, runoff, and erosion (Imfeld et al. 2021). In addition, nutrients from fertilisers are washed in, changing the chemical composition of the water up to the point of eutrophication (Schoumans et al. 2014). In the context of climate change, water levels and the frequency and intensity of flooding can change with regional patterns (Blöschl et al. 2019), whereas still waters in floodplain areas depend on water input from flooding. Aquatic macroinvertebrates can initially experience a density increase when the water volume is reduced, but prolonged drought conditions then lead to a decline in the abundance of more sensitive species, presumably due to habitat degradation (Herbst et al. 2019). In addition, anthropogenic water regulation, such as dam operation, can intensify drought conditions during dry periods (Khelifa et al. 2021). Floodplain water bodies show a strong and highly dynamic relationship with the river, for example through exchanging chemically different water depending on the river water regime (Gmitrowicz-Iwan et al. 2020). Consequently, climate change may alter the input of micropollutants into floodplain water bodies by modifying the frequency and extend of river water exchange with these systems. Another anthropogenically introduced stressor is Bti (*Bacillus thuringiensis israelensis*) to control mosquitoes, which can also impact the aquatic-terrestrial ecosystem (eg through the reduction of chironomids; Brühl et al. 2020). Although no direct effect of Bti on damselfly larvae is known, it was shown to alter the benthic macroinvertebrate community composition driven by a reduction of the number of chironomid, libellulid, and coenagrionid larvae (Gerstle et al. 2023).

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As adults, damselflies prey upon flying insects and thus rely heavily on the functioning of their wings. Wing size and shape are environmentally mediated traits that directly influence flight performance, thereby determining a species' capacity for foraging, predator avoidance, and dispersal (Rocha-Ortega et al. 2022). The fluctuating asymmetry (FA) of wings is the deviation from the perfect bilateral symmetry of wings (eg size and shape difference between left and right wings) and is used as a proxy of development stability (Benítez et al. 2020). Stress has been associated with increased fluctuating asymmetry of insect wings, including honey bees (Friedli et al. 2020), bumblebees (Hierlmeier et al. 2022) as well as dragonflies and damselflies (Stoks 2001). Environmental stress during development can disrupt gene regulatory networks, morphogen gradients, and cellular processes such as proliferation or apoptosis, thereby reducing developmental buffering and leading to increased FA (Takahashi et al. 2010, Juarez-Carreño et al. 2018, Blanco-Obregon et al. 2022). Since wing development in damselflies takes place in the aquatic larval stage, asymmetrical wings in response to aquatic stress could alter the role of damselflies in the terrestrial system. Although FA has been used as a proxy for environmental stress in damselflies, its suitability as a biomonitoring tool is still controversial due to behavioral influences and complex developmental dynamics (Hardersen 2000a, Ryazanova and Polygalov 2013). Moreover, short-term disturbances such as parasitism during sensitive developmental windows may also increase FA independently of environmental quality, as shown by a significant correlation between mite load and wing asymmetry in *Coenagrion puella* (Bonn et al. 1996). In addition, a study examining FA of another coenagrionid species (*Coenagrion scitulum*) in relation to fitness traits such as mating success, as well as genetic parameters like heterozygosity, have yielded in no significant associations (Carchini et al. 2001). However, FA can still be considered sensitive to environmental stress in insects, especially when the stressor's biological relevance is confirmed through negative effects on fitness traits and when confounding environmental variation is minimized (Beasley et al. 2013). These methodological challenges and the complexity of stressor interactions highlight the need for more comprehensive and standardized approaches across experimental contexts differing in environmental complexity and control. Addressing such questions requires both controlled laboratory settings to disentangle direct physiological effects and field-based approaches that capture broader, community-mediated processes.

In studies of FA, traditional approaches have typically focused on straightforward morphological traits, such as wing length, wing area, and overall shape asymmetry, usually derived from linear measurements or landmark-based methods (Graham et al. 2010). Advances in computer vision now offer the potential for a more precise and reliable 2D-assessment of the whole wing in combination with analyzing a large sample size. Modern algorithms can automatically detect boundaries, identify junctions, and extract vein patterns (Eshghi et al. 2024) thereby extending FA analyses to asymmetries in cell count, cell shape, and vein-junction positions. These features build a complex network that provides support and rigidity to the wing membrane (Rajabi et al. 2016). Wing venation can considerably affect both the dynamic deformation of insect wings during flight (Rajabi et al. 2016) and their susceptibility to mechanical damage and abrasive wear (Rudolf et al. 2019).

The scrambling damselfly *C. puella* is a common species in central Europe, mostly univoltine and present in natural standing waters as well as in urban and agricultural areas (Wildermuth and Martens 2019). The ability of this generalist species to inhabit different habitats (Muséum National d'Histoire Naturelle and Office Français de la Biodiversité 2025) makes it a potential indicator for numerous anthropogenic stressors in freshwater ecosystems. Its broad distribution, high local abundance, wide ecological tolerance, and well-documented taxonomy and life-history fulfil key criteria of effective bioindicators, such as cost-efficient sampling and a consistent response at the population level (Holt and Miller 2010).

We investigated the effect of 3 anthropogenic stressors on the fluctuating wing asymmetry of *C. puella* in a laboratory and field perspective by (i) exposing larvae to the insecticide chlorantraniliprole in a climate chamber experiment, by (ii) treating ponds with Bti, and (iii) altering their hydrological regime in a field experiment using artificial outdoor floodplain mesocosms (FPM). We hypothesized (i) that the insecticide chlorantraniliprole negatively affects wing size and FA due to its neurotoxic mode of action, which may impair larval development and thereby reduce larval weight gain as well as wing size and wing symmetry of the emerged imagines. For the mosquito larvicide Bti, we expected that (ii) it has a negative impact on the wing size and FA due to food web interference as it causes a decline in mosquito and chironomid larvae. We further hypothesized (iii) a negative impact on the wing size and FA due to strong water level fluctuations and their disturbance of the benthic communities and crowding effects during low water. Since damselfly wings have a complex structure, we also investigate whether any stress dependent changes in the symmetry of the wing are uniformly spread or spatially dependent. Based on sex-specific differences in larval activity, life-history traits, and development (Mikolajewski et al. 2005), we included potential differences in wing symmetry between male and female damselflies in our study.

Materials and Methods

Climate-Controlled Chamber Experiment: Insecticide Chlorantraniliprole Exposure

Chlorantraniliprole (CAS Number 500008-45-7) is an anthranilic diamide insecticide, introduced in 2007. It acts on insect ryanodine receptors and causes excessive release of intracellular Ca^{2+} stores, resulting in death by paralyzing muscles and inhibiting feeding (Lahm et al. 2007). Its degradation is considered slow with a 50% dissipation time (DT_{50}) in the water phase of 23.5 d, water-sediment DT_{50} = 170 d and soil DT_{50} (field) = 204 d (Pesticide Properties Database [PPDB] 2024). Globally, chlorantraniliprole plays an important role as it is routinely used in rice fields (Wei et al. 2019, CIBRC 2024). In Germany, it is approved for the use in different kind of vegetables, pome fruit and grape (BVL Bundesamt für Verbraucherschutz und Lebensmittelsicherheit 2015) against potato beetles (*Leptinotarsa decemlineata*), leafroller moths (Tortricidae), snout moths (Pyralidae), and other caterpillars (Lepidoptera). It has an acute 96-h 50% lethal concentration (LC_{50}) of 4 µg/L for *Chironomus dilutus* and chronic 28 d no-observed-effect concentration (NOEC) in static water of 2.5 µg/L for *Chironomus riparius* (PPDB 2024). For *Daphnia magna*, an acute 48 h half maximal effective concentration (EC_{50}) of 11.6 µg/L and chronic 21 d

NOEC of 4.47 µg/L was determined (PPDB 2024). Its predicted environmental concentrations in freshwaters vary between 0.04 and 9.12 µg/L (European Food Safety Authority 2013). In a large-scale study across 101 sites of small lowland streams in Germany, chlorantraniliprole was detected in 68% of event-driven samples and 38% of grab samples with a mean concentration of 0.0008 and 0.0007 µg/L, and max concentration of 0.0225 and 0.1031 µg/L, respectively (Liess et al. 2021). A recent study in the Upper Rhine Valley in Germany, measuring different available water sources at non-target sites (eg puddles and small streams) showed 4 detections in off-field puddles with concentrations between 0.014 µg/L and 0.002 µg/L (Mauser et al. 2025b). On the basis of available effect data and measured environmental concentration data, we opted for the following 4 concentrations to ensure non-lethal effects: 0 µg/L, 2.5 µg/L, 10 µg/L, and 40 µg/L (0, 0.0025, 0.01, and 0.04 mg/L). To prepare a stock solution, chlorantraniliprole was first dissolved in acetone before tap water was added. The amount of acetone present in the solution with the highest target concentration was also added to the control. This resulted in an acetone concentration <100 µl/L in all groups, in accordance with the Organization for Economic Co-operation and Development (OECD) Guideline 211 (OECD 2012) which describes the standard test for assessing the reproduction and survival of *Daphnia magna* under chronic exposure conditions.

Larvae of *C. puella* (*n* = 160) were collected on 13 April 2023 with a net (mesh size = 1 mm) from ponds in Trippstadt (Environmental Education Centre LIBELLULA, 49°21'30.5″N 7°44'36.0″E) within the Palatinate Forest in Southwest Germany. Only healthy larvae (based on their moving and lack of observable injuries) were taken which had not reached the final instar (F-0) yet. Larvae were immediately transferred to the lab in multiple 10 L buckets with previously aerated tap water (aeration time at least 24 h) and then split individually to 250 ml glass beakers with 100 ml of similar prepared medium and randomized IDs. Before weighing (± 0.01 mg), individuals were dried thoroughly with laboratory tissues (Carl Roth GmbH + Co. KG, Germany) to remove as much contact water as possible. To prevent exposing the larvae to an abrupt temperature increase, all beakers were first kept at constant room temperature (20 °C) for 24 h to allow acclimatization. All beakers with larvae were then transferred to a WK 19'/+15-35 climate chamber (Weiss Umwelttechnik GmbH, Germany). For the duration of the experiment, the parameters in the climate chamber were set as follows: temperature of 23 °C, humidity of 80% and a lighting time from 7 am to 11 pm, maintaining a day/night cycle of 16/8 h. The temperature of 23 °C was chosen based on previously reported optimal larval growth rates for *C. puella* (Suhling et al. 2015).

At the start of the exposure phase on 21 April 2023, the larvae were transferred to previously prepared 250 ml beakers with 100 ml of aerated tap water and the chlorantraniliprole concentrations of 0, 0.0025, 0.01, and 0.04 mg/l (40 beakers each). A plastic net stripe (length = 15 cm, width = 1.5 cm, mesh width = 0.2 cm) was added to the beakers as climbing assistance for emerging damselfly larvae. The beakers were covered with a piece of fly screen net (1 mm mesh width) and rubber band. Initially, the water exhibited a pH of 7.45, dissolved oxygen content of 10.33 mg/L and conductivity of 129 µS/cm at measured temperatures of 23.4 °C (Multi 340i, WTW, Germany). To compensate for evaporated water, the beakers were filled up to 100 ml with aerated tap water after 7 d. Every day of the exposure, a 100 µl water sample was taken for every concentration, stored at −20 °C and later analyzed (Roodt et al. 2023) via high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS, Agilent 1260 Infinity II HPLC and Agilent 6495C MS, Agilent, United States). The 160 larvae were exposed until the 5 May 2023 for a total of 14 d. Four individuals emerged before reaching 14 d of exposure (7, 10, 10, and 12 d of exposure) but included in the analysis. After the exposure, the larvae were weighed again (±0.01 mg) (PBA224I-1x, VWR, United States) to calculate the wet weight gain. Until emergence, the larvae were fed daily with 2 ml Artemia suspension (prepared from ArtemiaVita eggs, Algova, Germany). The beakers were checked several times per day, and adults that had emerged on the plastic net strips and completed hardening were collected, placed in glass tubes, and frozen at −40 °C until further measurements. The imagines were weighed (±0.01 mg) (PBA224I-1x, VWR, United States) and species identity and sex were checked. Six adult individuals belonged to the closely related *Coenagrion pulchellum*, with very similar larval morphology, and were excluded from the analysis.

Floodplain Mesocosm: Indirect Chemical and Direct Hydrological Stress

The floodplain mesocosms (FPM) are located at the Eußerthal Ecosystem Research Station (49°15′14′′N, 7°57′42′′E; RPTU Kaiserslautern-Landau) within the Palatinate Forest in Southwest Germany. The studied FPMs were 12 units each 176 m^2 and rectangular in shape (23.5 × 7.5 m), constructed in 2017 along the stream Sulzbach, ensuring natural colonization of biota (Manfrin et al. 2023). Each unit has steep banks at 3 sides and a flat water–land floodplain riparian area at the inflow. All units are connected to the Sulzbach from where they can be supplied with water through a pipe to regulate water levels if needed. The FPMs progressively deepen toward the outflow section, following a bed slope of 1:20. They are inhabited by communities of merolimnic aquatic insect larvae (including Diptera, Ephemeroptera, Trichoptera, and Odonata) as well as various amphibians and terrestrial arthropods (Stehle et al. 2022). A detailed description of the FPM can be found in Stehle et al. (2022).

Since 2020, Bti was applied in 6 of the 12 FPM units (AP 2, 4, 6, 8, 10, 12) at maximum field rates (2.88 × 109 ITU/ha, Gerstle et al. 2023) annually in April, May, and June once a month using a VectoBac WDG solution (Valent BioSciences Corporation, Illinois, United States). To mimic a realistic Bti application, the FPM units were flooded 3 times by increasing the water level from 30 to 50 cm from mid-April to the end of May (only in the years 2020 and 2021, see Gerstle et al. 2023), before the implementation of the hydrological regime 2022. In 2023, the application rate was intensified, to increase hunger stress in the system, to 7 fortnightly applications starting from 14 April 2023 using the same field rates (Schöndorfer et al. 2025). Bti was only applied under windless conditions to avoid drift between ponds.

In 2022, 1 yr before the damselfly sampling, an altered hydrological regime was used in 6 of the 12 FPM units ("altered hydrology," AP 3,4,7,8,11,12) as additional stressor to simulate a climate change scenario during the developmental phase of the larvae (see Bauspiess et al. 2025). Briefly, from February to April 2022 (hydrological winter), the standard water level of the FPMs was set at 40 cm for both the control and altered hydrology FPMs. The water level of the deeper part of the FPM, opposite the inflow area, was used as a guide for water level changes. When the water level was low (20 cm), the inflow area fell dry (only half of the FPM under water). When the water level was high (70 cm) the inflow area was under water. Although top layer freezing of the FPM units can occur at the study site during winter, no ice formation was observed during the hydrological manipulation periods. Throughout the hydrological winter, the control FPMs were flooded 3 times (once every 4 wk), while the altered hydrology FPMs experienced 11 floodings (weekly), with water levels rising to 70 cm for 4 to 5 d during each flooding event. From May to August 2022 (hydrological summer), the standard water level for the altered hydrology FPMs was adjusted to 20 cm, while it remained at 40 cm for the control FPMs. During the hydrological summer, the control FPMs maintained the same hydrological regime as in winter, with a standard water level of 40 cm and floodings every 4 wk (4 floodings in total). In contrast, the hydrological regime for the climate change scenario FPMs was modified: the standard water level was reduced to 20 cm, and the flooding frequency was changed from weekly to every 4 wk, also resulting in 4 floodings. For both control and altered hydrology FPMs, summer floodings involved a rise in water level to 70 cm for 4 to 5 d.

To catch all insects, one large emergence tent (Greenhouse 4.5 × 3.0 × 2.0 m, vidaXL, Netherlands) was placed in the water–land floodplain area of each FPM from 22 May 2023 to 11 August 2023. In total, we collected 79 adult *C. puella* at the beginning of the emergence season on 13, 14, and 15 June 2023 with an insect net (mesh size = 1 mm). We selected damselflies depending on availability in the units and approximation of equal shares between treatments and sexes. This resulted in 40 individuals from Bti-treated FPMs (20 male, 20 female) and 39 from untreated FPMs (20 male, 19 female). In case of the altered hydrology, it resulted in 25 individuals from the altered hydrology FPMs (8 male, 17 female) and 54 from the control FPMs (32 male, 24 female). We then transferred the individuals into glass tubes and stored them in a cooling bag during the time in the field to reduce their activity and minimize wing damage. Subsequently, we froze them at −40 °C until further measurements.

Wing Photos

Photos of the imagines' wings were taken with a stereomicroscope and camera (Stemi 508, Carl Zeiss AG, Oberkochen, Germany, objective magnification: 0.63×, internal zoom: 1.0×, camera adapter magnification: 0.5×) and attention was paid to achieve the best possible contrast between veins and white background by adjusting the light and exposure time as well as limiting the histogram values. Twelve landmarks (Lm) were set with the software IdentiFly (version 1.5, Tofilski 2023) on the hind antenodal cross vein (Lm 1 and 2), a branch of the anal vein (Lm 3), the nodus (Lm 4), the cubital vein (Lm 7), 3 branches of a medium vein (Lm 8, 9, 10), and 2 outer sides of the pterostigma (Lm 11, 12). Landmarks at positions 5 and 6 covered the wing interior along the second radius (Fig. 1A, Supplementary Fig. S1). Before setting landmarks on the wing photos, it was trained on 60 independent wing photos. Created landmark files were transferred to *R* for further analysis.

After removing cut cells at the wing base and particles from the image, we adjusted the contrast by using the *brightness/contrast* and *levels* tool in GIMP 2.10 (The GIMP Development Team 2023) and reduced the resolution to 1,000 × 563 pixels for stable computation and effective cell detection (Supplementary Fig. S2).

Calculation of Geometric Features

We analyzed the wing images of front and hind wing pairs separately using WingAnalogy 4.0 (Eshghi et al. 2024), where vein coordinates were automatically aligned along the Y- and

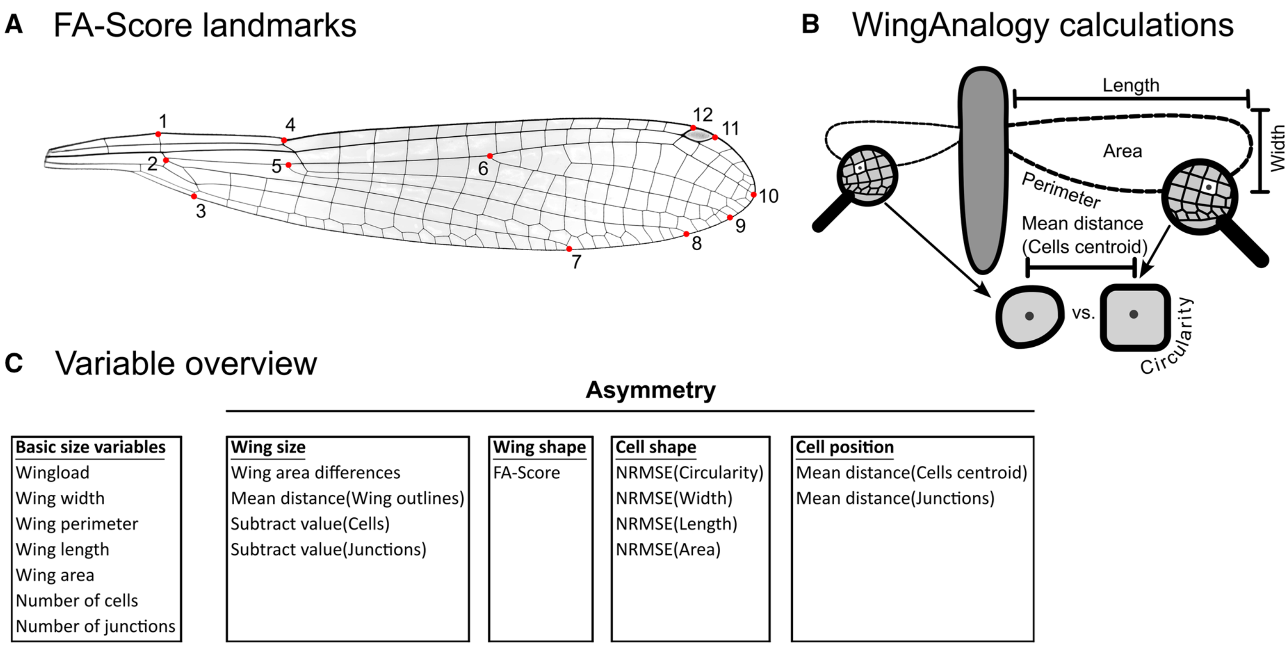

Fig. 1. Calculation of geometric features. (A) Landmark placement to calculate the shape FA-Score. (B) WingAnalogy calculation of geometric features. The overall wing area, length, width and perimeter were compared between the left and right wing. In addition, several cells feature like the circularity were compared between the 2 sister cells between the left and right wing. The distance between cells centroid of sister cells added another cell position asymmetry parameter. (C) Overview of all wing variables, including basic size variables and asymmetry variables.

X-Axis based on the average outline distance. Each wing was divided into 2 sections (proximal section: Set 1, distal section: Set 2) at the branching of the second and third radius, with the cell column containing the branch origin included in Set 1 (Supplementary Fig. S3). We extracted overall geometric features of the wings (Fig. 1B), including the area, perimeter, length, and width as the mean between the left and right wing of a pair. Before calculating the asymmetry, we extracted the circularity of cells as well as the location of junctions, and the number of cells and junctions. Then, the normalized root mean square error (NRMSE) between the area, length, width, and circularity of corresponding cells between the left and right wing were calculated to describe the wing pair asymmetry of an individual. The asymmetry data output also included the mean distance between wing outlines, junctions, and cell centroids, as well as the difference in the number of cells and junctions (subtract value), wing area, and perimeter differences. A mean from the asymmetry comparison in both directions (left/right and right/left) was used for the further analysis. To discuss the cell shape asymmetry of each individual between the left and right wing, we further use the cell shape parameters (NRMSE of cell area, length, width, and circularity). To discuss the cell position asymmetry of each individual, we further use the cell position parameters (mean distances of junctions and cell centroids). For an overview of compared wing variables, see Fig. 1C. We excluded the regression and standard deviation values of the WingAnalogy output to reduce the number of variables and potential Type II error.

To calculate the overall wing shape asymmetry, the previously mentioned landmarks were used. First, a procrustes fit was performed in *R* for females and males separately with *gpagen* (geomorph package, version 4.0.7, Adams et al. 2024). FA and shape components were calculated with bilateral symmetry (Adams et al. 2024) and an individual wing pair wise FA score was calculated as distance between FA component and mean symmetric shape with *calc_fageo* (facefuns package, version 0.0.0.9, Holzleitner and DeBruine 2020). FA-score outliers were visually inspected and all were integrated into the analysis after the possibility of measurement errors has been excluded.

To calculate the wing load, we divided the adult wet weight by the mean wing area of either the front or hind wings, resulting in 2 values per individual. A normal distribution of the measured values ruled out anti-symmetry and a zero-mean ruled out directional asymmetry (Supplementary Fig. S4).

Statistical Analyses

We used generalized linear models (stats package core, version 4.3.2, R Core Team 2023) for the response variables (see Supplementary Table S1) "Days until emergence," "Wet weight," "Wet weight gain," "Landmark FA-score," "Subtract value(Cells)," "Subtract value(Junctions)," "Wing area," "Wings area differences," "Wing length," "Wing perimeter," "Wing width," "Number of cells," "Number of junctions," "NRMSE(Area)," "NRMSE(Length)," "NRMSE(Width)", "NRMSE(Circularity)," "Mean distance(Cells centroid)," "Mean distance(Junctions)" and "Mean distance(Wing outlines)" and the explaining variable "concentration" (0 to 0.04 mg/l), and "sex" (f, m) to investigate the impact of the insecticide concentration. For the "Days until emergence," "Number of cells," "Number of junctions," "Subtract value(Cells)," and "Subtract value(Junctions)" a Poisson distribution with log link was used. All other variables were fitted with a gaussian distribution with an identity link. Significance of the generalized linear model was tested with a *t*-test for gaussian family models and Wald-test for Poisson family models (base package core, version 4.3.2, R Core Team 2023). For the mesocosm analysis, we took the stressors treatment (non-bti/bti), hydrology (control/altered hydrology), their interaction and sex (f/m) as explaining variables with the same response variables (except "Days until emergence" and "Wet weight gain," which were only available in the climate chamber experiment). Additionally, we applied likelihood-ratio χ^2 tests (car package, version 3.1.2, Fox and Weisberg 2019) to assess the overall effect of categorical predictors and interactions in the generalized linear models of the mesocosm. The normal distribution of the residuals was visually checked for the models using Q–Q plots and the homoscedasticity and linearity were checked using residuals versus fitted plots, whereas no major irregularities were observed (stats package core, version 4.3.2, R Core Team 2023). Percentage changes were calculated as the relative difference between the model-predicted mean of each group and the baseline group mean. Standard errors were derived from the standard deviation of the model predictions within each treatment combination, divided by the square root of the number of predictions per combination, and scaled relative to the baseline mean.

The spatial wing analysis was conducted to localize which regions of the wing contributed most to overall asymmetry. For this purpose, individual cell data and coordinates were extracted from the WingAnalogy project file. The shape difference (eg cell circularity) or distance (eg between cell centroids) between the individual sister cells in the left and right wing and vice versa was calculated before the mean was taken. Sister cells were assigned automatically by WingAnalogy. All wings were superimposed using rotation over the upper edge of the wing and the center of gravity before they were further superimposed on the X and Y axes using translation, ensuring a uniform coordinate system for all wings. For a more uniform visualization, the lower and upper 5% was excluded for the cell circularity and width asymmetry. A matrix corresponding to the coordinate system with 300 x 300 cells was then created as a mean for all the wings of a group and interpolated with *interp* (akima package, version 0.6.3.4, Akima and Gebhardt 2022). The matrices were subtracted to calculate the differences between stressor and control. To remove noise in the data for better visualization, matrices were gaussian blurred with *isoblur* and a sigma setting of 10 (imager package, version 0.45.8, Barthelme 2024).

Results

Insecticide

The measured concentrations of chlorantraniliprole were 0.053, 0.014, 0.003, and 0 mg/L at the beginning of the exposure and stayed relatively constant over the course of the exposure (Supplementary Fig. S5). Of the initial 160 individuals, a total of 125 successfully emerged (0 mg/L: 33, 0.0025 mg/L: 30, 0.01 mg/L: 28, 0.04 mg/L: 34) which resulted in 106 complete front wing pairs and 110 complete hind wing pairs after the exclusion of damaged wings due to injuries during the emergence. The larvae needed 28.2 d on average from the beginning of the experiment until emergence (0 mg/L: 28.3, 0.0025 mg/L: 28.0, 0.01 mg/L: 28.0, 0.04 mg/L: 28.5).

Figure 2 provides an overview of the predicted effect sizes expressed as averaged percentage changes relative to the

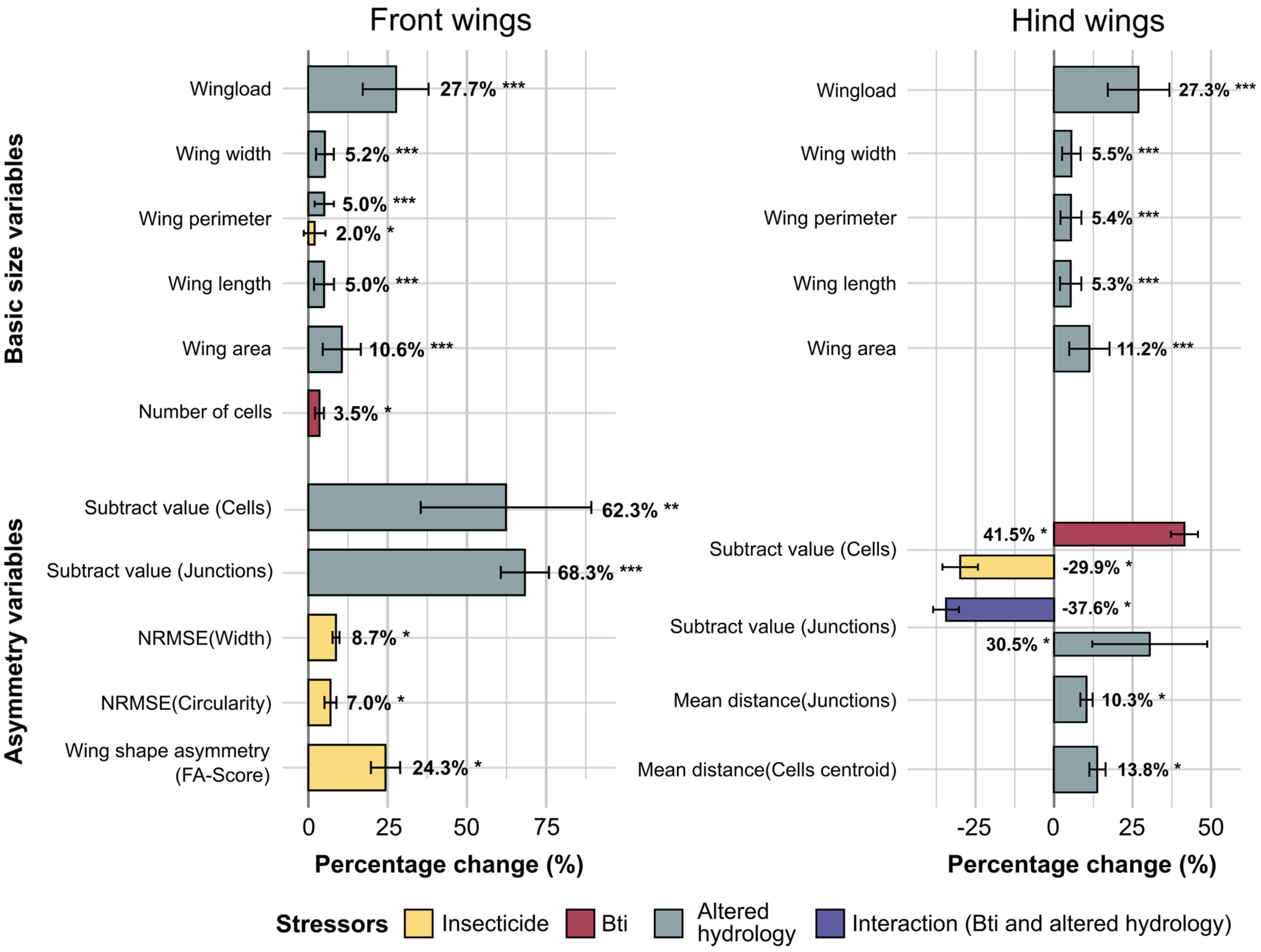

Fig. 2. The 3 stressors insecticide (yellow), Bti (red), altered hydrology (gray) and interaction of Bti and altered hydrology (purple), and their significant effect sizes are shown as predicted percentage changes relative to the respective control group. Respective percentage changes are averaged over the levels of the other factors, thus also representing both sexes. Error bars indicate standard errors based on variation in model predictions. Interaction values represent the effect of Bti compared to the control group, calculated under altered hydrology. Values refer to different response variables of the wing size (load, width, perimeter, length, area, and number of cells) and wing asymmetry (Cell shape asymmetry: NRMSE(Width), NRMSE(Circularity); cell position asymmetry: Mean distance of cells centroids and junctions, wing shape asymmetry: FA-score; count asymmetry: Subtract values). The predicted effect of the insecticide applies to a concentration of 0.04 mg/L relative to the control (0 mg/L). To test for effects of the insecticide, a generalized linear model was constructed for each response variable, with insecticide concentration and sex as explanatory variables. To test for effects of Bti and the altered hydrology, a likelihood-ratio χ^2 test was conducted for generalized linear models of each response variable, with Bti treatment, altered hydrology, their interaction and sex as explanatory variables. All model-based percentage changes, including nonsignificant ones, are shown in Supplementary Fig. S7.

control group, offering a comparative perspective across all significant variables and both sexes. No differences between insecticide concentrations were detected for the days until emergence ($z = 0.90$, $P = 0.369$) or wet weight gain during the exposure ($t_{1,122} = 0.06$, $P = 0.949$). Male individuals had overall less wet weight gain during the exposure, smaller wings, and lower wing load in addition to different wing asymmetry compared to females (see Supplementary Table S2). At 0.04 mg/L, front wing perimeter significantly increased by 0.77 mm (estimate, $t_{1,103} = 2.47$, $P = 0.015$), and cell shape asymmetry significantly increased with the front wing NRMSE(Width) increasing by 0.028 ($t_{1,103} = 2.08$, $P = 0.040$) and NRMSE (Circularity) by 0.024 ($t_{1,103} = 2.41$, $P = 0.018$). The mean predicted increase in significant cell shape asymmetry variables for both sexes ranged from 7.0% to 8.7% (Fig. 2). In addition, the subtract value of hind wing cells significantly decreased by 0.70 ($z = -2.40$, $P = 0.016$) at the highest concentration. Moreover, the wing shape asymmetry was higher at 0.04 mg/L with a significant increase in the FA score in front wings by 0.003 ($t_{1,103} = 2.49$, $P = 0.014$) resulting in a predicted increase of 24.3% (Fig. 2). Nonsignificant predicted percentage changes can be seen in Supplementary Fig. S7.

Bti and Altered Hydrology

After the exclusion of damaged wings, there were 26 front and 24 hind wing pairs of only Bti treated FPMs, 11 front and 10 hind wing pairs of only altered hydrology FPMs, 14 front and 13 hind wing pairs of the combined Bti and altered hydrology FPMs, and 52 front and 56 hind wing pairs of the control FPMs, which resulted in a total of 103 front and 103 hind wing pairs.

Mean predicted percentage changes of significant variables relative to the control groups for both sexes can be seen in Fig. 2. Similar to the insecticide, significant differences in several wing parameters were observed for Bti and altered hydrology. In Bti treatments, the number of cells in front wings significantly increased by 3.88 cells (estimate, χ^2 = 4.75, $P = 0.029$) in addition to the subtract value of cells in hind wings (χ^2 = 6.49, $P = 0.011$), resulting in a predicted increase of 3.5% and 41.5%, respectively (Fig. 2). There was also a trend of 1.17 mg heavier individuals (χ^2 = 2.98, $P = 0.084$) and a higher subtract value of hind wing junctions (χ^2 = 3.50, $P = 0.061$) in Bti-treated FPMs. Under altered hydrology, the body weight significantly increased by 3.36 mg (χ^2 = 12.04, $P < 0.001$), front wing load by 0.10 mg/mm^2 (χ^2 = 22.53, $P < 0.001$), wing area by 5.33 mm^2 (χ^2 = 20.42, $P < 0.001$), wing length by 1.00 mm (χ^2 = 19.11, $P < 0.001$), wing perimeter by 2.15 mm (χ^2 = 20.83, $P < 0.001$), and wing width by 0.13 mm (χ^2 = 18.32, $P < 0.001$). Predicted percentage changes due to altered hydrology for significant size variables ranged from 5% to 27.7% (Fig. 2). Similar significant effects of the altered hydrology on the wing size parameters were observed for the hind wings (Fig. 2, Supplementary Table S3). In addition, under altered hydrology, the wing asymmetry increased with significantly higher subtract value of cells in front wings (χ^2 = 31.25, $P = 0.001$), subtract value of junctions in front wings (χ^2 = 10.89, $P < 0.001$), subtract value of junctions in hind wings (χ^2 = 6.36, $P = 0.012$) as well as the mean distance of cell centroids in hind wings by 21.28 µm (χ^2 = 4.64, $P = 0.031$), and mean distance of junctions in hind wings by 16.37 µm (χ^2 = 3.94, $P = 0.047$). Predicted percentage changes due to altered hydrology for significant cell position asymmetry variables ranged from 10.3% to 13.8% (Fig. 2). Nonsignificant predicted percentage changes can be seen in Supplementary Fig. S7.

There only was one significant interaction between Bti treatment and the altered hydrology for the subtract value of hind wing junctions (χ^2 = 5.36, $P = 0.021$, Fig. 2). Post-hoc pairwise comparisons (Tukey adjustment) showed that individuals from the group with neither Bti nor altered hydrology differed significantly from all other groups. However, under Bti treatment, the positive effect of the altered hydrology was significantly lower (Bti and altered hydrology vs. non-Bti and altered hydrology, z = 2.969, $P = 0.016$). Male individuals showed smaller wings and less body weight and wing load in addition to a higher subtract value of front wing cells (see Supplementary Table S3).

Correlation and Localization of Structural Changes across the Wing

In both front and hind wings, significant positive correlations were observed between all cell shape (eg NRMSE [Circularity]) and all cell position asymmetry variables (eg Mean distance (Cells centroid), Supplementary Fig. S6). In addition, wing size parameters (eg wing area) correlated significantly positively with the body weight and number of cells and junctions. The cell position asymmetry correlated positively with several wing size parameters, see Supplementary Fig. S6 for more details.

Under insecticide exposure, Bti treatment and altered hydrology various changes in wing morphology and asymmetry were observed, with its effects varying both individually and spatially, depending on the specific area or section of the wing (Fig. 3). The distribution of these morphological changes showed the possibility of shifts: asymmetry increased and concentrated in specific areas within the wing while it decreased in others due to the environmental alterations. When looking at the spatial distribution of the insecticide affected cell circularity (Fig. 3A), the cell circularity difference (asymmetry) between left and right wings in control wings (0 mg/L) was concentrated at the edges of the wing. Compared to the control, the cell circularity difference of the insecticide treated individuals changed in a spatially scattered manner, with no distinct pattern, except for cells at the wing base, central cells at the branching of the second and third radius and cells next to the pterostigma. The cell width difference (asymmetry) between left and right wings in control wings was mostly present at the outer edge of the wing (Fig. 3B) and below the pterostigma. The asymmetry of the insecticide-treated individuals was higher at the central cells after the branching of the second and third radius and at the same outer edge area compared to the control. When looking at the spatial distribution of the cell centroid distance asymmetry (Fig. 3C), the control asymmetry was positioned at the center of the distal wing part and the rear outer edge. The asymmetry of the Bti treated individuals was higher at the front outer edge and lower in the proximal half of the wing compared to the control. The altered hydrology showed a concentrated higher asymmetry at the branching of the second and third radius and at the same outer edge area where large cells form. The individuals under combined Bti and altered hydrology stress showed higher asymmetry at the center of the distal wing part compared to the control. Compared to individuals of Bti only, the similar patterned area in the lower proximal half of the wing was higher with an opposite effect.

Discussion

We used a climate chamber and mesocosm experiment to study the effect of 3 different anthropogenic stressors on the wing morphology and asymmetry of the damselfly *C. puella*. The insecticide chlorantraniliprole caused an increase in asymmetry by means of the overall front wing shape (FA score) and cell shapes (NRMSE(width) and NRMSE(circularity)). The altered hydrology showed the strongest effects by increasing the wing size and load as well as the cell position asymmetry (mean distance of junctions and cells centroid) but not the overall wing shape. The mosquito control agent Bti increased the overall number of cells by 3.5%. The stressors affected individual traits and asymmetry to varying degrees, with spatially distinct effects across the wing. We conclude that anthropogenic stressors in aquatic environments influence damselfly wing morphology and asymmetry, suggesting a potential pathway of impacts into the terrestrial phase if these traits are functionally linked to terrestrial performance.

Insecticide Effects

Supporting our first hypotheses, the insecticide caused an increase in asymmetry of the front wing shape (FA score) and cell shapes (NRMSE(width) and NRMSE(circularity)). Unexpectedly, the insecticide increased overall front wing perimeter and left other wing size parameters rather unaffected. The larvae were exposed for 2 wk in their late larval stages which represents a relatively small time window in the life of the damselfly which is usually around 1 yr in duration under field conditions, with larvae hatching between mid-July and late August, overwintering in instars 6 to 9, and emerging the following summer (Waringer and Humpesch 1984). Nevertheless, damselfly larvae were exposed during the final steps of wing

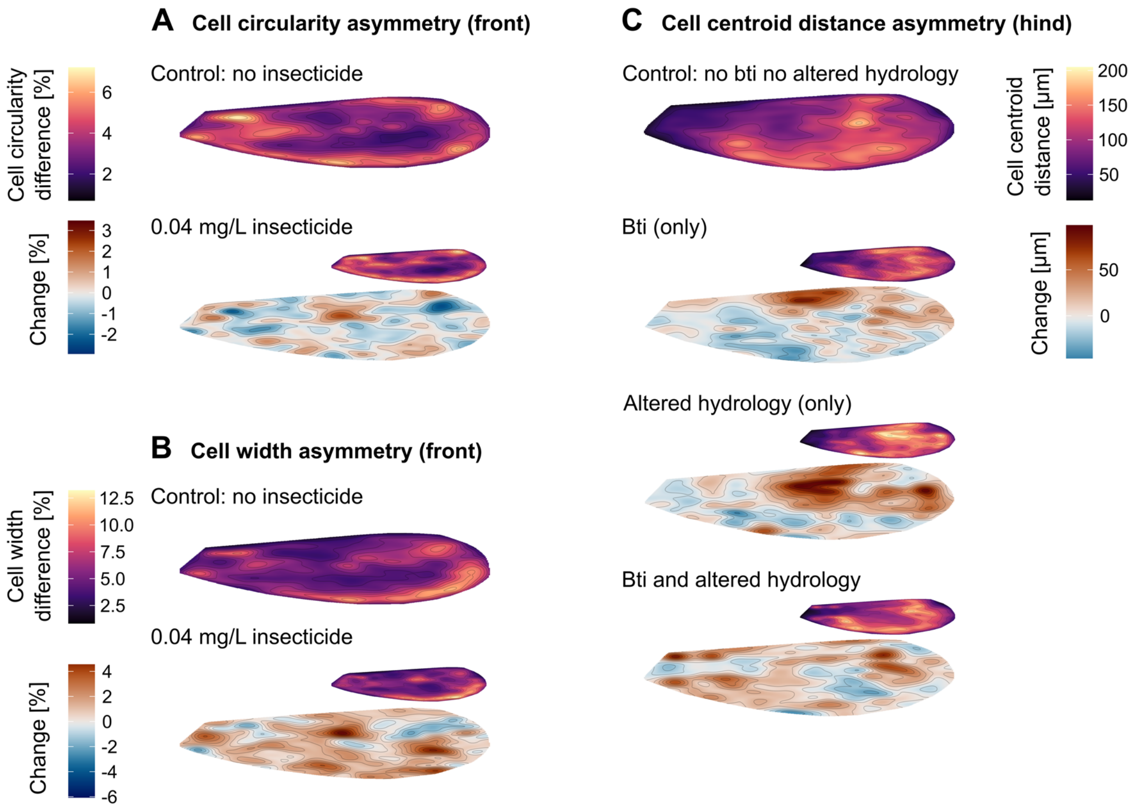

Fig. 3. Spatial expression of wing cell asymmetry in response to environmental stressors. Each panel visualizes asymmetry between left and right wings in different traits and treatment scenarios, focusing on the spatial structure of trait responses. (A) Front wing cell circularity difference (%) (asymmetry) under insecticide exposure. (B) Front wing cell width difference (%) (asymmetry) under insecticide exposure. (C) Hind wing cell centroid distance (µm) (asymmetry) in response to Bti application and/or altered hydrology. In each panel, using a black-purple-yellow scale, the upper colored wing shows asymmetry values for the respective control group, while the small colored wing shows the treatment group. The red–blue colored wing illustrates the change in asymmetry (%) between control and treatment, where red areas define an increase in asymmetry under stress.

development as an additional sensitive point of development at which stress can induce asymmetry: Larvae initiate critical pre-emergence processes such as wing sheath expansion and onset of melanization, indicating active wing tissue differentiation and structural maturation before emergence (Okude et al. 2021). This timing may amplify the effects of environmental stressors. The duration and timing of exposure are important. Prolonged exposure to a stressor can either cause an increase in asymmetry through sublethal, non-selective effects on development, or lead to lethal developmental selection for symmetry, which reduces levels of FA in the population (Beasley et al. 2013). The latter is especially the case for organisms with a stressful metamorphosis, which can erase the link between stress in the larval stage and adult asymmetry not by filtering out less fit individuals but by inducing an overall increase in asymmetry during metamorphosis itself (Campero et al. 2008). This pattern likely reflects the physiological challenges during emergence, where limited resources may be shifted toward processes of structural maturation, leaving reduced capacity to maintain developmental stability. As a result, metamorphosis acts as a levelling process that overrides prior stress signatures and leads to similar asymmetry levels across individuals regardless of their larval history. In our case, the insecticide exposure may have overlapped with a time window in which the development of the wings was already very advanced but the paralyzing mode of action may have complicated the final formation of cell/vein shape or overall wing shape by partially affecting the wing inflation process. Since in reality the larvae can be exposed to pesticides for longer periods of time, a possible effect on FA could be amplified under such conditions. Given the observed higher asymmetry of the front wings in *C. puella*, exposure to sublethal insecticide concentrations should be considered a factor that increases developmental stress and may directly affect the organism, either through morphological changes of important wing traits or through the higher energy demands needed to compensate for asymmetrical development. Pesticide exposure could have increased energy expenditure because of raised investment in detoxification and repair processes (Monteiro et al. 2019) and therefore shifted resources away from regulating developmental stability and maintaining bilateral symmetry. Maintaining a uniform cell size in all directions and maximizing area while minimizing material use is strongly determined by cell circularity (Rudolf et al. 2019). If maintaining equally high cell circularity—and thus material efficiency—across both wings is energetically demanding, in line with the theory of energetic costs of symmetry (Møller and Swaddle 2023), then deviations from symmetrical cell circularity could serve as indicators of larval developmental stress. Such stress might further trigger other behavioral responses associated with larval energy investment. An increase in asymmetry due to pesticide stress has been shown for coenagrionid larvae legs (Chang et al. 2007, Campero et al. 2008) but results for adult wings remain inconsistent (Hardersen et al. 1999, Hardersen 2000b, Campero et al. 2008). In our study, the insecticide exposure during the last larval stages led to a decrease of 0.70 subtract values of cells, with the effect being significant but confined to a small number of wing cell differences. Two individuals out of 29 control individuals with high subtract values of cells of 11 to 14 mainly drove this difference, probably explaining the observed effect. Individuals with high subtract values may have failed to emerge–similar to the negative effect on the subtract value observed under flooding conditions–which could suggest a selective mortality against highly asymmetric phenotypes due to the insecticide. However, this remains speculative, as no significant lethal effects were observed and the 6 mortalities at the highest concentration were comparable to those in lower concentrations and the control.

The increase in wing shape and cell shape asymmetry in our study was mainly driven by the highest insecticide concentration of 0.04 mg/L which is not in the range of environmental 1-time measurements in the same area (<0.014 µg/L, Mauser et al. 2025b). However, the environmental occurrence of this specific insecticide in combination with other complex pesticide mixtures over a longer time period could lead to sublethal effects on damselflies, that are not considered in pesticide authorization procedures. Nevertheless, they may alter key life-history traits, such as development, mobility, or reproduction success, which are essential for maintaining viable populations (Samways et al. 2025). Morphological alterations may have consequences for reproductive success, as recent work on *C. puella* demonstrated that wing morphology can be linked to short-term mating dynamics, although the direction and strength of this relationship may vary depending on local ecological conditions (Mauser et al. 2025a).

Altered Hydrology Effects

Contrary to our hypotheses, the altered hydrology showed the strongest effects by increasing the wing size and wing load instead of decreasing it. However, in accordance with our hypotheses, cell position asymmetry (mean distance of junctions and cells centroid) and cell count asymmetry (subtract value of cells and junctions) was higher in altered hydrology FPMs. Lower water depth and a reduced water volume, as it was simulated in the climate change scenario, may have led to higher temperatures and wider temperature fluctuations. A higher water body temperature can result in decreased egg development and larvae with smaller body size but increased growth rate after hatching (Frances et al. 2017). This increased growth rate could then be limited by a trade-off with asymmetrical development (De Block et al. 2008), as we observed it for the higher mean distance of junctions and cells centroid as well as the higher subtract value of cells in the altered hydrology. *C. puella* populations can be partly semivoltine (ie individuals may take more than 1 yr to complete their development), as observed for more northern populations or populations with high larvae density (Wildermuth and Martens 2019). Since a larger wing size was recorded in the altered hydrology, a limitation of growth due to temperature stress seems only likely as a result of larvae remaining in the ponds for another year and then emerge delayed with an extended growth phase and resulting larger wings. This would imply that water temperatures exceeded a supportive level, as moderately warmer conditions have been shown to promote univoltine over semivoltine development in damselflies (Raczyński et al. 2022). However, this scenario appears unlikely in our case, since no significant differences in the abundance of *C. puella* were recorded in the previous year (Schleihauf, not published), which would be expected if a substantial portion of the population had delayed emergence. Further changes in the water due to lower water levels could have led to a change in the development of *C. puella*: As volume declines, major changes can occur in habitat quality like the increase in individual density, temperature, conductivity (salinity), turbidity, and major nutrients (eg nitrogen and phosphorus) as well as the decrease of dissolved oxygen (Lake 2011). Habitat change can lead to adverse effects on food resources by altering their availability, quality, and spatial distribution within the water body, which in turn affect the consumers and community structure (Herbst et al. 2019). If prey density increased at lower water levels, *C. puella* could have benefited in their early larval stages in the year before emergence, which could explain the larger wing size due to fostered development. In addition to prey availability, the survival of *C. puella* larvae also strongly depends on shelter conditions: they typically inhabit shallow shoreline areas or vegetation where the availability of cover is critical for reducing mortality under high larval densities (Wildermuth and Martens 2019). Thus, both prey concentration, cannibalism and microhabitat structure may have influenced larval development under the altered hydrological regime, albeit via different mechanisms. In the FPMs with high water level dynamics, we observed change of the vegetation over the course of the flooding year with the shallow ends of the pools becoming more overgrown (floodplain area), especially with horsetail (*Equisetum*). Such changes in vegetation structure may have influenced microhabitat conditions for *C. puella*, potentially relating to altered patterns of developmental stability. Predator presence (eg Aeshnidae) is a common factor, determining the activity level and food foraging of coenagrionids (Mikolajewski et al. 2005). Reduced density of the top predator *Aeshna cyanea* in the altered hydrology, as it was recorded in the year before sampling (Schleihauf, not published), may further supported the development of *C. puella* and its wing size. A trade-off between growth and developmental instability in terms of increased fluctuating asymmetry (De Block et al. 2008) may explain the increase in both wing size and cell position asymmetry beyond their positive correlation due to scaling effects alone. In addition to larger wings in the altered hydrology, the wing load increased, which can be directly connected to the flight speed and endurance (Gyulavári et al. 2017). As shown for the scrambling damselfly *C. puella*, flight endurance, rather than flight speed, is the main target of sexual selection (Gyulavári et al. 2014). Individuals under hydrological alteration showed larger wings but also higher wing load, which has contradictory implications for flight performance and mating success. While larger wings may improve aerodynamic efficiency and potentially support flight endurance, higher wing loading generally increases flight speed but may reduce endurance (Gyulavári et al. 2014). If endurance is a key factor for mating success, higher wing load may not necessarily be beneficial. However, it is unclear to what extent the observed increase in wing size under altered hydrology may influence this relationship. Alternatively, if flight speed plays a larger role, higher wing loading could provide an advantage, highlighting potential differences in selective pressures. While altered hydrological conditions may result in short-term physiological shifts, such as increased body mass, wing size, and wing load, it is difficult to assess whether these changes are beneficial or detrimental for *C. puella*, as long-term selective consequences for flight performance and reproductive success remain uncertain. Even if *C. puella* benefits as a generalist species that copes better with hydrological alterations, a community shift in dragon and damselflies with a decline of more specialized species must be considered, as generalists tend to be favored under environmental change while specialists face increased extinction risk (Cerini et al. 2020). As shown, hydrological alterations can influence the morphological development of damselflies, which may also be linked to other indirect habitat alterations and species composition in the water body. Due to the overwintering of *C. puella*, hydrological alterations during early larval stages in spring and early summer resulted in morphological responses that became apparent upon adult emergence in the following year. This time-lag highlights the importance of considering delayed effects when assessing the ecological impacts of environmental change. In the context of climate change, the consequences of altered hydrology can extend beyond the aquatic environment, affecting adult stages after emergence. These cross-ecosystem impacts should therefore be explicitly considered in conservation planning.

Bti Effects

Contrary to our hypotheses, the mosquito control agent Bti significantly increased the mean number of cells per front wing by 3.5%, which is around 3 to 4 cells. Moreover, individuals emerging from Bti-treated FPMs tended to be heavier, with an average wet weight increase of 1.17 mg (~5% averaged over sexes). In addition, Bti increased the asymmetry by increasing the subtract value of cells, which would support our hypotheses. However, other asymmetry parameters did not significantly respond to the Bti treatment. Bti is known to not only impact the target mosquitoes but also to reduce the amount of non-biting midges (Chironomidae) (benthic kicknet sampling: Gerstle et al. 2023, emergence traps: Kolbenschlag et al. 2023) and overall Odonata abundance and species richness (Jakob and Poulin 2016). A reduction of Libellulidae by 54% and Coenagrionidae by 27% due to Bti was observed at the FPM study site by collecting exuviae 2 yr before our sampling (Gerstle et al. 2023). However, no significant effect of Bti on the abundance of *C. puella* was found in the year before sampling (emergence traps: Schleihauf, not published) and in the abundance of chironomids in the year of sampling and the year before (emergence traps: Schöndorfer et al. 2025). Aquatic odonate larvae can follow 2 different strategies: "sit-and-wait" species prioritize avoiding predators, resulting in slower growth, and actively foraging species that accept higher predation risks in exchange for faster development (Harvey and White 1990). The larvae of *C. puella* exhibit pronounced behavioral plasticity: depending on predation risk and the availability of perches, they reduce their active foraging to minimize the likelihood of detection and can therefore react flexibly to environmental conditions (Convey 1988). This underscores, that the impact of Bti on the complex aquatic food web of odonates and other participating predatory species (eg Nepomorpha, Hydrophilidae, and Hydrachnidiae) may depend on multiple factors and effects could occur immediately or with a delay. If Bti reduced chironomid abundance during the larval stages of sampled damselflies but it was not detectable via emergence traps, there could have been an increase in cannibalism or intraguild predation (see Gerstle et al. 2024). Smaller damselflies in early larval stages experience more cannibalism (Anholt 1994), which would have favored larger individuals by filtering out smaller ones and by reducing resource competition. This would be in line with our findings of an increased number of cells and body weight. More cells may require a higher number of supporting veins, thereby increasing material costs. Plastic responses due to resource availability have been observed in *Drosophila melanogaster*, where poor nutrition led to reduced wing size, which resulted from decreases in both cell size and number (Vijendravarma et al. 2011). From another perspective, Bti could also have had an effect independent of the direct abundance of chironomids in the system, such as changing the community composition of chironomids and thus the nutrient availability through prey for *C. puella*. In combination with the altered hydrology, however, further Bti effects may have been partly blurred, since both treatments may have influenced other species that are indirectly related to the development of *C. puella* or by simply washing out the Bti and other prey or competing organisms by draining the water. The observed significant interaction of the treatment and altered hydrology, showed a slight suppression of the effect of the scenario on the subtract value of junctions in combination with Bti. This interaction was likely driven by 2 control individuals with high subtract values of 11 to 14, which may have led the interaction to reach statistical significance.

Assessing Wing-Based Stress Indicators

The applied method for assessing wing morphology and asymmetry proved suitable for detecting the effects of multiple anthropogenic stressors, including the insecticide, Bti, and hydrological alterations, capturing both general and region-specific changes. Fluctuating asymmetry is often considered an indicator of environmental and genetic stress, but its predictive reliability depends on understanding evolutionary, ecological, and methodological contexts (Benítez et al. 2020). We showed, that stressors differentially affect specific facets of wing morphology and asymmetry, highlighting variation in the magnitude and nature of their impacts. In our case, simple measurements like the wing area or subtract value of the whole wings' cells but also the wing shape or cell shape asymmetry responded to environmental changes. Other parameters, like the cell position asymmetry parameters appeared to react only in the context of the altered hydrology but correlated with wing size. We demonstrated that, depending on the environmental context, wing size can be more strongly affected than asymmetry, indicating its value as a sensitive measurement parameter. In addition, our correlation analysis (Supplementary Table S6) suggests that some of the parameters examined, such as the mean distance between cell centroids, may be positively correlated with wing size. We further included adult wet weight as an additional parameter reflecting overall body size, which also showed a positive relationship with wing size (Supplementary Table S6). However, we did not measure other morphological body size metrics such as body length. This constitutes a potential limitation, as wet weight of insects can be influenced by nutritional status, body composition, or environmental conditions (Knapp and Knappová 2013), potentially confounding its interpretation as a body size proxy. The inclusion of additional structural size measurements, such as body length, could help refine the interpretation of size-related trait responses in future studies. However, relying solely on size measurements without considering asymmetry may lead to incomplete or misleading interpretations of developmental stress, as exemplified by the insecticide treatment, where wing and cell shape asymmetry increased while wing size remained unaffected. In addition, it also highlights the necessity of combining laboratory and field approaches to disentangle direct physiological effects from community-mediated or environmentally modulated responses and to avoid misinterpretation based on isolated experimental conditions.

The duration and timing of stress are important factors determining the development of asymmetry (Beasley et al. 2013). Damselfly larvae develop their wings throughout several instars but also during the last instar, larvae go through the important process of wing sheath expansion (Okude et al. 2021), which ultimately leads to many different possible targets for stress throughout the aquatic stage. When testing multiple response variables, risk of Type II errors increases. Ideally, the interpretation of stress effects should integrate information from multiple response variables simultaneously rather than overemphasizing isolated findings. Nevertheless, especially in methodologically modern studies, isolated findings can provide valuable insights into which aspects should be examined more closely in the future. Taking the analysis one step further, this includes not only comparing simple quantities but also considering patterns such as 2-dimensional spatial changes in asymmetry of a wing. As advancements in computer-assisted trait evaluation enable the generation of a vast array of potential trait measurements, a strategic and focused approach is necessary to effectively initiate analyses.

Conclusion

Our study demonstrates that computer-vision-assisted analysis of wing morphology and asymmetry is a suitable tool for detecting trait responses of damselflies to anthropogenic environmental changes. The insecticide increased wing shape and cell shape asymmetry, while altered hydrology affected overall wing size and cell position asymmetry. Bti exposure primarily increased cell number and tended to raise body weight. All 3 anthropogenic changes also influenced asymmetry via the subtract value of cells, although this metric was mainly driven by a few extreme values. Importantly, effects were not uniformly distributed across the wings but varied spatially and between traits. Overall wing size and asymmetry did not always respond in parallel, highlighting the need to assess multiple traits simultaneously.

As damselfly wings develop during the aquatic larval stage, their morphology can carry environmental signals into the terrestrial phase. This highlights their potential as indicators not only of aquatic ecosystem alteration but also of cross-ecosystem impacts, with possible consequences for terrestrial food webs and insect-mediated ecological functions.

Our findings contribute to a growing understanding of how agricultural pesticide use, climate-change-related hydrological shifts, and mosquito control agents may directly or indirectly affect trait expression in aquatic non-target insects. Future research should examine whether such trait alterations influence behavior, reproductive success, or species interactions and to what extent these responses are consistent across taxa and environmental gradients.

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Author Contributions

Ken M. Mauser (Conceptualization [equal], Data curation [equal], Formal analysis [equal], Investigation [lead], Methodology [lead], Writing—original draft [lead], Writing—review & editing [equal]), Samiksha Paudel (Investigation [equal], Methodology [equal], Writing—review & editing [equal]), Olivia Sigmund (Methodology [equal], Writing—review & editing [equal]), Martin H. Entling (Conceptualization [equal], Writing—review & editing [equal]), Jürgen Ott (Conceptualization [supporting], Methodology [supporting], Writing—review & editing [supporting]), and Carsten A. Brühl (Conceptualization [equal], Methodology [equal], Project administration [lead], Resources [equal], Supervision [lead], Writing—review & editing [equal])

Supplementary Material

Supplementary material is available at *Environmental Entomology* online.

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Conflicts of Interest

The authors declare no competing interests.

Data Availability

Data will be made available on request.

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14 APPENDIX V: PAPER 5

Short-term mating success in relation to fluctuating wing asymmetry in the male azure damselfly *Coenagrion puella*

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RESEARCH PAPER

Short-term mating success in relation to fluctuating wing asymmetry in the male azure damselfly *Coenagrion puella*

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ABSTRACT

As top flying predators, damselflies rely on wing symmetry and functionality. However, already during aquatic larval development, environmental stressors, including anthropogenic ones, can affect their development and impair wing morphology raising the question whether such alterations affect reproductive fitness. To investigate the role of wing morphology in mating success, we collected males of *Coenagrion puella* observed in copulation wheels (indicating short-term mating success) or actively chasing other wheel or tandem formations (unsuccessful mating attempts at the time of capture). Twenty individuals of each group were collected at two locations differing in environmental and anthropogenic pressures: one regularly used for recreational fishing and surrounded by agriculture in the Rhine Valley and one consisting of research ponds in the Palatinate Forest with restricted public access. Wing morphology and symmetry were assessed via computer-vision by comparing several cell shape and position dependent variables as well as wing size and number of cells and junctions using the recently developed software WingAnalogy. Mating males in the Palatinate Forest exhibited higher cell shape asymmetry than mating males in the Rhine Valley. In these individuals, the cell shape asymmetry was more pronounced in the distal part of the wing than in the proximal part. Mating males had lower body weight (-5%) and smaller wings (-3%) compared to chasing males at both locations. Our results challenge the general theory that stress-induced lower body weight and higher asymmetry diminish short-term mating success. Instead, they underscore the ecological importance of population-specific factors, like female availability or male-male competition, and environmental conditions that shape mating dynamics. Our results suggest that anthropogenic stressors in aquatic habitats have implications for terrestrial food webs by affecting the reproductive interactions of adult amphibiotic top predators, such as damselflies. This highlights the need to consider cross-ecosystem carry-over effects in ecological monitoring and conservation strategies.

Introduction

Dragonflies and damselflies link freshwater and terrestrial ecosystems as they have a larval stage in freshwaters and a terrestrial adult stage as flying top-predators. This amphibiotic life cycle has the ability to carry-over effects of larval conditions to shape adult fitness (Tüzün & Stoks, 2018). Environmental conditions and anthropogenically introduced stressors in surface waters like micropollutants, increased temperatures, or nutrient loads could affect Odonata development during their aquatic larval stage (Finckh et al., 2024; Meerhoff et al., 2022; Wolfram et al., 2023; Woolway et al., 2020). After metamorphosis, as terrestrial top-predators they rely on their wing structure and mobility (Bomphrey & Walker, 2022; Siepielski et al., 2022). Developmental alterations of wing morphology, e.g. wing symmetry, could lead to significant effects on adult behaviour. Understanding how stressors in aquatic environments shape the development and function of key traits in terrestrial adult stages is critical for linking habitat quality with ecosystem-level processes.

The fluctuating asymmetry of wings is the deviation from the perfect bilateral symmetry of wings and used as a proxy of development-stability (Benítez et al., 2020). Several studies showed, that stress can increase the fluctuating asymmetry (FA) of insect wings (Friedli et al., 2020; Hierlmeier et al., 2022), including damselfly wings (Hardersen & Frampton, 1999; Stoks, 2001). However, studies about the consequences

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on fitness and reproduction success as a result of reduced predation and flying ability, which is important during reproductive flights, led to contrasting results (Beck & Pruett-Jones, 2002; Carchini et al., 2000; Harvey & Walsh, 1993). Non-univocal results might be attributed to the assessment of simple morphological traits with high susceptibility to measurement error and lacking repeatability analysis (Simmons et al., 1999). In addition, the role of individual wing symmetry may be less essential for reproductive success if different local environmental conditions act as leading selective factors (Outomuro et al., 2016). Sexual selection, as a component of natural selection, can sometimes act in opposition to other selective pressures, as demonstrated in the case of wing shape in *Lestes sponsa* (Hansemann, 1823), where survival-based selection favoured individuals with long and slender forewings, whereas sexual selection favoured individuals with short and broad forewings (Outomuro et al., 2016). Many odonates are sensitive to environmental alterations in breeding habitats, such as pollution, eutrophication, or habitat loss, and are therefore often used as bioindicators to assess habitat quality (Šigutová et al., 2022). However, their role as valuable broad-spectrum indicator organisms highlights the complexity of understanding stress-reproduction relationships, as these can depend on various factors such as the type of stress, environmental conditions, and species-specific reproductive strategies (Samways et al., 2025). Morphological alterations of key species thus could help uncovering the response of coupled systems to complex, spatially and temporally variable anthropogenic pressures which is applicable to environmental management and policy (Schulz et al., 2024).

Unlike territorial species of damselflies, which rely on energetically costly flights for patrolling, fighting, and territorial defence, males of the family Coenagrionidae are non-territorial. They engage in scrambling competition, actively searching for females. This behaviour reflects their reproductive strategy, which differs from the courtship flights observed in territorial species (Fincke et al., 1997). While territorial species may rely on symmetry for stability and dominance in fights, non-territorial species depend more on speed and agility to locate females. Sexual selection by *C. puella* females on phenotypic traits such as markings or colorations may play a relatively minor role compared to the greater importance of scrambling intensity, especially when contrasted with territorial species (Thompson & Banks, 1989). After successful mating in the copulation wheel, males remain in tandem with the females to protect them from competitors. In species of the genus *Coenagrion*, unsuccessful competitors try to disturb pairs by chasing and assaulting (Cordero et al., 1995). The damselfly *C. puella* is a common species in central Europe, mostly univoltine and present in natural still waters as well as in urban- and agricultural areas (Wildermuth & Martens, 2019). This allows the assessment of its wing features, mating success in different habitats and environmental conditions.

In most studies, wing morphology was measured as a fraction of length and width or by positioning landmarks at vein intersections (Benítez et al., 2020). With the introduction of modern computing techniques, a direct conversion of wing images to wing feature and asymmetry data became possible, allowing a more detailed pattern exploration of deviations. Modern algorithms can automatically detect boundaries, identify junctions, and extract vein patterns (Eshghi, Rajabi, Poser, et al., 2024) which build a complex network that provides support and rigidity to the wing membrane (Rajabi et al., 2015). These interactions of veins, membranes and corrugations may considerably affect the dynamic deformation of insect wings during flight (Rajabi et al., 2015). Additionally it is suggested that insect wings are adapted not only for flight efficiency, but also for the tolerance of mechanical damage and abrasive wear (Rudolf et al., 2019). The 2D analysis of the entire wing in combination with the assessment of fluctuating asymmetry at different locations with different environmental conditions is an efficient way to assess the relationship between morphological traits and mating success in a more precise way. A standardised and accelerated collection of wing data could help to create comparability between studies and support the spatial data base for decision-makers.

In this study, we obtained complete 2D morphological data with the novel computer vision-based analysis software WingAnalogy (Eshghi, Rajabi, Matushkina, et al., 2024b) and compared body weight, wing area and cell shape and cell position asymmetry variables to the short-term mating success of the individuals at two sites: a site located in the Palatinate Forest, characterized by cooler temperatures and different anthropogenic activities (i.e., forested upland research site) compared to the site located in the Rhine Valley (i.e., anthropogenic lowland site). We photographed wings from 80 males of the damselfly *C. puella* which were either in a copulation wheel or unsuccessfully chasing other tandems or copulation wheels in the beginning of the mating season in May 2024. We hypothesized that the more intensive land use and anthropogenic pressures in the Rhine Valley – such as agriculture and fishing – would result in (1) higher wing asymmetry in the Rhine Valley compared to the Palatinate Forest. We further hypothesized that (2) mating males will show smaller wings, as the wing size positively correlates with the amount of asymmetry. In addition, (3) mating male damselflies, so presumably more fit than the chasing males, will show less wing asymmetry and (4) the same correlation pattern of short-term mating success and wing asymmetry will be observed at both locations. We also expect (5) higher asymmetry in the distal part of the wing compared to the proximal part, as the distal region is farther from the wing attachment point on the body. This greater distance likely creates more challenging growth conditions and involves a higher number of cells, which may contribute to increased asymmetry. Given the assumption that a separate consideration of wing parts and inclusion of several asymmetry parameters leads to a more detailed analysis, we follow the research question if positive and negative correlations between the included variables in this study exist.

Methods

Sampling locations

The study was conducted at two locations (Eußerthal and Sondernheim). The two sites were located 30 km apart in Rhineland-Palatinate in South-West Germany. The forested upland site in the Palatinate Forest is located at the Eußerthal research station's experimental ponds (FPM, Manfrin et al., 2023, N 49° 15′ 13.6836, E 7° 57′ 42.9984, 195 m a.s.l.) while the anthropogenic lowland site is located at the fishing ponds in Sondernheim in the agricultural Rhine Valley (N 49° 10′ 42.7188, E 8° 21′ 35.3628, 110 m a.s.l.). The sites were selected for their proximity, but contrasting landscape characteristics and degrees as well as the type of anthropogenic influence. Eußerthal is situated in the Palatinate Forest, a low-mountain area characterized by dense forests with limited human activity. Within a 500 m radius there is a cover of 97% forest and 3% urban areas, which proportions stay rather constant for a 2000 m radius with 94% forest, 1% agriculture and 3% urban area (Supplementary Fig. S1). The elevation and forest cover in Eußerthal led to slightly lower temperatures compared to Sondernheim (Mean air temperature March: Max 9 °C, Min 1 °C, 1980–2016, (Weather Spark, 2024)). Within a 500 m radius from Sondernheim there is a cover of 93% forest and 6% agriculture, which proportions change for a 2000 m radius to 52% forest, 26% agriculture and 4% urban area (Supplementary Fig. S1). Sondernheim is characterized by warmer temperatures compared to Eußerthal (Mean air temperature March: Max 11 °C, Min 3 °C, 1980–2016, (Weather Spark, 2024)). The ponds in Sondernheim were used as recreational fishing ponds with anthropogenic intervention and about 200 m to the north a continuous agricultural area begins. Furthermore, there is a cultivated field directly to the west of the ponds. In contrast, the 12 ponds in Eußerthal had no fish but six of them were treated with the mosquito control agent Bti (VectoBac WDG, 2.88 × 109 ITU/ha; Valent BioSciences, IL, USA) as part of ongoing research studies of the University of Kaiserslautern-Landau (Gerstle et al., 2023). In addition, the hydrological regime of 6 out of the 12 ponds was changed in the year before sampling in order of an ongoing climate change experiment, with the water levels being temporarily lowered to 20 cm in the summer months. Although minor temperature differences are likely between the locations based on elevation and land cover, our study did not directly assess thermal effects. However, sampling started later in the Palatinate Forest compared to Sondernheim, presumably due to the temperature-dependent delayed emergence of *C. puella.*

Sampling of male damselflies

We sampled a total of 80 male individuals of *C. puella* (Linnaeus, 1758) at the two locations. At each location, we captured 20 successfully mated and 20 unsuccessfully mated males using nets during their active period between 10am and 4pm. We identified males as successfully mated in short-term if they formed a copulation wheel with a female and unsuccessfully mated in short-term, but willingly to mate, if they chased the female after a copulation wheel or tandem (Carchini et al., 2000). Only males that fit in these two categories were included in this study, whereby individuals that did not participate in mating or chasing where not taken into account. We further refer to males observed in copulation wheels as “mating males”, and to males actively following or attempting to interrupt tandems or copulation wheels as “chasing males”. We transported individuals to the lab in 15 ml glass tubes in a cooling box and froze them at −20 °C after each sampling day. In the Rhine Valley the sampling took place from May 18th till May 26th 2023 and in the Palatinate Forest from June 5th till June 11th 2023.

Measuring of water parameters

At both sampling sites, we collected water samples (Rhine Valley: May 26, 2023; Palatinate Forest: June 11, 2023) in 250 ml plastic bottles. After collection, the bottles were frozen at −20 °C until further analysis. Prior to measurement, the samples were thawed and vortexed. We then determined the nitrate, phosphate, and copper content using test kits (NANOCOLOR® Nitrate 8, Phosphate 5 total, Copper 5, Macherey-Nagel GmbH & Co. KG) and measured pH and conductivity with a multiparameter analyser (Multi 340i, WTW, Weilheim, Germany).

Individual weighing and wing measurements

We weighed the individuals with a resolution of ± 0.01 mg (PBA224I-1x, VWR, Radnor, Pennsylvania, USA) after they were thawed for 5 min in a standardized way to minimize weight differences due to surface condensation. We did not measure dry body mass to preserve specimens for potential further morphological analyses. We then cut the front and hind wings at their base with small scissors. We took wing images with a stereomicroscope (Stemi 508, Carl Zeiss AG, Oberkochen, Germany, objective magnification: 0.63x, internal zoom: 1.0x, camera adapter magnification: 0.5x) with a resolution of 3840 × 2160 pixels. We paid attention to find the best possible vein contrast and a similar light setting for all wings. After we erased the cut cells at the wing base and impurities such as particles from the image, we increased the contrast in GIMP 2.10 (The GIMP Development Team, 2023) and reduced the resolution to 1000 × 563 pixels to achieve stable computing and cell detecting performance (Supplementary Fig. S2). We analysed front and hind wing pairs separately with WingAnalogy version 4.0 (Eshghi, Rajabi, Matushkina, et al., 2024a) and automatically superimposed the vein coordinates on the Y- and X-Axis via the mean distance of the wings outline. We separated each wing into two halves (proximal part of the wing: Set 1, distal part of the wing Set 2) at the split of the 2nd and 3rd branch of the radius, whereby we added the cell column with the origin of the split itself to the Set1 (Supplementary Fig. S3).

Calculation of geometric features

We extracted the geometric features of the wings including the area, length, width, and circularity of cells, the location of junctions, and the number of cells and junctions. WingAnalogy is a computer vision-based tool, developed in MATLAB to automate the process of wing image segmentations and to increase the precision by reducing human intervention in measuring the geometric features from the wing image. In the very first step, the embedded algorithms within WingAnalogy extract the above-mentioned geometric features from the wing. Two main algorithms encompass the region-growing for extracting the wing, and its cell boundaries, and the thinning method for detecting the place of junctions. Another tool is embedded in WingAnalogy for automated superimposing wings using Particle Swarm Optimization (PSO) until the overall distance between the wing outlines is minimized. Corresponding cells are found as cell couples, with minimum centroid distance. The normalized root mean square error (NRMSE), and the regressions between the area, length, width, and circularity of corresponding cells are some criteria of the wings’ asymmetry. The output also includes the mean distance and standard deviation between wing outlines, junctions, and cell centroids, as well as the difference in the number of cells and junctions, wing area, and perimeter differences. A mean from the comparison in both directions (left/right and right/left) was used for further analysis. For the mean shape difference of each individual wing cell between left and right wing, we used the cell shape parameters (cell area, length, width, and circularity). For the mean position difference of each individual wing cell between left and right wing, we used the further called cell position parameters (mean distance for outlines, junctions, and cell centroids). For each individual, the difference in the number of cells and junctions between the left and right wings was calculated as the variables “Subtract value (Cells)” and “Subtract value (Junctions)”, respectively. We excluded the values regression and standard deviation of the WingAnalogy output to minimize the number of variables and potential β-error. A normal distribution of the calculated asymmetry values ruled out anti-symmetry and a zero-mean ruled out directional asymmetry (Supplementary Figs. S3 and S4).

Statistical analysis

One front wing pair from the Rhine Valley was excluded due to strong vein mutations. We used linear models from the stats package core (version 4.3.2) for R (R Core Team, 2023) to assess the effect of location (Palatinate Forest/Rhine Valley), mating (mating males/chasing males) and their interaction on the following basic metrics: Body weight, Wing area, Wing length, Wing perimeter, Wing width, Number of cells, Number of junctions as well on the following asymmetry parameters: Subtract value(Cells), Subtract value(Junctions), Wings area differences, NRMSE(Area), NRMSE(Length), NRMSE(Width), NRMSE (Circularity), Mean distance(Cells centroid), Mean distance(Junctions) and Mean distance(Wing outlines) (see section above). Afterwards, we conducted an analysis of variance (ANOVA, F-statistic) using the stats package core (version 4.3.2, R Core Team, 2023) for the previously built linear models which assessed the effects of location and mating on the wing morphology variables. In case of significant factor interaction (location and mating) we used a pairwise analysis as post-hoc test from the (“emmeans” function) emmeans package (version 1.10.3, Lenth, 2024) for R, with Tukey adjustment for p-values to account for multiple testing. We used *t*-tests from the stats package for R to compare the asymmetry quantities between Set 1 (proximal wing) and Set 2 (distal wing). The normal distribution of the residuals was checked for all models visually using Q-Q plots and the homoscedasticity and linearity were checked using residuals vs fitted plots. For the variables “Subtract value (Cells)” and “Subtract value (Junctions)” a $\log(x + 1)$ transformation was applied to approach normal distribution for the linear model fit. We conducted a Spearman correlation using the stats package for R between all wing metrics and the body weight. Plots were built with the package ggplot2 (version 3.5.1, Wickham, 2016) for R.

Results

Water parameters

In the Palatinate Forest, we measured a low nitrate concentration of 2.4 mg/L, while the phosphate and copper concentrations were both below detection limits (<0.2 mg/L for phosphate and <0.1 mg/L for copper). The Palatinate Forest showed a pH of 9.28 and a conductivity of 116 μS/cm. In the Rhine Valley, nitrate levels were below the detection limit (<1.3 mg/L), as were phosphate and copper concentrations (<0.2 mg/L and <0.1 mg/L, respectively) and we recorded a pH of 9.08 and a conductivity of 465 μS/cm there.

Basic values: body weight and wing size

Independently of the location, mating males had a significantly lower (−1.29 mg) body wet weight than chasing males ($F_{1,79} = 6.04$, $p = 0.016$). Males collected at the Palatinate Forest were 4.63 mg lighter than males from the Rhine Valley ($F_{1,79} = 67.80$, $p < 0.001$). Mating males compared to the chasing ones had −2.87 mm^2 smaller front wing area ($F_{1,78} = 4.60$, $p = 0.035$) and −2.74 mm^2 smaller hind wing area ($F_{1,79} = 4.37$, $p = 0.040$). In the Palatinate Forest, we found that individuals had a smaller front wing area of −7.54 mm^2 ($F_{1,78} = 55.69$, $p < 0.001$) and smaller hind wing area of −6.89 mm^2 ($F_{1,79} = 50.23$, $p < 0.001$), compared to the Rhine Valley. The wing lengths, perimeters and widths showed lower values in the Palatinate Forest compared to the Rhine Valley and in mating compared to chasing males, independently of the location (Fig. 1, Supplementary Tables S1 and S2). For the front wings, a significant interaction between mating and location was found for the number of junctions ($F_{1,78} = 13.14$, $p = 0.001$) and cells ($F_{1,78} = 9.44$, $p = 0.003$) (Fig. 2A, B). Hereby, it was significant that mating males, compared to chasing males in the Palatinate Forest had −10.28 junctions ($t_{75} = -2.92$, $p = 0.024$) and −6.53 cells less ($t_{75} = 2.77$, $p = 0.035$) and mating males in the Palatinate Forest compared to mating males in the Rhine Valley had −10.25 junctions ($t_{75} = -2.91$, $p = 0.024$) and −7.30 cells less ($t_{75} = -3.10$, $p = 0.014$). This interaction could not be observed for the front wing area ($F_{1,78} = 0.97$, $p = 0.327$) (Fig. 2C). With the same pattern in hind wings, significant interaction between mating and location was found for the number of junctions ($F_{1,79} = 10.52$, $p = 0.002$) and cells ($F_{1,79} = 17.55$, $p < 0.001$) but not for the area ($F_{1,79} = 1.45$, $p = 0.232$). Additional information is given in Supplementary Table S3 and Supplementary Fig. S6.

Cell shape asymmetry

Individuals from the Palatinate Forest showed higher asymmetry in cell shapes between left and right front wings compared to the Rhine Valley: The NRMSE of the width was significantly 9.6% higher in the

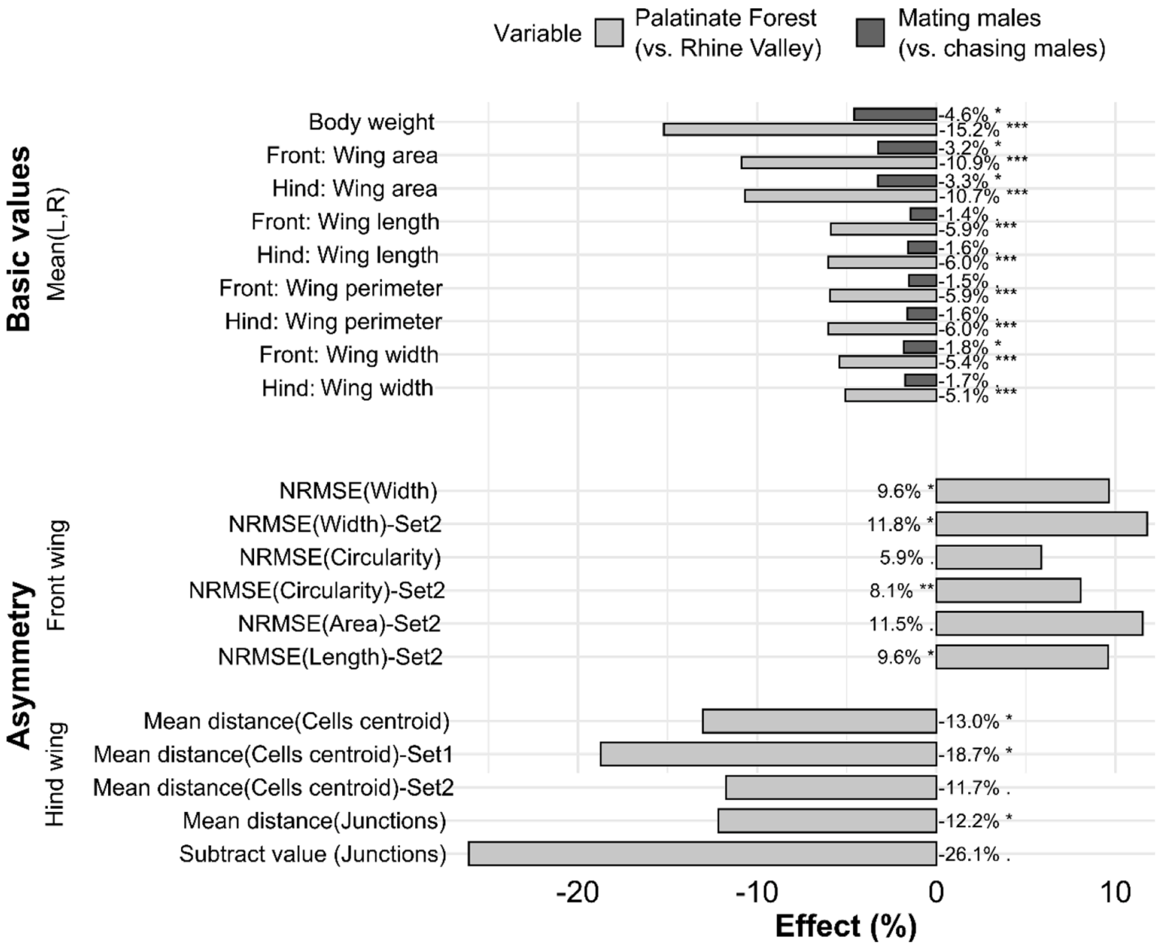

Fig. 1. Barplot of significant effects of mating (mating males vs. chasing males) and location (Palatinate Forest vs. Rhine Valley) on different response variables: the basic values describe the mean of left and right wing regarding weight and size variables, the Normalized Root Mean Squared Error (NRMSE) describes the mean cell shape asymmetry (difference between left and right wing); the mean distance of cells centroid or junctions describes the cell position asymmetry. Significant effects indicated with an asterisk; trends indicated with a dot. Response variables without significant effects of mating or location excluded from this figure. Results obtained from a linear model using mating, location and their interaction as variables. Total number of analysed front wings = 79 and of hind wings = 80. Wings were separated in two halves (proximal: Set 1, distal: Set 2).

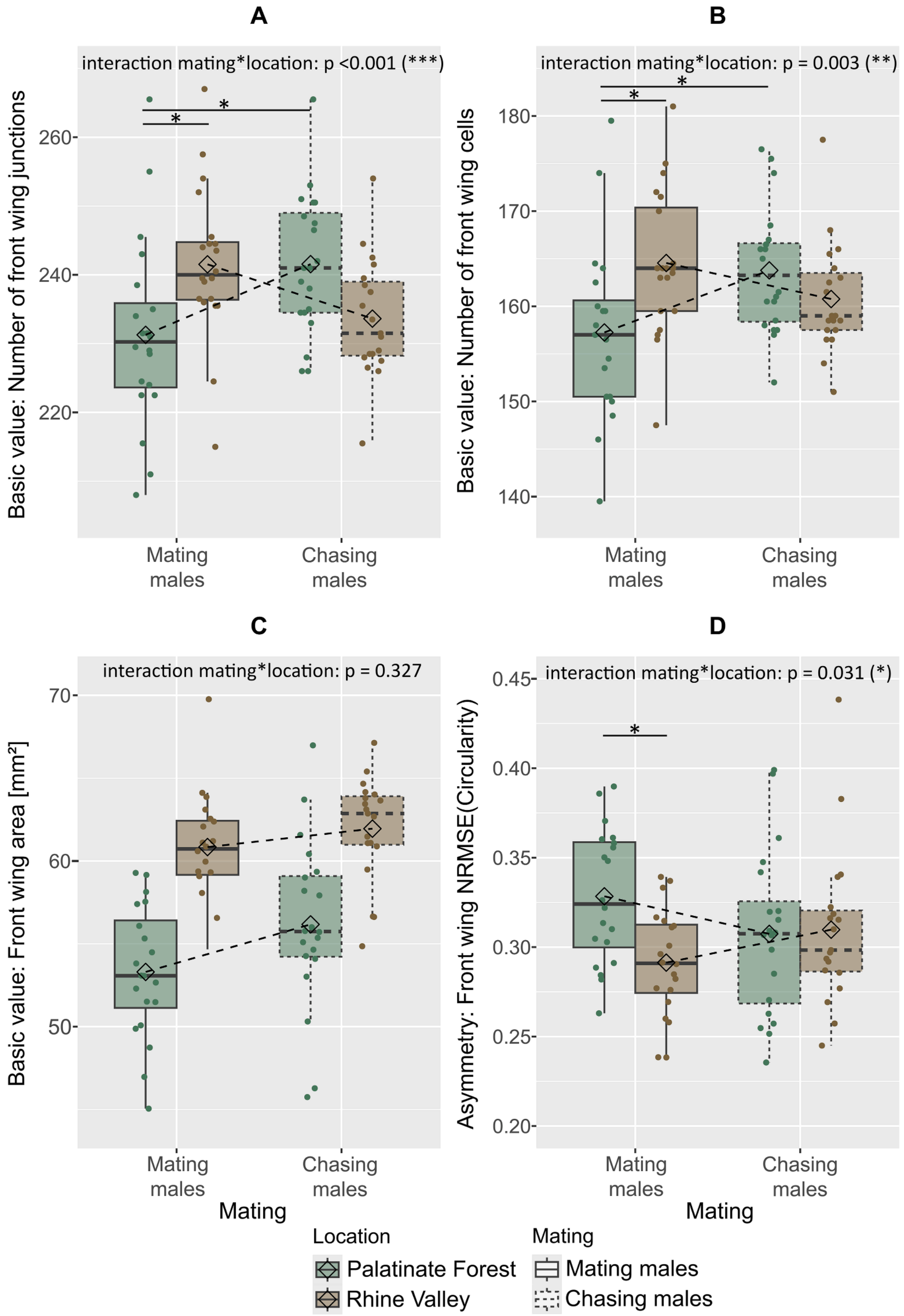

Fig. 2. Boxplots showing the basic front wing values: number of junctions (A), number of cells (B), and area (C), as well as cell shape asymmetry quantified as NRSME of circularity (D) for the mating males (solid outer line) and chasing males (dashed outer line) male damselflies. Location Palatinate Forest indicated with green colour; location Rhine Valley indicated with brown colour. Each dot represents a damselfly with a total of 40 damselflies in the Palatinate Forest and 40 in the Rhine Valley. One front wing pair was excluded due to strong vein mutations. Trendline indicated with a dashed line, means indicated with squares. Interaction p-value obtained from an ANOVA of a linear model of the response variable and the explaining variables are mating, location and their interaction. Statistical significances are indicated with significance levels.

Palatinate Forest than in the Rhine Valley ($F_{1,78} = 3.85$, $p = 0.049$). Similarly, The NRMSE of the circularity was 5.9% higher in the Palatinate Forest than in the Rhine Valley ($F_{1,78} = 3.85$, $p = 0.053$) although the difference was just not statistically significant. We found for the distal front wing (Set 2), in the Palatinate Forest compared to the Rhine Valley, 11.8% higher NRMSE of the cell width ($F_{1,78} = 4.16$, $p = 0.045$), 8.1% higher NRMSE circularity ($F_{1,78} = 7.52$, $p = 0.008$) and 9.6% higher NRMSE length ($F_{1,78} = 4.05$, $p = 0.048$) and as a trend +11.5% of the NRMSE area ($F_{1,78} = 2.79$, $p = 0.099$). This pattern was not observed for the complete or proximal (Set 1) and distal (Set 2) parts of hind wings (Supplementary Tables S1 and S2). There was a significant interaction between mating and location for the NRMSE (circularity) of the front wings ($F_{1,78} = 4.82$, $p = 0.031$) (Fig. 2D) and as a trend in hind wings ($F_{1,78} = 3.03$, $p = 0.086$). Hereby in front wings, mating males in the Palatinate Forest showed compared to mating males in the Rhine Valley a 12.72% higher NRMSE (circularity) ($t_{75} = 2.94$, $p = 0.022$). Although not significant, mating males in the Palatinate Forest showed compared to chasing males in the Palatinate Forest a 6.83% higher NRMSE (circularity) ($t_{75} = 1.66$, $p = 0.349$) and mating males in the Rhine Valley showed compared to chasing males in the Rhine Valley a −6.83% lower NRMSE (circularity) ($t_{75} = 1.66$, $p = 0.478$), resulting in a reversed but not significant effect of mating. An interaction of location and mating with focus on Set 2 of the front wings was also significantly present ($F_{1,78} = 6.68$, $p = 0.012$). Hereby, mating males in the Palatinate Forest showed a 15.96% higher NRMSE (circularity) in Set 2 ($t_{75} = 3.77$, $p = 0.002$) compared to chasing males in the Rhine Valley. Although not significant, mating males in the Palatinate Forest showed compared to chasing males in the Palatinate Forest a 9.53% higher NRMSE (circularity) in Set 2 ($t_{75} = 2.38$, $p = 0.09$) and mating males in the Rhine Valley showed compared to chasing males in the Rhine Valley a −5.22% lower NRMSE (circularity) in Set 2 ($t_{75} = -1.28$, $p = 0.577$). Additional information is given in Supplementary Table S3 and Fig. S6.

Cell position asymmetry

Individuals from the Palatinate Forest showed compared to the Rhine Valley significantly lower asymmetry in cell position between left and right hind wings: The mean distance of cell centroids and junctions was in the Palatinate Forest compared to the Rhine Valley significantly lower with −11.63 µm ($F_{1,79} = 4.28$, $p = 0.042$) and −10.44 µm ($F_{1,79} = 5.53$, $p = 0.021$), respectively. We also found a significantly lower asymmetry in the Palatinate Forest than in the Rhine Valley in proximal part of the hind wing (Set 1) for the mean distance of cell centroids with −21.05 µm ($F_{1,79} = 5.49$, $p = 0.022$). Although not significant, we found that the mean distance of cell centroids in the distal part of the hind wing (Set 2) was compared to the proximal hind wing (Set 1) −7.17 µm ($F_{1,79} = 3.12$, $p = 0.081$) smaller in the Palatinate Forest compared to the Rhine Valley (Supplementary Tables S1 and S2). No significant effects of mating were found for the cell position asymmetry values.

Proximal vs. distal part of the wing

A mean significant effect of +51.79% higher asymmetry was found in the distal half (Set 2) of front wings, compared to the proximal half (Set 1) for the NRMSE of cell area (+44.8%, $t_{78} = -9.60$, $p < 0.001$), length (+78.51%, $t_{78} = -31.50$, $p < 0.001$), width (+42.13%, $t_{78} = -12.77$, $p < 0.001$), circularity (+69.77%, $t_{78} = -42.70$, $p < 0.001$), and the mean distance of cells centroids (+12.03%, $t_{78} = -3.24$, $p = 0.002$). A similar significant pattern with 51.40% higher asymmetry in the distal half compared to the proximal half was observed for the hind wings (Supplementary Table S4).

Correlation of asymmetry parameters

All *cell shape* asymmetry values (the NRMSE of the cell area, length, width and circularity) were positively correlated in both front and hind wings (Fig. 3). The same positive correlation applied to the *cell position values* (the mean distance of cell centroids and junctions). Positive correlations were also found for the *cell shape* and *cell position* asymmetry values. The mean distance of wing outline only correlated positively with the cell shape asymmetry values. With a greater hind wing size (area, length, perimeter and width) a higher asymmetry of the mean distance of cell centroids and junctions in hind wings was found. Additionally, heavier individuals had a greater front and hind wing size as well as more cells and junctions.

Discussion

We showed that body weight, wing morphology and symmetry of emergent damselflies varied among locations, suggesting that environmental conditions during the larval stage influence development. We identified lower body weight and smaller wing size (area, length,

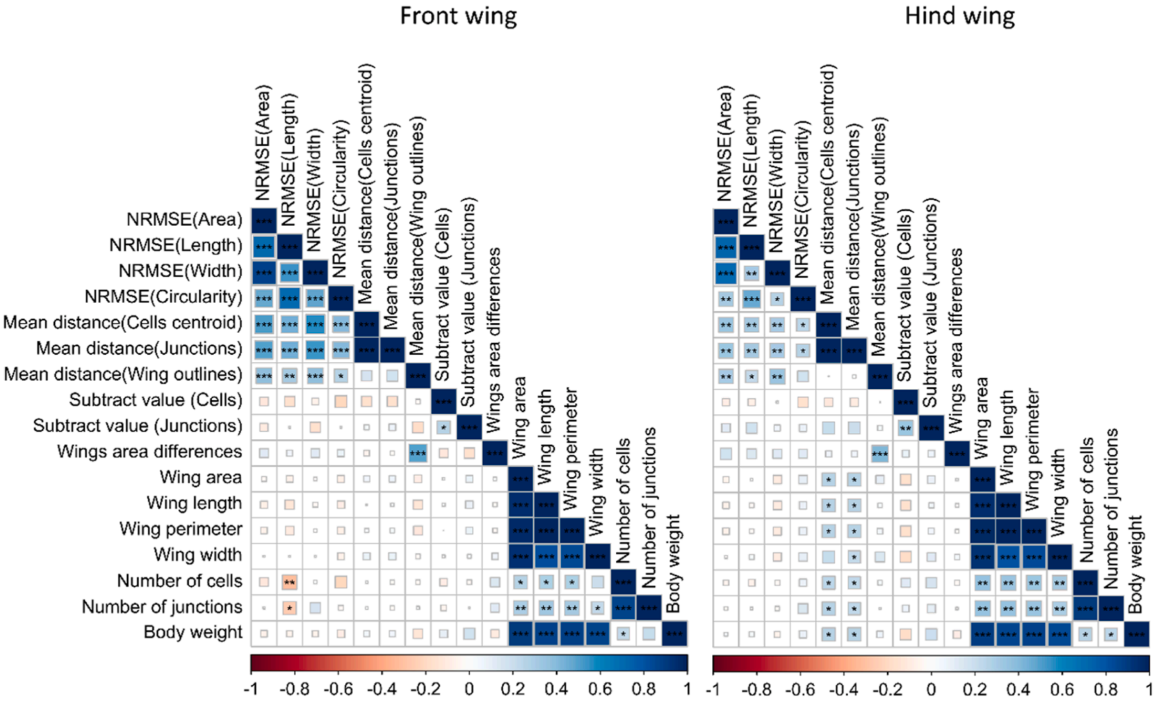

Fig. 3. Spearman correlation plot for the variables NRMSE of cell area, length, width and circularity, mean distance of cells centroid, junctions and wing outlines, the subtract values of cells and junctions, wing area differences wing area, length, perimeter and width, number of cells and junctions and body weight. Reddish colour indicates a negative, blueish a positive Spearman correlation. Squares with a star indicate a significant spearman correlation depending on the significance level ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***).

perimeter and width) in a population as predictors of short-term male mating success. Additionally, the general number of cells and junctions interacted independently from wing size parameters with the location and mating. Contrary to our expectations, we observed higher cell shape/circularity asymmetry of mating males in the Palatinate Forest than in the Rhine Valley site which we assumed to be more strongly influenced by anthropogenic pressures due to its intensive land use and recreational use. We could elaborate the advantages of computer-vision based analyses and relevance of wing features for short-term male mating success by separate consideration of the whole, proximal and distal wing as well as identification of correlated and uncorrelated features.

Both locations showed values of nitrate, phosphorus and copper below or close to the measuring range. The pH and conductivity were also within the tolerance range for *C. puella* (Schlüpmann, 1995). Although the measured water chemistry was rather similar at both sites during sampling, anthropogenic pressure may be not solely reflected in these parameters. The Rhine Valley is part of vast agricultural and urban activities, whereas the Palatinate Forest is a forested, less fragmented landscape. We therefore interpret anthropogenic influence as a broader context of land-use, habitat fragmentation, and potential long-term exposure, beyond momentary water parameters. Despite the similarity in the analysed environmental parameters and contrary to our first hypotheses of an overall higher asymmetry in the more anthropogenically influenced Rhine Valley, we found trait-specific responses to location: individuals from the Palatinate Forest had lower body weight and wing size but higher cell shape asymmetry, whereas Rhine Valley individuals showed higher cell position asymmetry. These differences might reflect genetic variation between the populations, local adaptations to their respective habitats, or phenotypic plasticity, which may in turn contribute to asymmetry under environmental stress. Damselflies have been shown to develop larger wings with a decreasing mean seasonal temperature over time whereas variability in wing structure is also sex- and species-specific (Wonglersak et al., 2020). The body weight of *C. puella* was shown to increase with latitude along a 640 km transect through the United Kingdom, confirming a negative correlation with temperature and aligning with the general theory of allometric scaling (Hassall et al., 2008). Contrasting to this, the Palatinate Forest, which is assumed to have a lower average temperature, showed lower body weight and wing size presumably due to other factors than allometric scaling. Lower body weight and delayed emergence can be caused by low food availability, as it has been previously shown in a laboratory experiment (Anholt, 1990). This could be due to the Bti treatment of 6 out of the 12 ponds, which, as shown in a previous experiment at the same location, led to a reduction of chironomid larvae, a potential food source for Odonata larvae (Gerstle et al., 2023). Additional to smaller wings, individuals in the Palatinate Forest showed lower cell position asymmetry compared to the Rhine Valley, probably due to the measured positive correlation with wing size. However, there was no significant correlation between cell shape asymmetry and wing size, indicating a more size independent asymmetry formation. If the higher cell shape asymmetry that we found in the Palatinate Forest compared to the Rhine Valley was more related to stress response it could be explained by the hydromorphological conditions of the artificial ponds. Although we did not directly measure temperature fluctuations, the small size of the ponds and therefore less water mass may induced stress due to more frequent and intense temperature fluctuations compared to the larger ponds in the Rhine Valley as smaller water bodies show less thermal buffering (Martin, 1972). The effect of temperature variability may have been further amplified by hydrological manipulation in which water levels in 6 out of the 12 ponds were temporarily lowered to 20 cm as part of another study. Warm waters can result in smaller larvae with increased growth (Frances et al., 2017), whereas an increased growth rate could be then limited by a trade-off with asymmetrical development as it could be observed for female damselflies (De Block et al., 2008). The delayed emergence in the Palatinate Forest, which also restricted our sampling, suggests a shorter larval development window, which may have limited growth rates, potentially due to site-specific conditions such as lower mean temperatures or forest shading. Aquatic *C. puella* larvae are able to accelerate their development under time constraints simulated with shorter photoperiods and pre-winter food shortages (Mikolajewski et al., 2015) possibly because late transitions toward the adult stage result in worse conditions for survival of organisms with complex life cycles (Altwegg & Reyer, 2003). While it is well established that environmental stress can influence the symmetry of traits, some aspects of this relationship remain debated (Benítez et al., 2020). For instance, it has been theorized that a comparatively lower asymmetry might paradoxically indicate higher stress levels—particularly if stress occurs before the developmental window during which asymmetries can form, thus selectively removing the most developmentally unstable individuals from the population (Hardersen & Frampton, 1999). In our data, such a mechanism might underlie some of the observed patterns, as the effect of location on asymmetry differed between traits—suggesting that selective mortality could have acted more strongly on specific aspects of morphology. Nonetheless, while odonate community-level shifts have proven useful to monitor degradation (Datto-Liberato et al., 2024), our detected morphological differences between the location may add species-specific resolution as early stress signals in the Palatinate Forest and Rhine Valley.

Supporting our second hypotheses of a negative correlation of body weight or size and short-term mating success, mating males were lighter and smaller. However, there was an interaction between the number of cells and junctions, which independently interacted with the location. An early study on *C. puella* already showed that body size is positively correlated with the lifespan in males and females, but negatively correlated with the daily mating rate in males (Thompson, 1989). Consistent with these observations, we additionally found that smaller wing area, length, width and perimeter correlate with smaller body weight and predict higher short-term mating success. Low energy consumption and high flight manoeuvrability is suspected to be the result of low body weights (De Block & Stoks, 2007). Allometric scaling laws dictate that the surface area of an animal’s body scales to the 2/3 power of its body volume/mass, while linear measurements scale to the 1/3 power of body volume/mass (Schmidt-Nielsen, 1984) which means that smaller individuals have unproportionally larger wing area in comparison to their mass. Also, smaller individuals have unproportionally larger cross-sectional area of their muscles in comparison to their body mass, generating more potential for force production (Schmidt-Nielsen, 1984). The observed interactions of mating and location on the number of cells and junctions, represent new parameters that apparently are related to short-term mating success but their contribution to this success was locality-dependent. This underlines the need to consider population-specific trait-environment interactions in ecological forecasting and species conservation, especially under scenarios of increasing land use heterogeneity and climate-driven habitat shifts (Urban et al., 2016). For dragonfly and damselfly wings there is a functional trade-off between cell size and wing size: smaller cells may lead to better crack resistance due to increased structural compartmentalization, but also require a higher number of supporting veins, thereby increasing material costs (Eshghi, Rajabi, Shafaghi, et al., 2024; Rajabi et al., 2017). Successful males of the different populations in the Palatinate Forest or Rhine Valley, could have developed different strategies. If larval resource availability was lower in the Palatinate Forest – due to factors such as temperature-driven growth constraints or limited prey - it is conceivable that males with reduced wing cell number may be at an mating advantage due to lower developmental costs. Similar plastic responses to poor nutrition have been observed in *Drosophila melanogaster*, where reduced wing size results from decreases in both cell size and number (Vijendravarma et al., 2011). It is also possible that the observed interpopulation differences in the cellular basis of mating reflect underlying genetic divergence. For example, the developmental response to environmental cues might be genetically canalized in one population but more plastic in another (Wagner et al., 1997), leading to distinct trait combinations associated with mating advantage. The observed population-specific associations between traits and mating might be shaped not only by environmental or plastic responses, but also by epistatic interactions, which are non-additive genetic effects that cause identical environmental inputs to result in different phenotypic outcomes depending on the genetic background (Schmidt, 2023). In our study, this implies that males from the Palatinate Forest or Rhine Valley may exhibit distinct developmental responses to similar environmental conditions, resulting in population-specific wing size and cell number combinations being favoured in the context of mating. It should be noted that we recorded the mating and wing features at the beginning of the season due to the need for undamaged wings, which does not reflect life time mating success. Sexually selected traits that are beneficial in the short-term could be detrimental in the long-term, especially in unstable environmental conditions. For example, high activity levels associated with mate searching in *C. puella* could be advantageous for short-term mating success but detrimental in the long-term, where endurance-related traits are more important, as observed in marginal *Coenagrion mercuriale* populations (Purse & Thompson, 2005). In case of our results, traits like reduced wing cell number and less material cost may correlate with short-term mating advantages but further studies are needed to investigate the relation of those traits with life time mating success. Since such trait-level alterations at the individual level can reflect sublethal stress responses before community-level changes occur (Dallas & Ross-Gillespie, 2015), further studies could investigate if they may serve as early-warning indicators of environmental stress.

Contradictory to our third hypotheses that mating males show less asymmetry and our fourth hypotheses of a similar pattern at both locations, we observed higher cell shape/circularity asymmetry of mating males only in the Palatinate Forest location. For maintaining an even cell size in all directions and to assure maximum area while using less material, the circularity of cells is an important factor (Rudolf et al., 2019). We presume that this effectiveness can be achieved more symmetrically for the wing pair in conjunction with stable organism development and may predict stress during larval stage. This stress may induce behavioural effects of a terminal investment in reproduction. Under infection stress, mature damselflies have been shown to increase their investment in mating efforts, even at the expense of their energy reserves, supporting the terminal investment hypothesis (González-Tokman et al., 2013). In addition, behaviour can be closely linked to environmental conditions (Purse & Thompson, 2005; Stoks et al., 2005). Larvae that experience food stress show increased foraging activity (Stoks et al., 2005) that could be carried-over to adult stages. Larger larvae are typically more aggressive toward conspecifics as well as potential predators (Sentis et al., 2022). A competitive environment, like it exists during mating season, may favour individuals that can endure higher levels of stress as they already experienced it previously in higher levels compared to other competitors. *C. puella* is considered a non-territorial damselfly that actively has to “scramble” for females (Fincke, 1986). Scrambling competition is suggested to arise from species with high-density populations, making the duration, timing and location of male mate searching crucial factors (Kokko & Rankin, 2006). A potential difference in male density and competition between the Palatinate Forest and Rhine Valley could therefore further explain why we found location dependent effects of asymmetry on mating due to different demands on the scrambling activity of males. Under certain conditions, behavioural traits such as increased activity, assertiveness, or endurance may be more relevant than optimal morphological configuration (Gyulavári et al., 2014) but correlating with asymmetry. This would allow asymmetry to not directly impact fitness but act as a marker for other advantageous traits (e.g. scrambling intensity) that contribute to short-term mating success. Similar patterns have been observed in other insect species, where fluctuating asymmetry increased alongside behavioural traits such as boldness and activity under environmental stress, without a direct causal link to fitness being established (Rech et al., 2022). In addition, natural selection tends to decrease levels of FA in evolutionarily relevant traits (Nunes & Souto, 2022). Therefore, in the Palatinate Forest, where anthropogenic disturbance through intensive land use is presumably lower, a different environmental selection pressure may allow for higher levels of cell shape asymmetry in successful males. In contrast, in the Rhine Valley, greater overall developmental stress might select and form more uniform levels of asymmetry, reducing its relevance as a signal or predictor of mating. Successful males in the Rhine Valley could rely more on behavioural flexibility to cope with varying conditions than on specific morphological or behavioural expressions, shaping mating success regardless of wing symmetry (see Perry & Chittka, 2019). In addition, the behavioural flexibility of individuals can change when environmental conditions are introduced, which is called adaptive flexibility (Wright et al., 2010). In line with this theory, the ability of *C. puella* to maintain short-term mating success in environments with anthropogenic stress, independent of wing asymmetry, suggests the adaptability to human-modified habitats (Wildermuth & Martens, 2019). This could partly explain the broad distribution of the damselfly, including its presence in urban and agricultural areas as a winner species (Wildermuth & Martens, 2019). Care should be taken when drawing conclusions about the relationship between asymmetry and mating from studies focused on single populations as we demonstrated the dependency on environmental context, location-specific selection pressures, and potentially the genetic pool of the populations.

Computer-vision based analyses, separate consideration of the whole, proximal and distal wing as well as identification of correlations was shown to generate a detailed data set. A classic 2-D evaluation of wing shape asymmetry through setting a specific number of landmarks is restricted by the number of regularly and consistently occurring vein junctions and their distribution on a wing. This results in a limited analysable wing area and focus on specific wing parts is barely possible due to the further reduction of available landmark positions. In accordance with our hypotheses, we consistently found higher asymmetry in the distal wing compared to the proximal wing, which suggests that analysing split wing regions separately can reveal spatial patterns of asymmetry that might be masked when averaging over the entire wing. Future research could thus focus on these sub-individual trait regions as sensitive markers, that may provide an even earlier signal of ecological disturbance than whole-organism metrics — for example, changes in wing cell shape asymmetry in distal wing areas may precede measurable shifts in body size or population structure.

Additionally, we show that a full computer-vision based 2D wing analysis enables the detection of subtle shape and asymmetry traits, some of which were significantly associated with mating. These findings suggest that high-resolution geometric morphometric approaches can uncover potential links between reproductive dynamics and morphological traits, although further studies are needed to directly relate these features to long-term reproductive success such as oviposition or offspring survival. In future, wing features (e.g. cell size, cell shape, junctions amount and symmetry) could be better understood by combining them with their mechanical properties needed for flight efficiency, e.g. deformation capability (Rajabi et al., 2015) or fracture resistance (Rudolf et al., 2019). The analysis showed a positive correlation between several *cell shape* and *cell position* variables. Additionally, cell position asymmetry showed positive correlation with body weight and wing size dependent variables in hind wings which was not the case for cell shape asymmetry. Damselfly wings should be considered as important morphologically and functionally differentiated structures, in which localized patterns of variation—especially in the context of asymmetry—may reflect region-specific responses to anthropogenic selection pressures.

Study limitations

We studied two locations with estimated differences in environmental and anthropogenic pressures, which limits the generalizability of the results due to only one location per condition. Therefore, it is not possible to disentangle site-specific from condition-specific effects. Although the measured water chemistry between the sites was rather similar, and the broader landscape-level anthropogenic pressure is not easily quantified, this limits the ability to pinpoint causal environmental drivers. In addition, our study site in the Palatinate Forest was considered as the more “natural” site due to less urban and agricultural surroundings. However, the application of *Bacillus thuringiensis israelensis* (Bti) and the alteration of the hydrological regime in 6 of the 12 ponds as part of two other studies represent a form of anthropogenic disturbance. Although *C. puella* was not specifically assessed, Coenagrionidae larvae were among the affected families observed in Bti-treated ponds (Gerstle et al., 2023), suggesting potential indirect effects on damselflies through altered prey availability or competition. Furthermore, we did not assess the microhabitat differences and fine-scale biometric and ecological variables which may partly explain the observed trait variation. These factors could have been influenced by the hydrological alteration applied to 6 out of 12 ponds as part of another study. Consequently, the site should not be considered entirely pristine, and Bti exposure and hydrological alterations are acknowledged as a relevant environmental factor in interpreting the morphological variation. While we focused on the assessment of early short-term mating success via male participation in a copulation wheel, true long-term success in mating and successful reproduction cannot be directly deduced (Banks & Thompson, 1985). We therefore call for a cautious interpretation of the results, which nonetheless suggest meaningful patterns and may guide future studies on long-term reproductive success across multiple locations, ideally in combination with a precise assessment of selection pressures.

Conclusion

The results indicate that a direct or indirect link between asymmetry and short-term mating success can differ between damselfly populations. As damselfly wing structures are formed during the larval phase, environmental changes in the water can imprint morphological traits that may functionally link aquatic stress to ecological processes in the terrestrial ecosystem that scale up to influence food webs. This raises the question whether variation in mating-related morphological traits may scale up to influence the role of damselflies in structuring terrestrial food webs. A corresponding negative correlation between asymmetry and fitness is often presumed after stress response of traits was observed. However, these relationships are complex. While we observed significant associations between asymmetry and mating males, the direction and strength of these associations differed between the two populations studied. This indicates, that the functional relevance of asymmetry may be modulated by population-specific ecological or social parameters. Other elements such as sexual selection due to fitness-independent traits or the sheer availability and number of male competitors within a population could play a role. We emphasize the potential of computer-vision based analyses to detect the fine-scale shape and asymmetry of wings, revealing subtle links to mating dynamics.

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Data availability

Data will be made available on request.

CRediT authorship contribution statement

Ken M. Mauser: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Jann Baumeyer:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Shahab Eshghi:** Writing – review & editing, Software, Methodology, Investigation. **Stanislav N. Gorb:** Writing – review & editing, Methodology, Investigation. **Alessandro Manfrin:** Writing – review & editing, Investigation. **Carsten A. Brühl:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization.

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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.baae.2025.05.002.

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15 APPENDIX VI: PAPER 6

Flooding affects fluctuating asymmetry but not growth of a riparian orbweaving spider

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ORIGINAL ARTICLE

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Flooding affects fluctuating asymmetry but not growth of a riparian orbweaving spider

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Abstract

1. Aquatic emerging insects play a critical role in riparian ecosystems by supporting terrestrial consumers, particularly generalist predators like spiders. Their emergence is highly sensitive to environmental changes such as river regulation and climate change.
2. While previous studies have demonstrated that fluctuations in aquatic insect subsidies influence spider abundance and diversity, little is known about how these changes affect spider morphology and physiology.
3. This study aimed to investigate how flooding affects both aquatic insect emergence and morphological responses of the web-building spider *Tetragnatha extensa* (Linnaeus, 1785).
4. We conducted an experiment using a riparian stream mesocosm facility, simulating four flooding events between May and September. Aquatic insects were sampled using emergence traps during all flooding events, while spiders were collected at the beginning and end of the experiment. To assess developmental stress, we measured spider head size and fluctuating asymmetry using landmarking and geometric morphometrics.
5. Spiders in flooded mesocosms exhibited ∼15% lower fluctuating asymmetry than those in controls, suggesting reduced developmental stress. This likely reflects the 45% higher emergence of chironomids, the main prey of *T. extensa*. These results indicate that spider developmental stability is responsive to prey availability and that geometric morphometrics is a sensitive tool for detecting such prey-mediated ecological effects.
6. Climate change, water use and river regulation, through altered flooding regimes, can disrupt cross-ecosystem fluxes by affecting the emergence of aquatic insects. Reduced prey availability may, in turn, impact spider physiology and propagate into

the riparian ecosystem through altered fitness, reproductive success and population dynamics of terrestrial consumers.

KEYWORDS
aquatic emerging insects, aquatic-terrestrial linkages, cascading effects, developmental stress, geometric-morphometric, physiology

INTRODUCTION

Aquatic and terrestrial ecosystems are interconnected by exchanging nutrients, organisms and energy (Bartels et al., 2012; Loreau et al., 2003). In freshwater systems, emerging aquatic insects are an important food source for terrestrial predators, including spiders (Middendorf et al., 2025; Paetzold et al., 2005). Climate change and altered hydrological regimes pose increasing threats to the dynamics of these fluxes (Häder & Barnes, 2019; Schulz et al., 2015). Frequent and unpredictable flooding events can affect insect emergence dynamics (Kennedy et al., 2016), altering aquatic insect availability for riparian spiders (Whiles & Goldowitz, 2001) and potentially impacting metabolism or activity level (Jensen et al., 2010; Koemel et al., 2019).

The emergence of chironomids is known to depend on various environmental factors, with changes in water flow, temperature and shoreline structure identified as major factors influencing emergence rates and timing (Vinnersten et al., 2014). Tronstad et al. (2010) demonstrated that chironomid larvae in inundated floodplains had higher growth rates benefiting from warmer water and higher food availability on flooded terrestrial areas, leading to faster emergence. As a key food source subsidizing many riparian predators, shifts in chironomid emergence can have cascading effects (Graf et al., 2017): Lower numbers of emerging insects have been linked with reduced abundance of riparian web-building spiders (Baxter et al., 2004), spiders' diet switch (Huszarik et al., 2024; Manfrin et al., 2017) or top-down effects on the riparian food web (Henschel et al., 2001).

Riparian web-building spiders such as *Tetragnatha* sp. strongly rely on aquatic insects as prey (Middendorf et al., 2025; Uno, 2016). Aquatic insect subsidies are highly nutritious due to their content of polyunsaturated fatty acids (PUFA) (Hixson et al., 2015). A diet rich in high-quality aquatic prey can improve growth (Pietz et al., 2023), reproductive success and increase overall fitness (Mestre & Bonte, 2012). An increase in these subsidies, particularly during critical life stages such as early growth, moulting or egg production (Wen et al., 2020), may enhance developmental stability in spiders. Developmental stability refers to an organism's ability to undergo normal growth and produce a consistent, symmetrical phenotype (Møller, 1997). Nutritional stress, pollution or other environmental pressures can alter developmental stability, leading to deviations from bilateral symmetry, known as fluctuating asymmetry (FA). Access to a high-quality diet could support healthier riparian predator populations and contribute to maintaining ecosystem functionality (Laws & Joern, 2013; Ohler et al., 2024). Conversely, reduced aquatic subsidies may force spiders to invest more energy into foraging, leaving fewer resources available for growth and the maintenance of developmental stability (Uetz et al., 2009). Such constraints can affect developmental stability and ultimately increase phenotypic asymmetry (Hendrickx et al., 2003).

Geometric morphometrics can be a powerful tool to analyse developmental instability due to environmental stress (Graham et al., 2010; Leung et al., 2000; Scalici et al., 2018). It analyses body shape variations and has been widely applied in studies of sexual dimorphism, taxonomy and symmetry across various taxa, including spiders (Brandt et al., 2023; Fernández-Montraveta & Marugán-Lobón, 2017; Klingenberg, 2015; Spani et al., 2025). More recently, geometric morphometrics has been used to assess environmental stress responses to pesticide exposure in insects or bivalves (Gerard et al., 2018; Scalici et al., 2017; Scalici et al., 2020). Based on measurements of fluctuating asymmetry, geometric morphometrics can quantify small deviations from perfect bilateral symmetry. Fluctuating asymmetry can be interpreted as an indicator of developmental stress, reflecting altered physiology during growth that can be induced by adverse environmental states, such as chemical, physical or pathogen effects. Fluctuating asymmetry has been applied to ecological questions in arthropods, but the number of studies remains low, and responses can be variable because fluctuating asymmetry is affected by interactions between environmental stress and individual genetic expression (Graham et al., 2010). Chang et al. (2007) observed increased asymmetry in the wings and legs of damselflies exposed to pesticides or thermal stress during their larval development. Mauser et al. (2025) observed differences in wing asymmetry between damselflies with different mating success and exposed to different environmental conditions. Higher fluctuating asymmetry was found in isopods following dam construction, which disrupted natural flooding and led to drier soils and a major shift in vegetation (Vilisics et al., 2005). Carabid beetles collected in agricultural sites (olives and vineyard) showed higher fluctuating asymmetry than those collected from Mediterranean maquis and garrigue pristine habitats (Ivanković Tatalović et al., 2020). While geometric morphometrics in arachnology has primarily focused on species delimitation and taxonomic classification (Brandt et al., 2023; Kallal et al., 2019; Kallal & Wood, 2022), its application to studying environmental stress in spiders remains rare. Hendrickx et al. (2003), investigated reproductive performance and fluctuating asymmetry in spiders exposed to heavy metals and Uetz et al. (2009) detected higher fluctuating asymmetry in the tuft area of males that had faced a catastrophic flood. Compared to the often-used measurements of size or body mass (Wimp et al., 2021), fluctuating asymmetry offers a more integrative approach, detecting latent or chronic environmental impacts that may not be apparent through conventional morphological measurements (Parsons, 1992; Zakharov & Trofimov, 2022). Moreover, assessing the morphology and symmetry of specific body structures can provide direct insight into their developmental performance (Ivanković Tatalović et al., 2020) or reproduction (Danielson-François & Sullivan, 2021). While traditional fitness assessments often rely on size as a proxy, fluctuating asymmetry analysis offers more specific information as a sensitive marker of environmental stress (De Anna et al., 2013). This study aims to investigate how flooding regimes influence aquatic insect emergence and, consequently, the development of *Tetragnatha extensa*. Contrary to traditional studies that rely on size-based measures of fitness, we use geometric morphometrics and fluctuating asymmetry to assess the ecological responses of *T. extensa* to flooding regimes, via changes in prey availability and developmental stress.

To assess the cascading impact of flood on riparian spider size and fluctuating asymmetry, we induced 14 days of flood in replicated riparian mesocosms and compared to non-flooded controls. The flooding design was repeated in four subsequent flooding events between May and September. We collected *T. extensa* after the first and the fourth flooding event and measured the effect of flooding and potential varying availability of aquatic subsidies on *T. extensa* fluctuating asymmetry. *Tetragnatha extensa* adult females can be found between May and August (Bellmann, 2016). Therefore, individuals collected in May had largely completed their development before the experiment. In contrast, the immature individuals collected in September had developed within the mesocosms and experienced several moulting events during the experiment. We hypothesized that (1) flooding will increase the number of chironomids emerging from the flooded mesocosm and therefore prey availability for *T. extensa*. (2) We expect that at the end of the experiment, spiders from flooded mesocosms will exhibit larger head sizes compared to those from non-flooded mesocosms. We also expect that enhanced access to high-quality polyunsaturated fatty acids (PUFAs) from chironomids prey in flooded conditions will promote better developmental stability. Consequently, (3) we predict that spiders from flooded mesocosms will show lower fluctuating asymmetry than those from non-flooded mesocosms.

METHODS

Mesocosm facility description

The experiment was carried out at the Riparian Stream Mesocosm (RSM) facility in Landau, southwestern Germany (49°12′03.9″ N, 8°08′19.9″ E); the facility consists of 16 flow-through mesocosms (Manfrin et al., 2023), each designed to simulate a riparian ecosystem with both an artificial stream and an adjacent riparian zone (Figure 1a). The streams are approximately 0.73 ± 0.08 m wide and 15.15 ± 0.07 m long, with a 1% slope, while the grassland riparian area extends 3.74 ± 0.09 m along the left bank. A narrow maintenance pathway runs along the right bank (Figure 1b). To simulate a natural riverbed, each stream contains six alternating gravel bars.

Water for the streams was continuously supplied from the nearby River Queich using pumps (BADU® Eco Flex, max. 40 m^3/h, 2.20 kW) at a flow rate of 4.2 ± 0.9 L/s (Rovelli et al., 2024). Each mesocosm was enclosed within a greenhouse-like structure covered with fine (1 mm) mesh gauze, preventing flying insects from entering or leaving the mesocosm as well as most ground-dwelling species (Figure 1a). During non-experimental periods (e.g., autumn to spring) the enclosures were removed, and the stream channels were left to dry, enabling natural colonization by plants and arthropods from the surrounding grassland.

To establish a natural macroinvertebrate community, we turned on the pumps one month before the start of the experiment. The pumps were equipped with a 6-mm filter that prevented large organisms such as fish and crayfish from entering our mesocosms but allowed for passive colonization of macroinvertebrates that passed through the filter to enter the mesocosms, ensuring emergence through the entire experiment. In addition, we actively introduced macroinvertebrates from the adjacent river Queich into the artificial streams after catching them with artificial substrates. A total of 80 wooden brooms (7 cm in diameter, 65 cm in length) were used as artificial substrates. The brooms were placed in a 100-m restored section of the river Queich for 16 days (from 19 April 2023 to 4 May 2023) to allow macroinvertebrate colonization. After broom collection, five brooms were randomly assigned to each mesocosm. All macroinvertebrates contained in the brooms were washed out and collected in trays. Unwanted material such as fish, crayfish, macrophytes and wood debris was removed before introducing the remaining organisms into each mesocosm stream. Although macroinvertebrates were not identified to lower taxonomic levels, the community was largely dominated by chironomid larvae and gammarids.

Flooding

For this experiment we used eight mesocosms. Four mesocosms were exposed to flooding for 14 days, and the other four were considered as controls and were never flooded during the experiment. The flooding was repeated in May, June, August and September 2023. Each flooding event was characterized by a slow increase of the water level inside the stream part until the inundation of 60% of the terrestrial area. The flood reached a maximum depth of 10 cm in the downstream section and gradually decreased toward the edges of the refuge area (Figure 1b), where only a few millimetres of water covered the soil surface.

Emerging insect collection

Aquatic emerging insects were sampled in both flooded and control mesocosms during defined periods surrounding each flooding event. In each mesocosm, emerging insects were collected using pyramidal emergence traps (Figure 1b), following the model of Cadmus et al. (2016), made from 20-mm PVC pipes, each with a 0.25-m^2 base, a height of 30 cm and covered with 500-μm mesh (Figure S1). A total

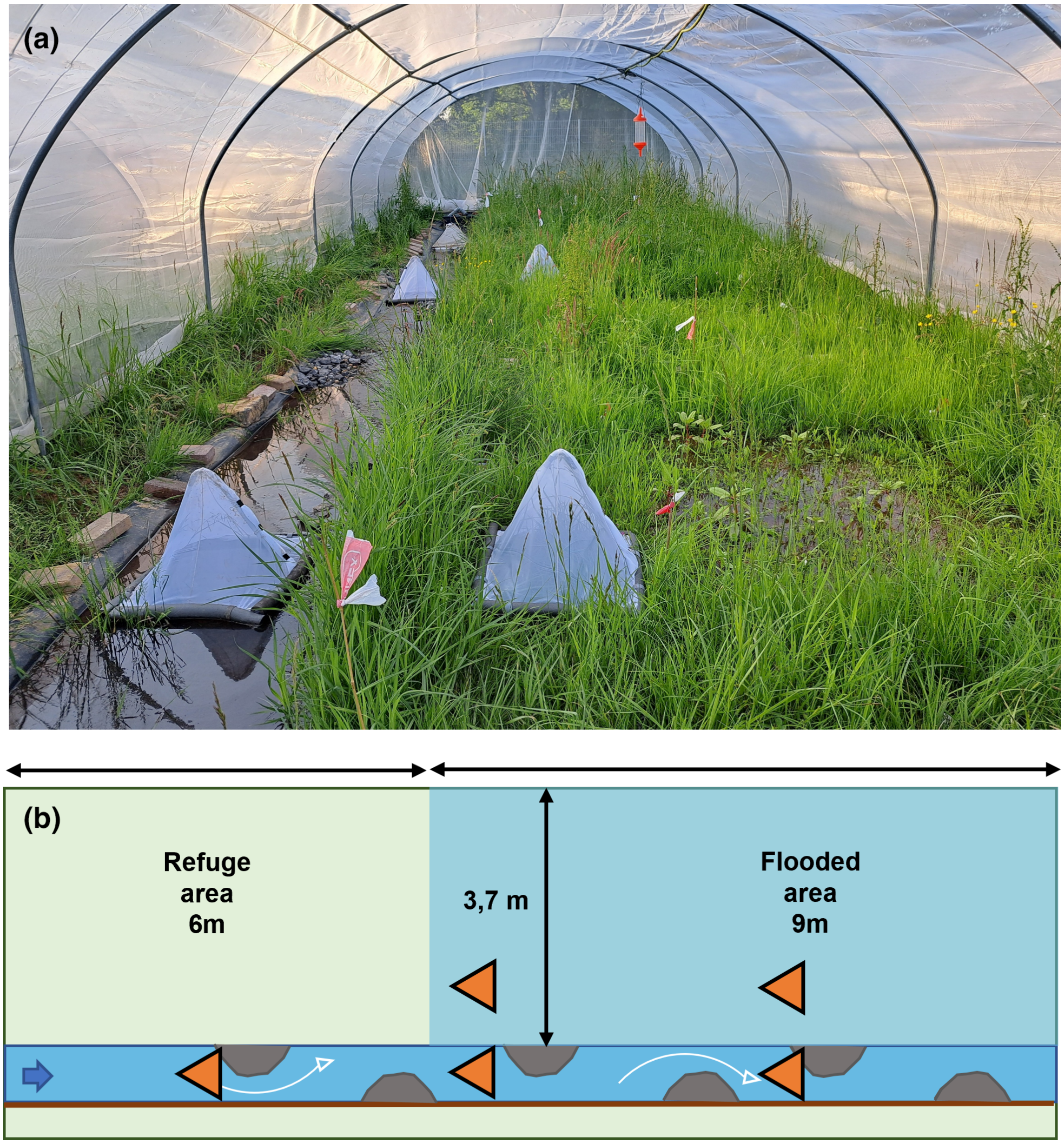

FIGURE 1 The Riparian Stream Mesocosm, Landau, Germany. (a) Inside view of one mesocosm equipped with greenhouse-like frame and covered by a 1 mm mesh gauze with emergence trap deployed (Picture by Stephane Mutel). (b) Schematic of one flooded mesocosm: stream (in blue), lateral bars (grey), adjacent riparian grassland with upstream 'refuge area' left unflooded (light green) and a 'downstream flooded area' (light blue); emergence traps position (orange triangles).

of five traps were deployed in each mesocosm. Three floating traps were positioned in the stream at upstream, middle and downstream positions, and two were placed on the terrestrial part, 1 m from the stream edge and parallel to the two most downstream traps (Figure 1b). To ensure consistent sampling across treatments, traps were deployed 3 days before the onset of each flooding event and remained in place until 4 days after its conclusion, regardless of whether the mesocosm was actually flooded. This resulted in a total of 21 days of sampling for each of the flooding events (Table S1). Outside of these defined sampling windows, no emergence traps were deployed. Traps were not equipped with catching bottles and preservative fluid but were emptied every 3 days using a mechanical pooter connected to a 150-mL Falcon tube. Captured insects were then immediately frozen at −20°C for preservation. Frozen insects were then counted and identified to family (Nilsson, 1997).

Spider collection, photography and landmarking

For imaging and landmarking, we analysed only female spiders from Flood 1 and Flood 4 to capture treatment extremes. Flood 1 spiders, already adults in May, served as controls, while Flood 4 spiders who developed under experimental conditions reflected cumulative flooding effects. Spiders from floods 2 and 3 were omitted due to logistical constraints and because the most pronounced effects were expected after Flood 4. *Tetragnatha extensa* females were handpicked along the stream edge, kept in individual containers for 24 h before being frozen at −20°C.

To obtain consistent images of the prosoma and to accurately capture its three-dimensional structure in two-dimensional photographs, spider legs were removed by cutting at the trochanter articulation to ensure the prosoma could rest flat on the sternum (Figure 2). The operation was conducted using micro scissors and tweezers. To improve stability and enhance the visibility of the lower part of the prosoma, the opisthosoma was removed prior to taking the photo. All images were captured with a stereomicroscope (Stemi 508, Carl Zeiss AG, Oberkochen, Germany, objective magnification: 0.63×, internal zoom set at 4.0×) using a ZEISS Axiocam 208c camera at a resolution of 2160 × 3840 pixels, resulting in a scale of 1.37 μm/px; exposure, colour range and white balance were adjusted individually to obtain best clarity and sharpness on each photograph.

To quantify fluctuating asymmetry (FA), we placed 16 landmarks on homologous points of the prosoma using the software tpsDig264 (version 2.32). The landmarking of the points was performed in a randomized order and by a single operator to prevent operator biases. Two central landmarks were positioned to define vertical symmetry (number 5 and 13, Figure 2), while the remaining seven pairs were set to capture the structural characteristics of the prosoma on both sides (Figure 2). After aligning all specimens to a common mean shape through a Generalized Procrustes Analysis (GPA), we quantified the FA component by comparing the coordinates of each left–right landmark pair, which provides a direct measure of how much the two sides deviate from perfect symmetry. The measurement error was assessed by randomly selecting 20 spiders from flood 1, capturing two images of each specimen, applying landmarks and performing a GPA followed by a bilateral symmetry analysis to quantify and compare variation, ensuring in this way that the measurement error was smaller than the variance. The Procrustes analysis of variance (ANOVA) revealed greater biological variation than landmarking error, confirming the reliability of our method for detecting potential treatment effects (Table S2).

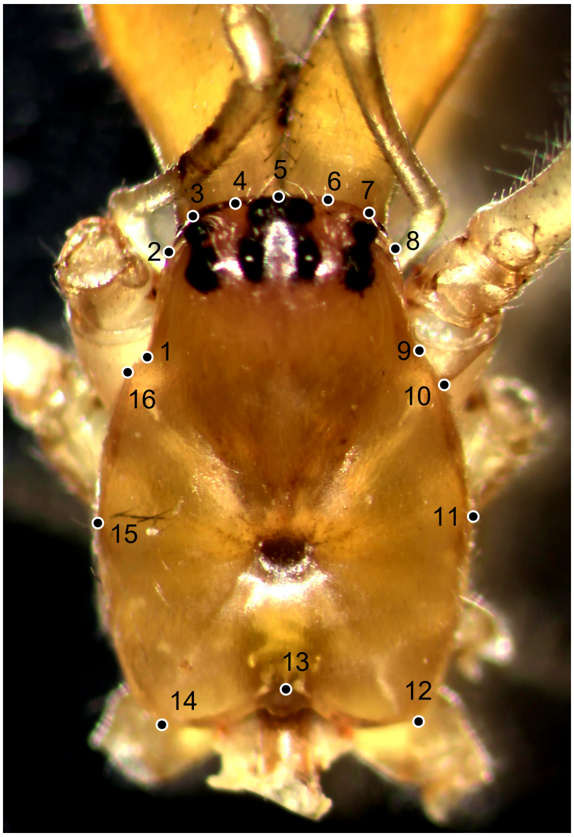

FIGURE 2 Dorsal view of *Tetragnatha extensa*'s prosoma landmarked on 16 homologous points (picture by Celia Thomas and Stephane Mutel).

Analysis

Chironomids represented 98% of the total insect emergence caught. For this reason, we focused our analysis exclusively on chironomids. For each mesocosm and flooding event, we summed the numbers of chironomids caught across the three emergence traps during the 21 days of sampling and standardized the values per day and square meter of sampling as Catch Per Unit Effort (CPUE, chironomids day^{-1} m^{-2}). We tested the effects of flooding event and flooding on the number of chironomids using a generalized linear model (GLM) with a Gamma distribution to account for the non-normal distribution of the residuals. The factors in the models included flooding event (flood 1, 2, 3 and 4), flooding (flood and no flood) and their interaction.

We analysed the landmark data using the 'geomorph' package in R (Adams & Otárola-Castillo, 2013; Baken et al., 2021). First, the tps files containing landmark coordinates were compiled into a geomorph data frame, incorporating specimen information. We then performed a GPA to align, scale and remove non-biological variation in shape due to size, orientation and positioning differences. We calculated Centroid Size as a proxy of head size (Curran, 2018) using the 'shapes' package (Dryden, 2023). Centroid size values (pixel) were then converted to head size (μm^2) by applying the scaling factor based on the magnification and resolution of the images. The FA score, an indicator of developmental instability, was calculated as the square root of the sum of the squared differences between the FA component and the mean shape as following the method described by Holzleitner and DeBruine (2020). Higher FA scores indicate greater asymmetry and, consequently, higher developmental instability.

To investigate the effect of flooding on the spider morphometry, we used GLMs on head size and on FA. The models included flooding (flood and no flood), flooding event (flood 1 and 4) and their interaction as fixed factors. Data distributions and homogeneity of variance were assessed using Shapiro–Wilk tests for normality and Levene's tests for variance homogeneity. Both variables (head size and FA) exhibited non-normal distributions of the residuals. While the assumption of homogeneity of variance was met by FA, it was not met for CS, nor for the interaction of flooding and flooding event and the individual factors. Hence, we chose to fit the GLM with a Gamma distribution for both variables after removing potential outliers identified using Rosner's test from the 'EnvStats' package (version 3.0.0, Millard, 2013); only one outlier was detected for FA and removed before model fitting. Because Flood 1 and Flood 4 spiders belong to different age classes, a smaller head size is ecologically expected in the Flood 4 individuals. We did not interpret the main effect of flood event on head size as biologically meaningful but retained the factor in the model to examine the interaction between flooding treatment and flooding event.

All statistical analyses were conducted using R (version 4.3.1; R Core Team, 2023), with a significance threshold set at $p < 0.05$. Model validation was performed using the DHARMa package (version 0.4.6, Hartig, 2022) by examining residual-fitted values plots to assess model assumptions. To test for the significance of the factors and their interaction, we performed an ANOVA on the fitted models using the 'Anova' function from the 'car' package (version 3.1-2; Fox & Weisberg, 2018). In case of significant interaction in our model, we used Estimated Marginal Means (EMMs) to perform pairwise comparisons between treatment groups as post hoc tests, using the 'emmeans' package (version 1.10.4, Lenth, 2024).

RESULTS

Aquatic emerging insects

The number of emerging chironomids was consistently higher in flooded mesocosms than in control mesocosms ($\chi^2 = 10.5$, df = 1, $p = 0.001$) across all flooding events, from May until September (Figure 3). We also observed a seasonal variation in emergence ($\chi^2 = 204.4$, df = 3, $p < 0.001$) with the highest numbers during May in flooded mesocosms. Chironomid CPUE decreased in June, reaching the lower value in August before rebounding in September (Figure 3). During the first flooding event, when emergence peaked, flooded mesocosms produced 27% more chironomids than the control mesocosms. In subsequent flooding events, emergence declined but flooded mesocosms still yielded 34% and 39% more chironomids than the controls during the second and third flooding events, respectively. During the fourth flooding event, emergence numbers rebounded, with 42% more chironomids emerging from the flooded mesocosms than from controls.

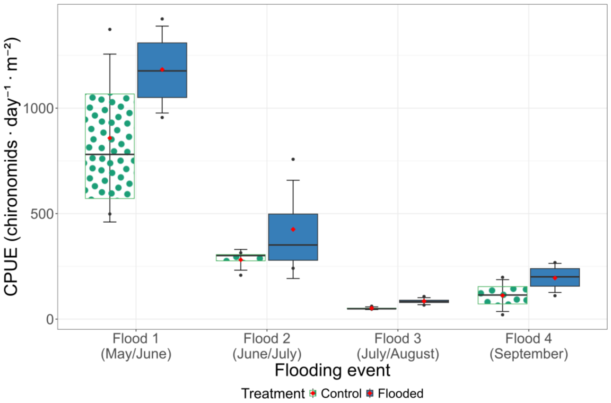

FIGURE 3 Number of chironomid individuals per square meter of trap surface (catch per unit effort [CPUE]) during each of the four flooding events according to flood exposure (control in green, 14 days flood in blue). Lower and upper box boundaries 25th and 75th percentiles, respectively. Lines inside the box represent the median, red dots the mean values, whiskers the standard deviation and black dots the outliers.

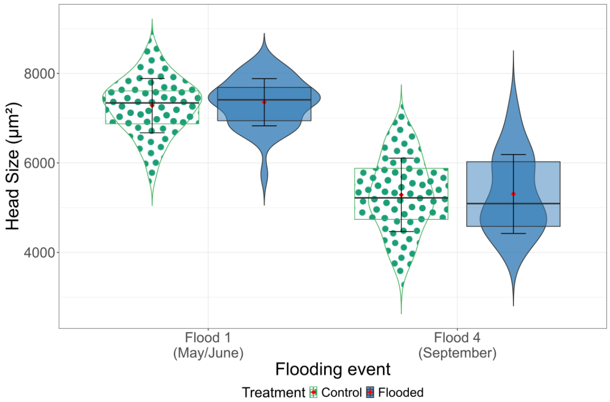

FIGURE 4 Head size of *Tetragnatha extensa* in control (green) versus flooded (blue) mesocosms after the first and fourth flood event (control flood 1: $n = 40$, control flood 4: $n = 43$, flooded flood 1: $n = 38$; flooded flood 4: $n = 40$). Lower and upper box boundaries 25th and 75th percentiles, respectively. Lines inside the box represent the median, red dots the mean values, whiskers the standard deviation.

Head size and FA

After flood 1, we captured 78 female spiders, with 40 individuals from the control mesocosms and 38 individuals from the flooded ones. Similarly, after flood 4, 83 female spiders were captured, with 43 individuals from the control and 40 individuals from the flooded mesocosms.

Analysis of head size revealed a significant seasonal effect, with individuals from flood 1 displaying significantly larger heads compared to their immature offspring from flood 4 (Figure 4; $\chi^2 = 258.5$, df = 1, $p < 0.001$). However, there was no significant effect of the flooding ($\chi^2 = 0.12$, df = 1, $p = 0.7$) or the interaction of flooding and season on head size ($\chi^2 = 0.03$, df = 1, $p = 0.9$).

The GLM on the FA revealed a significant flooding × flooding event interaction (Figure 5; $\chi^2 = 4.9$, df = 1, $p = 0.04$). We found that spiders collected after flood 4, that had developed under non-flooded conditions, showed 15% higher FA compared to those from the flooded mesocosms (df = 156, $t = 2.05$, $p = 0.04$).

DISCUSSION

The experiment showed that flooding of riparian grassland has an effect on the development of *T. extensa* spiders, influencing their

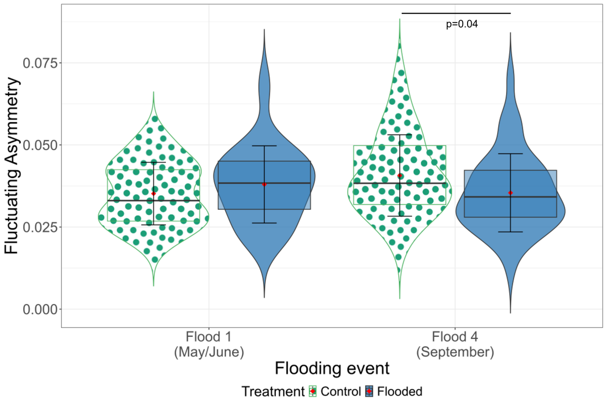

FIGURE 5 Fluctuating Asymmetry score in *Tetragnatha extensa*'s head in control (green) versus flooded (blue) mesocosms after the first and fourth flood event (control flood 1: $n = 40$, control flood 4: $n = 43$, flooded flood 1: $n = 38$; flooded flood 4: $n = 39$). Lower and upper box boundaries 25th and 75th percentiles, respectively. Lines inside the box represent the median, red dots the mean values, whiskers the standard deviation. The outlier was removed.

fluctuating asymmetry, but not their size. Following the first flooding event, there were no differences in either fluctuating asymmetry or size between spiders from flooded and non-flooded mesocosms. At the end of the season, after flood 4, individuals that had developed under flooded conditions exhibited higher symmetry, indicating improved developmental stability despite similar growth outcomes. Since flooding consistently led to higher chironomid emergence throughout the season compared to the controls, the increase in aquatic prey availability may have contributed to a higher diet quality and thus to a more stable spider development. At the same time, other effects of flooding, such as the creation of a more favourable microclimate with increased moisture and lower temperatures or shifts in terrestrial insect communities and predator–prey interactions, cannot be ruled out. Additionally, higher water levels may bring emerging aquatic insects into closer proximity to *Tetragnatha* webs spun on the riparian vegetation, potentially increasing prey capture success.

Our findings suggest that seasonal flooding events, when moderate, can enhance the availability of aquatic prey such as chironomids, which in turn may benefit terrestrial predators like spiders by improving foraging conditions (Henschel et al., 2001). Chironomids are well adapted to moderate environmental disturbances like flooding (Anderson & Ferrington, 2013; Dorić et al., 2023). Regular and moderate flooding events can create shallow habitats over terrestrial areas that warm more rapidly, potentially triggering earlier hatching or a more prolonged hatching period (Lytle, 2002). Our experimental setup allowed drifting macroinvertebrates, such as Chironomidae larvae at various developmental stages, to enter throughout the experiment. We suggest that flooding in our system created more favourable conditions for larval survival and emergence by increasing water volume and surface area, prolonging retention time, reducing flow velocity and slightly raising temperatures (personal observations). Moreover, these hydromorphological conditions might have reduced larval drift out of the system, enhancing their retention. Thus, the increased emergence observed may result from both improved larval survival and altered drift dynamics under flooded conditions. Additionally, flooding increases the total water coverage over a given area, expanding the habitat available for aquatic insect emergence, thereby increasing the potential number of emerging insects for terrestrial predators. However, in our mesocosms, the expanded flooded terrestrial areas did not contribute substantially to chironomid emergence: only a few individuals (less than 0.5% of the total) emerged from these areas. While this suggests that most emergence occurred in the stream section, it is possible that the longer-term consequences of flooding could create more favourable conditions for chironomid larvae developing in wet soils. Our experimental design, with relatively short floods and intervals between flooding cycles, may have limited the opportunity for specialized chironomids to complete development and hatch (Frouz et al., 2003). In more natural settings, where longer periods elapse between floods, wetter soils could support enhanced larval growth and emergence, potentially sustaining aquatic insect flux over extended periods.

Prey availability and the presence of flooding did not affect spider head size. Despite lower emergence of aquatic insects in non-flooded mesocosms, spiders achieved similar growth, suggesting that either the reduced aquatic input was still sufficient or that spiders effectively supplemented their diet with terrestrial prey to maintain development (Radermacher et al., 2020; Takada et al., 2014). While previous studies have shown that prosoma size can reflect food limitation (Vollrath, 1988), our results indicate no significant difference in growth between spiders from control and flooded mesocosms. This suggests that overall food availability was sufficient in both cases. However, Pietz et al. (2023) demonstrated in a laboratory experiment that reduced intake of physiologically important fatty acids from the same ingested biomass reduced the growth of *T. extensa*. This suggests that in the control mesocosms the spiders may have compensated for the fatty acid deficit from aquatic insects by consuming more terrestrial prey in order to maintain the same growth rate. Nevertheless, variations in the composition and ratio of the spiders' diet of aquatic and terrestrial prey likely led to differences in the fatty acids ingested.

Aquatic insects, such as chironomids, are richer in PUFAs than terrestrial insects (Hixson et al., 2015; Kowarik et al., 2021). PUFAs play a fundamental role in optimal growth and development, maintaining cell membrane function, hormone regulation and organism detoxification (Fritz et al., 2017), processes that are particularly sensitive during early developmental stages. In spiders, fluctuating asymmetry can be linked to developmental stress and be influenced by various factors, including food availability and quality or exposure to disturbances (Uetz et al., 2009). In our experiment, flooding increased aquatic insect availability, and spiders from flooded mesocosms showed lower asymmetry than those from non-flooded ones. Although we did not measure diet directly, greater access to PUFA-rich aquatic prey may have contributed to more stable development. Conversely, spiders in non-flooded mesocosms may have needed to forage more actively, increasing energetic demands, exposure to predators and reliance on lower-quality prey. Under such conditions, regulatory compounds required for maintaining symmetrical prosoma development may instead be diverted to other stress-mitigating functions, leading to small but detectable increases in fluctuating asymmetry. Experimental studies often show that strong stress can cause mortality before fluctuating asymmetry becomes measurable, whereas moderate stress allows subtle deviations to emerge (Symanski & Redak, 2021). The absolute differences we measured were minor and do not necessarily translate into direct measurable fitness consequences (e.g., head size). These minor deviations can nonetheless serve as sensitive markers of elevated developmental stress, reflecting subtle physiological or behavioural costs (De Coster et al., 2013). Similar patterns have been observed in other arthropods, such as grasshoppers stressed along an urbanization gradient (Rech et al., 2022). Since no comparative fluctuating asymmetry values are available from experimental studies that also measured fitness, our results do not allow us to conclude about fitness consequences. We encourage future studies to assess fitness consequences (e.g., mating success, egg clutch size, offspring survival) alongside asymmetry and size to test whether fluctuating asymmetry provides additional ecological insight beyond traditional size-based approaches.

As a result, floods may not only increase emergence but also shift the ratio of terrestrial to aquatic prey in spider diets (Henschel et al., 2001), impacting developmental stability. To our knowledge, no studies have directly linked dietary variation to fluctuating asymmetry in spiders, highlighting the need for future research in this direction as an indicator of nutritional stress.

Moulting is a critical phase in spiders' development, particularly sensitive to environmental conditions and nutritional quality, making it a key window where developmental instability, and thus fluctuating asymmetry, can arise. In our experiment, flooding may have impacted this specific aspect of spider ontogeny through two pathways: nutritional composition and moisture. Specific PUFAs have been linked to moulting efficiency and success as well as developmental stability (Wen et al., 2024), suggesting that shifts in prey composition could further influence fluctuating asymmetry. In addition, floods increased moisture, an essential parameter in spider moulting performance (DeVito & Formanowicz, 2003). Together, these factors suggest that flooding can influence fluctuating asymmetry not only by affecting aquatic prey availability but also by directly supporting the physiological demands of moulting.

The experiment highlights the critical role of aquatic subsidies in shaping riparian predator development. While river regulation has historically decreased flooding of riparian grassland, climate change will likely amplify drought stress while intensifying flood events at longer intervals, altering aquatic insect emergence dynamics. Predators relying on emergent aquatic insects may experience shifts in growth, symmetry and overall fitness, as well as a potential decoupling of prey availability during critical life stages. Such changes could alter food web dynamics through cascading effects, leading to shifts in predator diet, competition and prey selection. While we did not directly link fluctuating asymmetry to fitness response such as offspring production, our results show that asymmetry is sensitive to environmental variation that could not be detected with traditional size-based measurements. Although the study does not provide mechanistic conclusions, it provides a novel assessment of spider responses to dietary and hydrological stress, suggesting that changes in prey availability can influence developmental stability. Given that riparian zones are key ecosystems supporting biodiversity and trophic interactions, further research into both prey quantity and quality is crucial, as shifts in aquatic insect diversity and nutritional value could have an even greater impact on predator development and terrestrial food web dynamics.

AUTHOR CONTRIBUTIONS

Stephane Mutel: Conceptualization; data curation; formal analysis; investigation; methodology; project administration; resources; validation; writing – original draft; writing – review and editing; visualization. **Martin H. Entling:** Conceptualization; funding acquisition; supervision; validation; writing – review and editing. **Ken M. Mauser:** Conceptualization; formal analysis; validation; writing – review and editing. **Federica Spani:** Methodology; software; validation; writing – review and editing. **Celia Thomas:** Formal analysis; investigation; writing – review and editing. **Collins Ogbeide:** Resources; investigation; writing – review and editing. **Mirco Bundschuh:** Funding acquisition; supervision; writing – review and editing. **Franziska Fiolka:** Investigation; writing – review and editing. **Franziska Middendorf:** Investigation; writing – review and editing. **Ralf Schulz:** Funding acquisition; supervision; writing – review and editing. **Alessandro Manfrin:** Conceptualization; methodology; validation; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study (Mutel et al. 2025) are openly available in Zenodo at http://doi.org/10.5281/zenodo.17360335.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Floating emergence trap made with PVC tube—50 cm × 50 cm base covered by foam isolation tubing (Picture: Pradeep Chintapalli).

Table S1. Calendar of the RSM 2023 experiment—flooding period in blue, emergence sampling period in light brown and *Tetragnatha extensa* handpicking in green (1 June and 23 September).

Table S2. Statistics of Procrustes ANOVA evaluating potential shape variations generated by landmarking errors on 20 random spider's prosoma from flood 1. The table reports the degrees of freedom (df), sum of squares (SS), mean square (MS), proportion of total variance (R^2), *F*-ratio (*F*), and *p*-value.

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16 DECLARATION OF GENERATIVE AI TECHNOLOGIES IN THE WRITING PROCESS

During the revision of the abstract, introduction, methods, discussion, synthesis and outlook chapters, the author made selective use of DeepL, ChatGPT-4 and Claude to improve grammar, spelling, and phrasing in English. ChatGPT-4 was used to create an icon of a damselfly used in Figure 3 and Figure 16 as well as an icon of grass, soil and a water drop used in Figure 6 and Figure 7. All content generated with these tools was carefully reviewed and edited by the author, who takes full responsibility for the final version of the text.

17 DECLARATION

I, the undersigned author of this work, hereby declare that I independently wrote this thesis with the title "*Micropollutants as stressors in aquatic and terrestrial systems: their spatial distribution and effects on non-target organisms, biodiversity and ecosystem functions*". All used resources and references are specified in this work. Contributions of coworkers and coauthors have been clearly identified.

I did not use the assistance of a doctoral consultant (or a similar person) in return for payment.

I did not, and have never, submit this PhD thesis in identical or similar form elsewhere for a scientific examination.

I am aware that a violation of the above-mentioned points can lead to a withdrawal of the doctoral degree and additional legal consequences.

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2017 – 2018	**University of Koblenz-Landau** Student assistant, Working group Environmental & Soil Chemistry *Chemical and physico-chemical parameters of soil* Student assistant, Working group Ecotoxicology & Environment *Construction of an experimental watercourse*

Peer-reviewed publications

Mauser, K.M., Brühl, C.A., & Zaller, J. G. (2024). Herbicide Effects on Nontarget Organisms, Biodiversity and Ecosystem Functions. *Encyclopedia of Biodiversity (Third Edition)* (S. 239–257). Academic Press. https://doi.org/10.1016/B978-0-12-822562-2.00080-3

Mauser, K.M., Wolfram, J., Spaak, J.W., Honert, C., & Brühl, C.A. (2025). Current-use pesticides in vegetation, topsoil and water reveal contaminated landscapes of the Upper Rhine Valley, Germany. *Communications Earth & Environment*, *6*(1), 166. https://doi.org/10.1038/s43247-025-02118-2

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Mauser, K.M., Paudel, S., Sigmund, O., Entling, M.H., Ott, J., Brühl, C.A., (2025). Effects of chemical and hydrological stress on the wing morphology of a damselfly. *Environmental Entomology* 54, 1335–1347. https://doi.org/10.1093/ee/nvaf112

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Contributions to scientific conferences

05/2024	**SETAC Europe 34th Annual Meeting** Sevilla (Spain) *Topic: “Contaminated Landscapes – Current Use Pesticides Residue Measurement in the Upper Rhine Valley”*
03/2024	**Society of German-speaking Odonatologists (GdO) e.V. Annual Conference** Bonn (Germany) *Topic: "Effects of water pollution on wing symmetry in the Azure Damselfly (Coenagrion puella)"*
09/2023	**SETAC GLB e.V. GDCh-FG Environmental Chemistry & Ecotoxicology Joint Annual Conference** Muttenz (Switzerland) *Topic: “Contaminated Landscapes – Upper Rhine Valley’s Pesticide-Forecast”*
11/2022	**GfÖ Conference** Metz (France)

Supervised theses and research project courses

Thesis, M. Sc. Environmental Pollution Management (Ecotoxicology)

Effects of micropollutants in small agricultural water bodies on the wing morphology of the azure damselfly Coenagrion puella

Thesis, M. Sc. Environmental Pollution Management (Ecotoxicology)

Pesticide Contamination of Riparian Non-Target Zones Between Agricultural Fields and Streams

Thesis, M. Sc. Environmental Sciences

Effects of Reduced Prey Availability on Dragon- and Damselflies in a Mesocosm Experiment

Thesis, B. Sc. Environmental Sciences

Influence of wing asymmetry on the mating success of male Coenagrion puella

Thesis, B. Sc. Environmental Sciences

Effects of micropollutants in Rhineland-Palatinate small water bodies on the fitness of the azure damselfly (Coenagrion puella): An analysis of polyunsaturated fatty acids (PUFAs)

Research project course (RPC), M. Sc. Environmental Pollution Management (Ecotoxicology)

Effects of Pesticides in Aquatic Systems on the Fluctuating Asymmetry of Damselflies' Wings

Research project course (RPC), M. Sc. Environmental Pollution Management (Ecotoxicology)

The Influence of Forest Edge Proximity and Canopy Cover on Pesticide Contamination of Non-Target Topsoil and Vegetation

ETX 5b: Effects of Chemical Stressors II, M. Sc. Environmental Pollution Management (Ecotoxicology)

Method Development and Investigation: Influence of Luna® Experience on the Wing Symmetry of Parnassius apollo ssp. Vinningensis

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Research collaborations

02/2025	**Kiel University (CAU), Functional Morphology and Biomechanics, Prof. Dr. Stanislav Gorb,** Research Project: *"Short-Term Mating Success in Relation to Computer Vision Analysed Fluctuating Wing Asymmetry in the Male Azure Damselfly Coenagrion puella"*
07/2024	**Kiel University (CAU), Functional Morphology and Biomechanics, Prof. Dr. Stanislav Gorb,** Research Project: *"Contribution to the Automated Detection of Wing Asymmetry"*
02/2024	**University of Hohenheim, Applied Entomology, Prof. Dr. Georg Petschenka,** *Topic: Parnassius apollo ssp. Vinningensis (Moselle Apollo)*

Courses

09/2024	Intensive scientific writing course (Dr. Manfrin)
04/2024	Geometric Morphometrics (Physalia Courses)
04/2024	SystemLink Research Workshop in Landau, Germany
04/2023	SystemLink, DFG course: Ethics in science/GSP (Verse)

03/2023	SystemLink Research Workshop in Annweiler, Germany
12/2022	SystemLink, DFG course: Unconscious Gender Bias (Wronker)
11/2022	GfÖ in Metz, France: Transfer of anthropogenic stress from aquatic to terrestrial ecosystems – perspectives from young researchers
09/2017	Botanical and ornithological field course in the Lake Baikal region, Irkutsk State Agricultural University, Russia

Certificates

06/2025	BANU Bronze Certificate in Field Ornithology with Distinction, Rhineland Palatinate, Germany

Awards

03/2024	**Lopi Award**, Society of German-speaking Odonatologists (GdO) e.V. *Award for the best poster presentation on the wing asymmetry of the damselfly Coenagrion puella in relation to anthropogenic stressors*

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