ECOLOGICAL EFFECTS OF BTI-BASED MOSQUITO CONTROL ON AQUATIC-TERRESTRIAL LINKAGES

by

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» *The earth is what we all have in common.* «

– Wendell Berry

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LIST OF PUBLICATIONS

Gerstle V, Manfrin A, Kolbenschlag S, Gerken M, Islam ASMMU, Entling MH, Bundschuh M, Brühl CA (2023). Benthic macroinvertebrate community shifts based on Bti-induced chironomid reduction also decrease Odonata emergence. *Environmental Pollution*, 316, 120488.

Gerstle V, Solanki P, Manfrin A, Kobenschlag S, Brühl CA (2023). Stress response of European common frog (*Rana temporaria*) tadpoles to Bti exposure in an outdoor pond mesocosm. *Bulletin of Environmental Contamination and Toxicology, 110*(4), 70.

Gerstle V, Bollinger E, Manfrin A, Pietz S, Kolbenschlag S, Feckler A, Entling MH, Brühl CA (submitted). Trophic effects of Bti-based mosquito control on two top predators in floodplain pond mesocosms.

LIST OF ABBREVIATIONS

Bti	*Bacillus thuringiensis* var. *israelensis*
EERES	Eußerthal Ecosystem Research Station
FA	Fatty acids
FPM	Floodplain pond mesocosm
FR	Field rate
GST	Glutathione-S-transferase
ITU	International toxic units
NLFA	Neutral lipid fatty acids
PUFA	Polyunsaturated fatty acids
TEF	Trophic enrichment factor
KABS	Kommunale Aktionsgemeinschaft zur Bekämpfung der Schnakenplage e.V. (english: German mosquito control agency, GMCA)

Contents

ABSTRACT

Many amphibians and insects have a biphasic life cycle, linking aquatic and terrestrial ecosystems. In temperate wetlands, insect communities are largely dominated by midges, such as non-biting chironomids and mosquitoes. Particularly chironomids and their aquatic larvae play a key role for both aquatic and terrestrial predators, e.g., dragonflies and damselflies (Odonata), birds, riparian spiders and amphibians. Therefore, adverse effects on chironomid larvae induced by pesticides or biocides can have implications on food webs across ecosystem boundaries.

In floodplains of the Upper Rhine Valley in southwest Germany, the biocide *Bacillus thuringiensis* var. *israelensis* (Bti) has been applied for over 40 years to reduce nuisance by mass emergence of mosquitoes. Due to its specific mode of action, Bti is presumed to be a more environmentally friendly alternative to non-selective, highly toxic pesticides used in the past. However, research on indirect effects of Bti on non-target organisms inhabiting these wetlands is still relatively scarce. The aim of this thesis was the investigation of direct and indirect effects of Bti on non-target organisms and, consequently, bottom-up effects on aquatic food webs and propagation to the terrestrial ecosystem. Effects were examined in outdoor floodplain pond mesocosms (FPMs) with natural flora and fauna communities.

Benthic macroinvertebrate communities were significantly altered in Bti-treated FPMs, largely due to the reduction of chironomid density by over 40% compared to untreated FPMs. Sampling of exuviae indicated that the emergence of Libellulidae (Odonata) was reduced by Bti, while larger Aeshnidae were not affected. This finding suggested increased intraguild predation (predation of competing predators) in Bti-treated FPMs as a result of decreased prey availability, i.e. chironomid larvae. This conclusion was partly confirmed in food web analyses using stable isotopes of C and N and fatty acids, with Aeshnidae experiencing a slight diet shift towards larger prey (i.e., newts, Aeshnidae) in Bti-treated FPMs. In contrast, the diet proportions of newt larvae were not affected by Bti treatment, but showed a marginal trend in lower omega-6 fatty acid content. Analyses of oxidative stress biomarkers did not reveal any direct effects of Bti on common frog tadpoles under natural climatic conditions.

This thesis emphasizes that adverse effects of Bti on the base of aquatic-terrestrial food webs, i.e., reduction of larval chironomids, can have implications for higher trophic levels and cascade to terrestrial ecosystems. Affected organisms also include species of concern, such as protected Odonata species. In view of the global insect and amphibian decline, the large-scale use of Bti in (partially protected) wetlands should be carefully considered.

ZUSAMMENFASSUNG

Viele Amphibien und Insekten durchlaufen einen zweiphasigen Lebenszyklus, der aquatische und terrestrische Ökosysteme verbindet. In Feuchtgebieten gemäßigter Zonen werden Insektengemeinschaften, neben Stechmücken, weitestgehend von nicht-stechenden Zuckmücken dominiert. Zuckmücken und ihre aquatischen Larven spielen eine Schlüsselrolle für aquatische und terrestrische Räuber wie Libellen, Vögel, Ufer-Spinnen und Amphibien. Daher können nachteilige Effekte auf Zuckmückenlarven, die zum Beispiel durch Pestizide oder Biozide hervorgerufen werden, Auswirkungen auf Nahrungsnetze – auch über Ökosystemgrenzen hinweg – haben.

In den Überschwemmungsgebieten des Oberrheintals im Südwesten Deutschlands wird das Biozid *Bacillus thuringiensis* var. *israelensis* (Bti) seit über 40 Jahren angewendet, um die Belästigung der Bevölkerung durch den massenhaften Schlupf von Stechmücken zu reduzieren. Aufgrund seiner spezifischen Wirkungsweise gilt Bti als umweltfreundlichere Alternative zu nicht-selektiven, toxischen Pestiziden, die in der Vergangenheit verwendet wurden. Trotzdem ist die Forschung zu den indirekten Auswirkungen von Bti auf Nicht-Zielorganismen in diesen Feuchtgebieten relativ begrenzt. Das Ziel dieser Arbeit war die Untersuchung der direkten und indirekten Auswirkungen von Bti auf Nicht-Zielorganismen und folglich auch „Bottom-up-Effekten" auf aquatische Nahrungsnetze und deren Ausbreitung in das terrestrische Ökosystem. Die Auswirkungen wurden in künstlich angelegten Freiland-Mesokosmen (FPMs), die Überschwemmungsgebiete mit natürlichen Flora- und Faunagemeinschaften imitieren, untersucht. Die benthischen Makroinvertebraten-Gemeinschaften wurden in Bti-behandelten FPMs signifikant verändert, hauptsächlich aufgrund der Reduzierung der Zuckmücken-Abundanz von über 40% im Vergleich zu unbehandelten FPMs. Die Quantifizierung von Libellen-Exuvien zeigten, dass die Emergenz von Libellen (Libellulidae) durch Bti reduziert wurde, während größere Aeshnidae nicht betroffen waren. Dieses Ergebnis deutet auf eine erhöhte „Intraguild-Predation" (Prädation zwischen konkurrierenden Räubern) in Bti-behandelten FPMs als Folge der verringerten Beuteverfügbarkeit (v.a. Zuckmückenlarven) hin. Dies wurde teilweise in Nahrungsnetzanalysen, mittels stabilen Isotopen von Kohlenstoff und Stickstoff, bestätigt, infolgedessen die Diät von Aeshnidae in Bti-behandelten FPMs im Vergleich zur Kontrolle eher aus größeren Beutetieren (v.a. Molche und Aeshnidae) bestand. Im Gegensatz dazu wurde die Nahrungsaufnahme der Molchlaven nicht durch die Bti-Behandlung beeinflusst, welche aber in Fettsäureanalysen einen leicht niedrigeren Gehalt von Omega-6 Fettsäuren aufwiesen. Die Analysen von Biomarkern für oxidativen Stress zeigten keine direkten Auswirkungen von Bti auf verschiedene Lebensstadien von Grasfrosch-Kaulquappen unter natürlichen klimatischen Bedingungen.

Diese Arbeit betont, dass nachteilige Auswirkungen von Bti an der Basis aquatisch-terrestrischer Nahrungsnetze, also die Reduzierung von Zuckmückenlarven, Auswirkungen auf höhere trophische Ebenen haben und bis in terrestrische Ökosysteme vordringen können. Die hier untersuchten Organismen umfassen auch besonders geschützte Arten, weshalb angesichts des weltweiten Rückgangs von Insekten und Amphibien der großflächige Einsatz von Bti in (teilweise geschützten) Feuchtgebieten sorgfältig überdacht werden sollte.

1 INTRODUCTION

1.1 Aquatic-terrestrial ecosystems

Aquatic and terrestrial ecosystems are strongly linked via reciprocal transfer of organisms and matter (Soininen et al., 2015). While fluxes from terrestrial to aquatic systems mainly includes terrestrial-derived organic matter input, opposite resource fluxes are often associated with processes like insect emergence (as reviewed by Schulz et al., 2015). In this context, insect and amphibian species with a biphasic life cycle (aquatic larvae and terrestrial adults), such as dragon- and damselflies (Odonata), mosquitoes (Culicidae), chironomids (Chironomidae), frogs (Ranidae), and newts (Salamandridae), play a crucial role for energy flow between ecosystems (Sánchez-Hernández, 2020). Benthic macroinvertebrates are an integral part of complex aquatic food webs (Figure 1), which can serve both as prey and consumers, but also emerge to the terrestrial habitat. Understanding the connections between aquatic and terrestrial ecosystems, and the interactions of the organisms that inhabit them, is crucial for effective management and conservation of these important habitats. For instance, anthropogenic disturbances to aquatic communities, e.g., through pesticide pollution, can directly propagate to terrestrial habitats via altered insect emergence (e.g., Gerstle et al., 2023a; Kolbenschlag et al., 2023a; Pietz et al., 2023) or time-to-metamorphosis, in case of amphibians (Hayes et al., 2006).

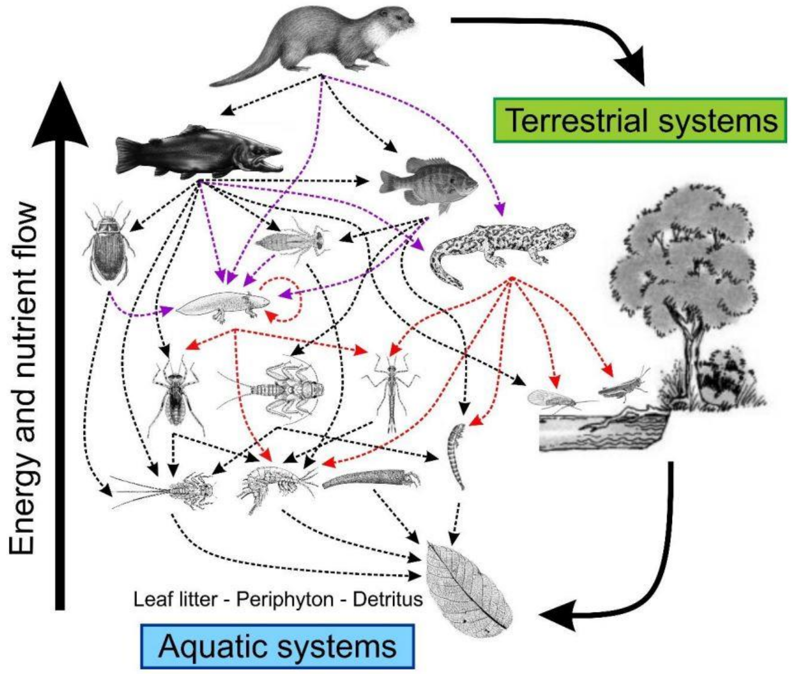


Figure 1: Conceptual view of an aquatic food web dominated by semi-aquatic vertebrate and invertebrate predators. Arrows indicate the direction of predator–prey interactions and energy flow. (Figure taken from Sánchez-Hernández, 2020).

1.2 Global biodiversity loss

Types of human-induced changes in the environment, e.g. global climate warming, nutrient and pesticide pollution, are a threat to organisms inhabiting these ecosystems. Consequently, this can have implications for ecosystem functioning, in turn, affecting the human population through alterations of nutrient cycling and biomass production (Chapin III et al., 2000). Therefore, global biodiversity loss constitutes the most critical problems of modern society (Cardinale et al., 2012). It is predicted that, if these trends continue, the rates of species loss will result in the sixth mass extinction in 230 years (Barnosky et al., 2011; Ceballos et al., 2015). According to the Red List of threatened species by the International Union for Conservation of Nature (IUCN), in 2022, more than 42,100 species a threatened with extinction, which constitutes 28% of all assessed species (IUCN, 2022). Among these species, amphibians are the vertebrate group facing the highest risk with 41% of species being endangered (IUCN, 2022; Stuart et al., 2004). Main reasons linked to decline of local amphibian populations are habitat fragmentation, climate change, diseases, and environmental contamination (Sparling et al., 2001; Stuart et al., 2004). While previously the loss of vertebrate species was in the center of attention, we face an even more drastic decline of insect diversity and abundance, with more than 75% decreased total flying insect biomass in the past 27 years (Hallmann et al., 2017). This pattern is well studied in pollinator insects like bees (Goulson et al., 2015; Vray et al., 2019) and mainly attributed to agricultural land use, involving habitat destruction and use of pesticides (products used for plan protection). Pesticides can unintentionally pollute aquatic habitats in agricultural landscapes via entry of contaminants spray-drift or run-off (Bereswill et al., 2012; Carter, 2000). However, there are products that are intentionally applied to water bodies, for instance, biocides (products used for protection of human health and well-being) targeting larvae of mosquitoes.

1.3 Mosquitoes and their control

There are two main reasons why mosquitoes (Culicidae) are considered a threat to human health and well-being: Mosquitoes are able to transmit vector-borne diseases such as malaria, dengue, Zika, Chikungunya, West Nile fever, Japanese encephalitis or lymphatic filariasis (Becker et al., 2010b). Therefore, mosquitoes are considered to be the deadliest animal in the world, with malaria being responsible for 619,000 deaths in 2021 (WHO, 2021). The majority of disease-carrying mosquito species, most importantly the yellow fever mosquito (*Aedes aegypti*), African malaria mosquito (*Anopheles gambiae*), and Asian tiger mosquito (*Aedes* *albopictus*), occur in tropical and subtropical regions (Becker et al., 2010b). Nowadays, humans inhabiting temperate latitudes in Europe are, besides some cases in the Mediterranean area (Calba et al., 2017; Rezza et al., 2007; Succo et al., 2016), largely spared from these diseases.

Exclusively female mosquitoes bite, as they need blood to produce eggs. Oviposition usually takes place in stagnant water bodies, ranging from ephemeral ponds, ditches, containers, and floodplains. However, above-mentioned disease-carrying mosquito species often breed in artificial habitats, such as catch basins, tires, bins, and knotholes (Becker et al., 2010b) close to urban areas, making mosquito control in these regions more targeted. In contrast, females of the floodwater mosquito species *Aedes vexans* occurring in temperate regions lay their eggs into moist soil of regularly inundated areas, such as floodplains. When these floodplains are inundated, e.g., through snowmelt in early spring or rainfall events, hatching of larvae is triggered, leading to mass emergence of mosquitoes a few weeks later (Becker, 1997). Mass emergence of mosquitoes poses a considerable problem for tourists and the local population. Despite the lack of disease-carrying competence, mosquitoes in temperate regions lower human life quality due to nuisance by mosquito bites, which can result in negative effects on the economy through discouraging tourism and outdoor labor (Halasa et al., 2014; von Hirsch & Becker, 2009).

Because of these consequences for human population, various mosquito control programs were implemented in the past. While mosquito control in the late 19th century was done biologically using predatory Odonata or fish (Lamborn, 1890), synthetic insecticides like chlorinated hydrocarbons, organophosphates, carbamates, and pyrethroids were increasingly used in the mid-20th century. The use of these persistent, non-specific compounds led to resistant mosquito populations in some regions (Becker et al., 2010). Resistances in mosquitoes and the tremendous contamination of surface waters increased the demand for a more environmentally acceptable alternative in mosquito control. For more than 40 years, the biological insecticide *Bacillus thuringiensis* var. *israelensis* (Bti) is extensively used, for instance, by the German Mosquito Control Agency (Kommunale Aktionsgemeinschaft zur Bekämpfung der Schnakenplage e.V., KABS) in floodplains of the Upper Rhine Valley in southwest Germany (Becker, 1997).

1.4 The biocide *Bacillus thuringiensis* var. *israelensis*

Bacillus thuringiensis var. *israelensis* is a gram-positive, endospore-forming soil bacterium. Its larvicidal activity against mosquito larvae was first discovered by Goldberg & Margalit (1977) in the Negev desert of Israel. This subspecies of *Bacillus thuringiensis* is considered to be target-specific and highly toxic towards the Dipteran suborder Nematocera. The mechanism of action is shown in Figure 2 (Brühl et al., 2020). Bti produces Cyt and Cry proteins during sporulation, which get, once ingested (1) and solubilized by the alkaline midgut milieu (pH > 10) of target species (2), activated by specific proteases (3). Ultimately, the toxins interact with receptors and lipid in the membranes of epithelial cells (4) and promote oligomerization (5). The oligomers are then inserted in the membranes forming pores (6). This breaks down the epithelial cells in the midgut leading to the rapid death of the larvae (Becker et al., 2010; Ben-Dov, 2014). The Cry and Cyt proteins exhibit different structural and functional differences. While the Cry toxin is the insecticidal part, the Cyt protein mainly increases the toxicity and prevents development of insect resistance against Bti toxins (Soberón et al., 2013; Wirth et al., 2005). In the field, resistances against mixtures of Bti toxins have not been observed after 37 years of application (Becker et al., 2018).

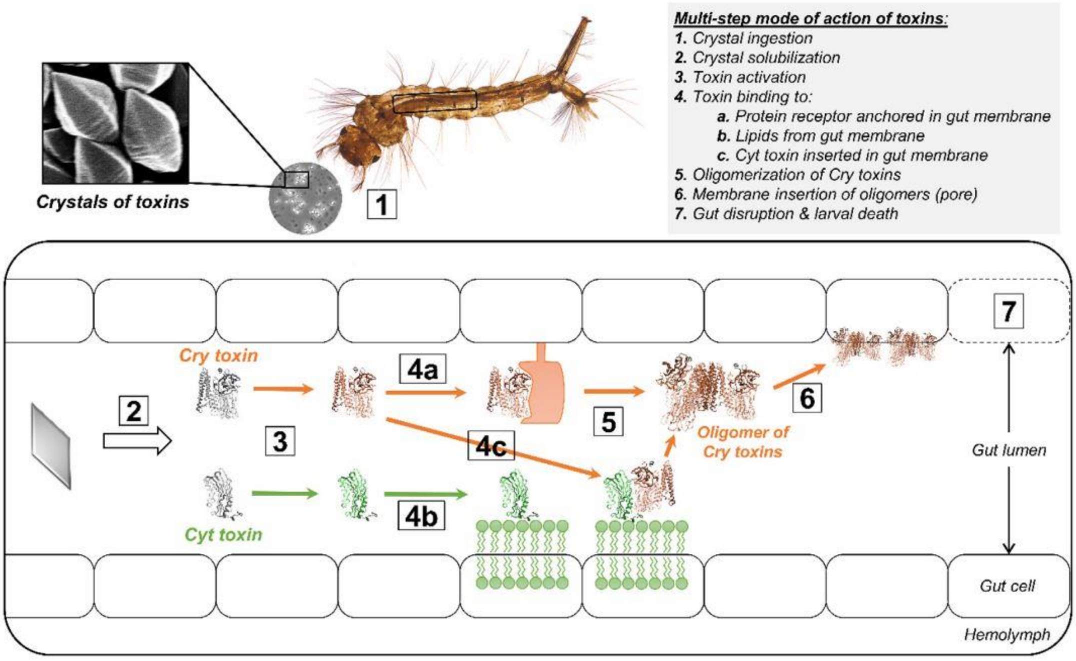


Figure 2: Schematic representation of the mode of action of Bti toxins in mosquito larvae (Figure from Brühl et al., 2020).

In the Upper Rhine valley, Bti (strain AM 65-52) is applied as liquid or granule formulations per hand or using a knapsack-sprayer, while Bti in form of ice pellets is applied from helicopters (Becker, 2003). Bti formulations in Germany contain a mixture of spores and toxic crystals, but viable spores must be sterilized by gamma-radiation before application (Becker, 2002). This prevents de novo sporulation and recycling of spores in the field (Becker et al., 2018; de Melo-Santos et al., 2009), reducing its persistency and proliferation in the environment. Depending on environmental conditions, Cry toxins can remain in the environment for a few years, while maintaining their toxic potential for up to three weeks (Dupont & Boisvert, 1986; Tetreau et al., 2012). Since Bti is an insect pathogen, it is unable to proliferate in soils without a host. However, in an insect cadaver, viable Bti spores can complete their entire growth cycle (Duchet et al., 2014). After extensive treatment of wetlands with Bti, it takes up to 3-4 years for spore density to return to normal levels after interruption of Bti spraying as observed in wetlands the French Camargue (Poulin et al., 2022). This suggests proliferation of Bti spores beyond the short-term efficacy against mosquitoes. The sterilization of Bti spores in Germany, which results in a decreased the efficacy against mosquito larvae of about 20 % (Becker, 2002), potentially reduces long-term effects through mitigated proliferation. In the Upper Rhine Valley, regular treatment of wetlands is still ongoing with five Bti applications per year on average. From 1981-2016, almost 5,000 tons of sterilized Bti formulations have been applied to approximately 400,000 ha (Becker et al., 2018). Therefore, even when sterilized Bti is used, organisms inhabiting these wetlands are continuously exposed to Bti toxins.

1.5 Effects of Bti on non-target fauna

Direct effects of Bti on non-target fauna are mostly limited to larvae of Nematocera (Belousova et al., 2021; Boisvert & Boisvert, 2000; Brühl et al., 2020), some Coleopterans (Tudoran et al., 2021), and to a smaller extent also on frog tadpoles via an increased biochemical stress response (Allgeier et al., 2018; Lajmanovich et al., 2015). Although chironomids are generally not targeted, in some regions Bti is applied to control mass emergence of chironomids which form huge swarms and disturb residents, for instance in South Wales (Vaughan et al., 2008). It is assumed that chironomid populations in the field are less sensitive to Bti and therefore not affected by field rates used for mosquito control (Becker & Lüthy, 2017; Lundström et al., 2010). However, due to the close phylogenetic relation of chironomids to mosquitoes, recent studies reported high susceptibility of chironomid larvae, also to field rates of Bti used for mosquito control (Allgeier et al., 2019a; Kästel et al., 2017; Stevens et al., 2005). The effect of Bti on chironomids has been a matter of debate for decades, as some studies recorded reduced chironomid abundance in regularly treated wetlands (Allgeier et al., 2019a; Hershey et al., 1998), while others did not report adverse effects of Bti on chironomids in the field (Duchet et al., 2015; Lundström et al., 2010; Wolfram et al., 2018). Since species and subfamilies differ in their sensitivity (Liber et al., 1998; Theissinger et al.,

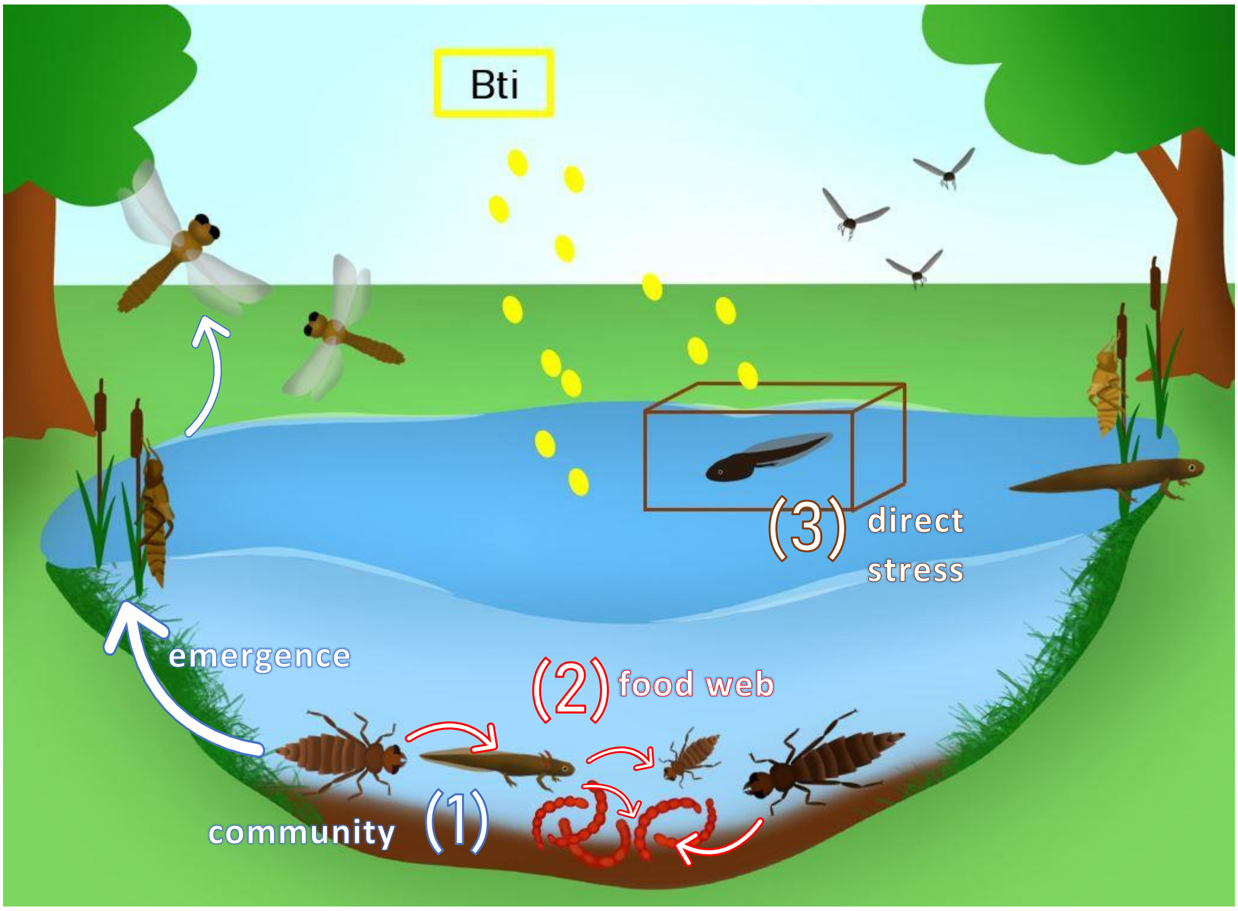
2019) and younger larvae are more susceptible towards Bti (Kästel et al., 2017), Bti can alter chironomid populations in the field regarding species community composition and age structure. Chironomids are present in high densities in most wetlands, representing a major component of macroinvertebrate communities as well as one of the most important linkages between aquatic-terrestrial habitats upon emergence (Armitage et al., 1995). Due to their nutritional quality and high digestibility, both larval and adult chironomids are a preferred food source for many aquatic (amphibians, Odonata and fish) and terrestrial predators (amphibians, Odonata, spiders and birds) (**Appendix A-2**; Allgeier et al., 2019b; Armitage et al., 1995; Gergs et al., 2014; Jakob & Poulin, 2016; Kolbenschlag et al., 2023b; Poulin et al., 2010). Bti-induced effects on larval chironomids are therefore not only limited to chironomid populations, but also influence aquatic and terrestrial food webs.

Bt toxins can also strongly affect soil microbiota (as reviewed in Belousova et al., 2021). Alterations in soil microbial communities can directly affect detritus-processing organisms, such as protozoans and nematodes, which may have effects on the food web as observed in McKie et al. (2023). Their findings suggest an increased food chain length in treated floodplains due to possible feeding of protozoans on decaying mosquito larvae adding an additional trophic level to the base of the food web. This effect may also cascade to other detritus-feeders, such as amphibian larvae (tadpoles). Direct sublethal effects on tadpoles have been investigated in recent laboratory studies. For instance, Allgeier et al. (2018) found an increase in biochemical stress biomarkers in tadpoles exposed to Bti. While this was not confirmed by other studies (Schweizer et al., 2019; Gerstle et al., 2023b), an overexpression of genes involved in detoxification processes was observed as well as altered intestine microbiota in tadpoles (Gutierrez-Villagomez et al., 2021). The latter may influence the sensitivity to Bti (Broderick et al., 2009) and the fitness of individuals, e.g., by facing a higher susceptibility to parasitic infections (Gutierrez-Villagomez et al., 2021). In food webs, Bti was found to affect top predators, for instance, via an increase of isotopic niche width in predatory Odonata and newt larvae because of reduced chironomid availability (Allgeier et al., 2019b). Additionally, in Bti-treated mesocosms, a decreased survival of newt larvae was observed in presence of Odonata larvae, indicating increase in intraguild predation. Similarly, Jakob & Poulin (2016) reported lower abundance of adult dragonflies in wetlands regularly treated with Bti, possibly due to reduced abundance of adult chironomids. Additionally, insectivorous birds breeding in Bti-treated areas consumed less Nematocera (both chironomids and mosquitoes) and their predators (spider and dragonflies), which resulted in a smaller clutch size and lower breeding success (Poulin et al., 2010). For riparian spiders, Bti treatment likely induced a diet shift from chironomids to higher proportions of mayflies (Ephemeroptera) and terrestrial prey as observed in Kolbenschlag et al., (2023b). In fact, regular use of Bti can affect wetland food webs. Therefore, understanding complex interactions is of critical importance, especially for protected areas, where until now large-scale spraying of Bti is still allowed.

2 OBJECTIVES AND THESIS OUTLINE

Since many researchers contributed to the work I present in this dissertation (see **Appendix A-4**: Author contributions), I will use the plural form in the following.

The main objective of this thesis is the investigation of stressor-induced effects on non-target organisms like chironomids, and consequences for higher trophic levels such as predatory Odonata and newts, in natural communities and possible propagation of effects to the terrestrial ecosystem. As a model stressor, we chose the biocide Bti because of its large-scale use, also in partially protected wetlands, and its specific mode of action, allowing for investigation of indirect effects on food webs, since predators in our test system are not directly susceptible to Bti (Empey et al., 2021; Painter et al., 1996). This thesis is divided in three chapters, each answering the overall research question (RQ) shown in Figure 3. Chapter 1 and 3 are peer-reviewed publications [**Appendices A-1 and A-3**], while chapter 2 is based on unpublished data [**Appendix A-2**].



(1) **RQ 1: Effects on benthic invertebrate communities and Odonata emergence**

Does multiple Bti treatment alter the benthic macroinvertebrate community composition and do effects propagate to the terrestrial ecosystem, e.g., via altered Odonata emergence?

[Scientific publication 1 – Appendix A-1]

(2) **RQ 2: Indirect effects on food webs**

How does the diet of predators (larval Aeshnidae and newts) change when prey density, i.e., chironomid larvae, is reduced by Bti treatment?

[Submitted manuscript – Appendix A-2]

(3) **RQ 3: Direct sublethal effects on tadpoles**

Does Bti treatment have direct sublethal effects, i.e., biochemical stress response, on common frog larvae (*Rana temporaria*) under natural climatic conditions?

[Scientific publication 2 – Appendix A-3]

Figure 3: Conceptual overview of the three research questions addressed in this thesis (RQ1–RQ3).

3 METHODOLOGICAL OVERVIEW

3.1 Study site

The field experiments were conducted in the floodplain pond mesocosms (FPMs; Figure 4) at the Eußerthal Ecosystem Research Station (EERES; 49°15′14′′N, 7°57′42′′E; RPTU Kaiserslautern-Landau) in the Palatinate forest in southwest Germany. The twelve FPMs were built in 2017 and since then naturally colonized by local flora and fauna (Stehle et al., 2022). One FPM measures 23.5 × 7.5 m and is 30 cm deep at the deepest point from where it gradually fades into a terrestrial shore at one side of the FPM. The FPMs can be flooded with stream water from the Sulzbach, an adjacent oligotrophic cold-water stream, until the terrestrial part is completely under water (at 50 cm water depth). When flooded, the water surface area is approximately 104 m^2. The deep aquatic part consists of coarse pebble gravel (1-3 cm diameter) and the shallow parts at the sides are mostly covered by submerged macrophytes (waterweeds and coontails) and emerged cattails. There are only minor anthropogenic influences in the upstream area and Bti was never applied to the FPMs or any nearby water body.

The FPMs function as breeding habitat for many amphibians, such as common frogs (*Rana temporaria*), common toads (*Bufo bufo*), palmate newts (*Lissotrion helveticus*), and alpine newts (*Ichthyosaura alpestris*). From January to April, adult amphibians migrate to their breeding ponds. Since their eggs and larvae can temporarily impact food webs, we set up an amphibian fence around the twelve FPMs to control mass immigration and oviposition inside

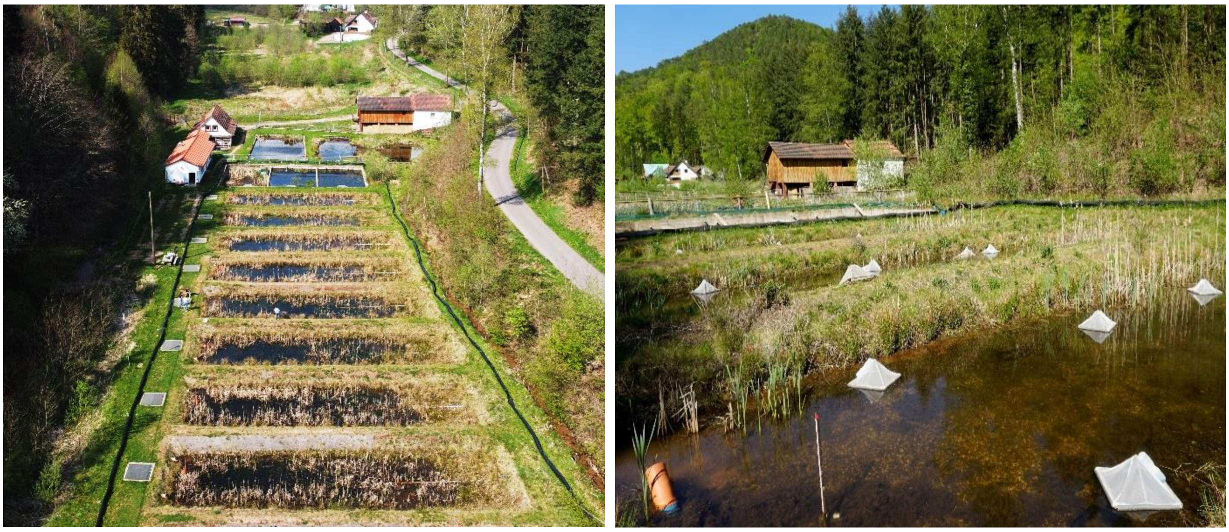


Figure 4: Study site of the field experiments. Floodplain pond mesocosms (A ≈ 104 m^2) at the Eußerthal Ecosystem Research Station in southwest Germany (photos by C. A. Brühl, May 2021).

the test system. Before test start, we introduced equal amounts of amphibian eggs and adult newts back into each FPM to not completely exclude them from the food web.

3.2 Bti application and flooding

Since Bti treatments in the Upper Rhine Valley start after temporary inundation of the floodplains, which triggers mass hatching of the mosquito larvae, Bti applications in these wetlands are usually linked to temporary flooding. To mimic a realistic Bti application scenario, we flooded the FPMs during spring three times from 30 cm to 50 cm in 2020 and three times in 2021 (for dates see Figure 7). On the third day of the flood, we applied the maximum field rate of VectoBac WDG (Valent BioSciences, Illinois, USA) to every second FPM using a knapsack sprayer (Prima 5, Gloria, Germany) (Figure 5). The toxic potency of Bti formulation is usually declared in international toxic units (ITU) per mg product. The potency of VectoBac WDG is 3000 ITU/mg, however, we calculated the amount per surface area with regards to the potency reduction of 20% due to gamma-radiation (Becker, 2002). The maximum field rate of Bti (2.88×10^9 ITU/ha) is applied when the water is deeper than 10 cm and/or older instar mosquito larvae are targeted (Becker, 1997). We suspended VectoBac WDG in filtered pond water (mesh size: 55 µm) and sprayed small droplets evenly on the water surface. We postponed the application when there were strong winds or heavy rain to avoid spray drift to adjacent control FPMs.

Figure 5: Top view of the floodplain pond mesocosm system, control (blue) and Bti-treated FPMs (red) (photo from C. A. Brühl, May 2021).

3.3 Methods

3.3.1 RQ-1: Effects on benthic invertebrate communities and Odonata emergence

To investigate effects of multiple Bti exposures on natural invertebrate communities, we sampled benthic macroinvertebrates three weeks after the last Bti application of the first year

(Figure 7, **Appendix A-1**). To determine if communities and Bti effects differ between habitats, we sampled two habitats which included gravel in the aquatic part, and macrophytes on the shallow sides of the FPMs (Figure 6). In each habitat, a 1-m transect was sampled with a kicknet (mesh size: 500 µm) dragged along the bottom of the FPMs, a total of three times. The sampled invertebrates were identified on family level and chironomid larvae on subfamily level.

In order to investigate possible propagation of Bti-induced effects on terrestrial ecosystems, we used sampling of Odonata exuviae as proxy for Odonata emergence. We collected exuviae weekly or bi-weekly in the second year of the experiment (Figure 7), because we expected effects on top predators to be more pronounced in the long-term (due to reduced prey availability), also with respect to semi-voltine Odonata species (aquatic larvae developing for two years). One person searched emerged plants for 20 min in each FPM. Exuviae were determined on family level.

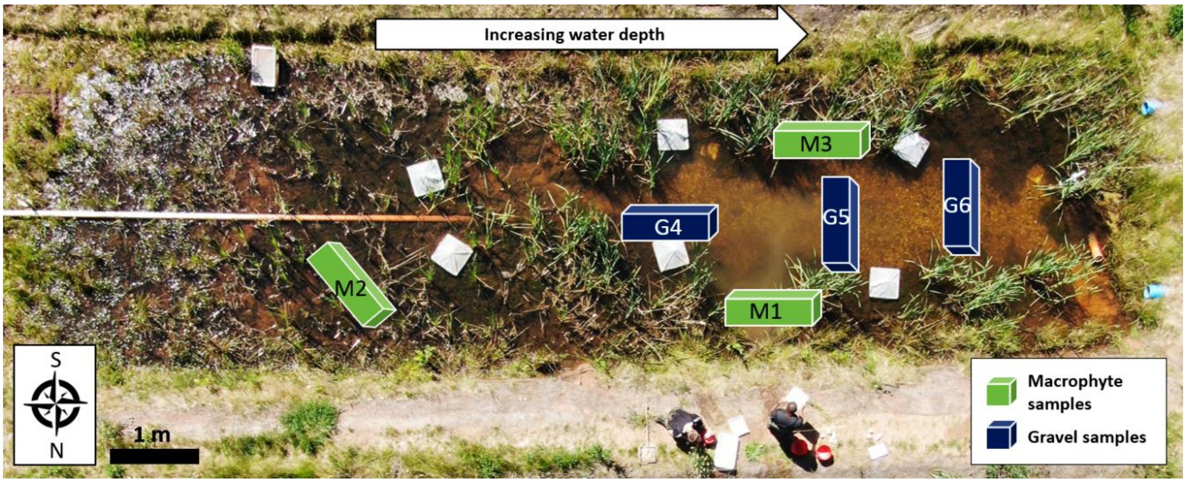


Figure 6: Top view of one floodplain pond mesocosm (A ≈ 104 m^2) with three sampling transects (1m) for each habitat (macrophytes as green polygons and gravel as blue polygons) using a standard kicknet sampler (25 x 25 cm; mesh size = 500 µm). The kicknet was dragged along the bottom of the mesocosm while disturbing the sediment by foot. (Figure taken from **Appendix A-1**)

3.3.2 RQ-2: Indirect effects on food webs

In recent years, stable isotope analyses of carbon (C) and nitrogen (N) have become increasingly important for understanding complex trophic interactions in food webs. To determine Bti-induced effects on the aquatic food web, we sampled top predators in our test system, such as larval dragonfly (Odonata: Aeshnidae) and newts (Urodela: Salamandridae), as well as their possible resources three weeks after the last Bti application in 2020. This was done simultaneously to the benthic invertebrate sampling in the FPMs (Figure 6). We used stable isotope analyses of C and N to determine whether a reduction in prey availability, i.e., reduced abundance of larval chironomids, induces a diet shift in predators. Fractionation of C depends on the source of food (terrestrial/aquatic or pelagic/benthic), therefore, enrichment of $\delta^{13}C$ in a consumer indicates where the predator is foraging. Fractionation of $\delta^{15}N$ usually happens during metabolism and assimilation of resources, hence, $\delta^{15}N$ enriches in consumers in a step-wise manner with an increasing trophic level. For stable isotope analyses, we used the tail from newt larvae and the thorax from Aeshnidae larvae, since these body parts contain the highest muscle content. Using a Bayesian approach, we determined the diet proportions, the relative niche size, and the trophic position in the food web of the two predators. Additionally, we determined neutral lipid fatty acid (NLFA) content in newt larvae from control and Bti-treated FPMs to estimate effects of diet on the body condition and nutritional value of newt larvae. We grouped the NLFAs into long-chain fatty acid groups: saturated fatty acids (SFA), monosaturated fatty acids (MUFA), and physiologically important polyunsaturated fatty acids (PUFA). Differences in NLFA content can indicate differences in the consumers' diet, which may not be detected using stable isotope analyses, as well as pointing to changes in nutritional quality of ingested prey and/or lipid resource use in the consumer. PUFAs, especially omega-3 PUFAs, are essential to maintain body functions, therefore, the depletion of PUFA content can have consequences on the nutritional quality for higher trophic levels.

3.3.3 RQ-3: Direct sublethal effects on tadpoles

We investigated the biochemical stress response of common frog larvae (*Rana temporaria*) to multiple Bti exposure under natural climatic conditions (Figure 7, **Appendix A-3**). *Rana temporaria* is a frog species widely distributed in Europe and a surrogate for herbivorous amphibian larvae, possibly ingesting large amounts of Bti during their development in treated wetlands. Due to contrasting results of previous investigation of biochemical biomarkers in *R. temporaria* larvae under laboratory conditions (Allgeier et al., 2018; Schweizer et al., 2019), we chose two biomarkers, i.e., glutathione-S-transferase (GST) and protein carbonyl content, to estimate oxidative stress induced by Bti in our outdoor FPM test system. Ambient water temperature can strongly influence enzymatic activity and therefore biochemical stress response to stressors, therefore, we also monitored water temperature continuously with loggers throughout the course of the experiment to determine the effect of water temperature on stress response in tadpoles. 48 h after each of the three Bti applications (see Figure 7), we sampled tadpoles in different developmental Gosner stages (GS; Gosner, 1960) and analyzed GST activity and protein carbonyl content in tissue homogenate (whole tadpoles). GST is a phase II detoxifying enzyme involved in the antioxidant system (Steinberg, 2012; Venturino & D'Angelo, 2005). In case the ROS defense mechanism by the antioxidant system inside the cells is insufficient, proteins can be oxidized generating protein carbonyls (Dalle-Donne et al., 2003). In contrast to ROS, protein carbonyls are stable making them a convenient biomarker for our study. Both biomarkers were investigated spectrophotometrically using a multiplate reader (Synergy HT-I, BioTek, USA).

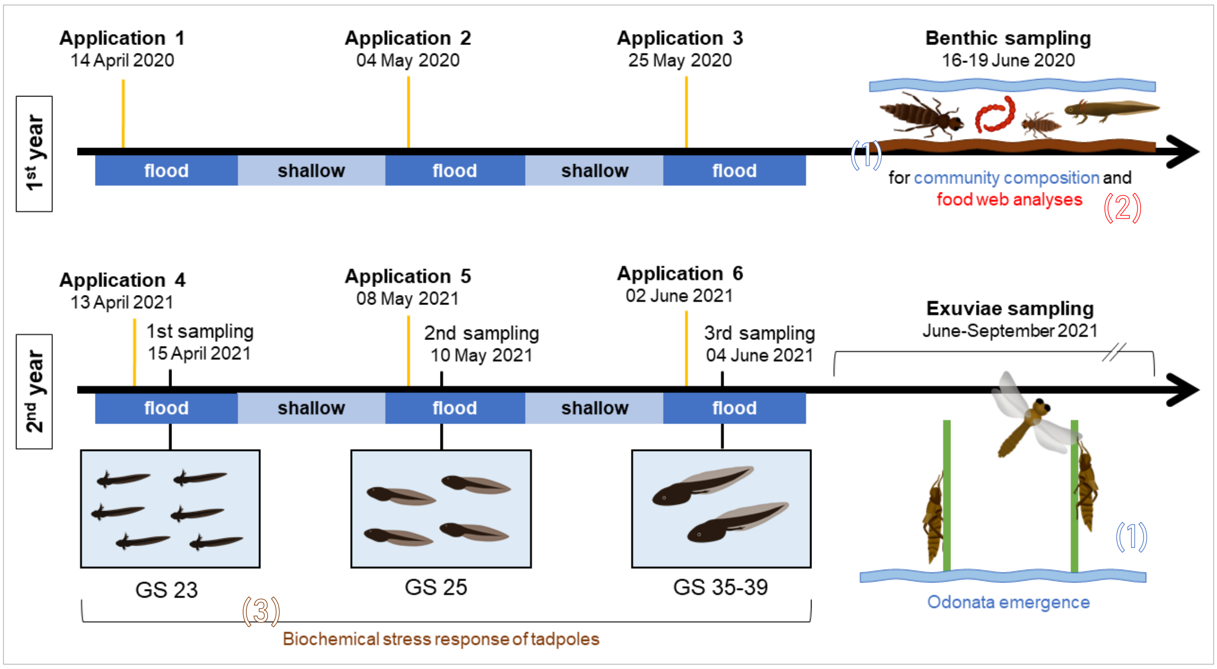


Figure 7: Schematic overview of the field experiments in the floodplain pond mesocosms from 2020 to 2021. Flood and shallow periods are shown as well as dates of Bti applications, samplings for community analyses and Odonata emergence (1), food web analyses (2), and direct effects on tadpoles (3).

4 SUMMARY AND GENERAL DISCUSSION

4.1 RQ-1: Effects on benthic invertebrate communities and Odonata emergence

The results of this chapter are based on **Appendix A-1**, which were published in a peer-reviewed journal (*Environmental Pollution*).

In June 2020, we collected and identified 13,688 benthic macroinvertebrates in total (**Appendix A-1**), three weeks after the last Bti application of the first year. Chironomids were the most abundant taxon accounting for 72% of total collected individuals, mayflies (Ephemeroptera) being the second most (12%) and Odonata the third most abundant taxon (11%). Bti treatment affected the abundance of macroinvertebrates significantly with approximately 36% fewer individuals compared to control FPMs. Also, the habitat type had a significant influence on the total macroinvertebrate abundance with 70% fewer individuals than in macrophytes habitats. Generally, macroinvertebrate communities differed significantly between treatment and habitats (Figure 8). Analyses of similarity percentage revealed which taxa contributed the most (cumulative > 70%) to the dissimilarities between treatments (Table 1). The three identified chironomid subfamilies Chironominae, Tanypodinae, and Orthocladiinae were driving the dissimilarities between control and Bti-treated FPMs, with each taxon contributing more than 10%. Overall, the abundance of chironomid larvae was significantly reduced by 41% in Bti-treated FPMs compared to controls.

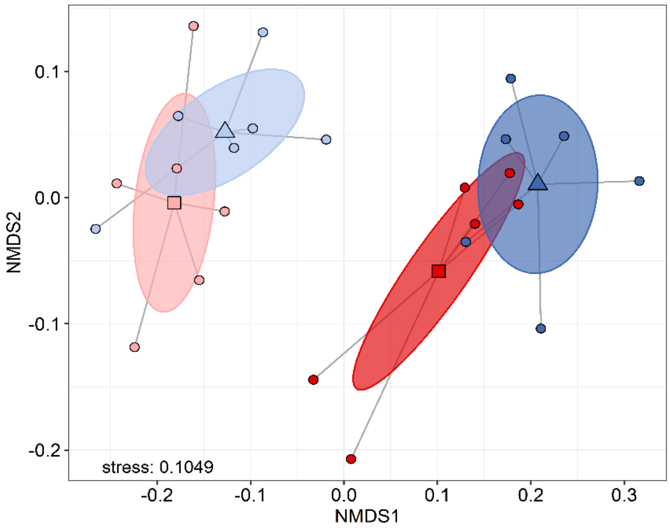


Figure 8: Non-metric multidimensional scaling (nMDS) ordination plot illustrating differences in benthic macroinvertebrate community composition between treatment (red squares; n=6) and control FPMs (blue triangles; n=6), and habitats gravel (light color; n=6) and macrophytes (dark color; n=6), including 95% confidence ellipses. (Figure taken from **Appendix A-1**)

Table 1: Taxa driving the dissimilarity (cumulative contribution ~70%) between control (n=6) and treatment FPMs (n=6) after SIMPER analysis on square root transformed densities. Average density (individuals per m^2) and respective square root transformed average densities (in brackets) are shown for control and treatment for each taxon, and the percent difference in Bti-treated FPMs compared to control FPMs. (Table taken from **Appendix A-1**)

	Average density (m^{-2})		Contributing	Percent
Taxa	Control	Treatment	percent	difference
Chironominae	324.8 (17.2)	173.1 (12.5)	13.5	↓ 47%
Tanypodinae	207.2 (13.2)	170.9 (12.0)	12.8	↓ 18%
Orthocladiinae	130.7 (8.8)	49.1 (5.5)	12.2	↓ 62%
Libellulidae	63.3 (6.7)	39.0 (5.3)	8.8	↓ 39%
Coenagrionidae	28.8 (4.2)	17.4 (3.6)	6.7	↓ 39%
Chironomidae sp.	30.2 (4.7)	14.3 (3.0)	6.5	↓ 53%
Ceratopogonidae	18.0 (3.1)	1.4 (0.6)	5.2	↓ 93%

Odonata exuviae were collected in the second year of Bti applications to the FPMs. During the sampling period from June to September 2021, we collected a total of 1,717 Odonata exuviae from the FPMs (**Appendix A-1**). Libellulidae were the most common family contributing 45% to the total number of exuviae, followed by Aeshnidae (40%) and Coenagrionidae (15%). The number of Libellulidae exuviae was significantly reduced by 54% in Bti-treated FPMs (Figure 9). Aeshnidae and Libellulidae were largely dominated by two genera, *Anax* and *Sympetrum*, respectively.

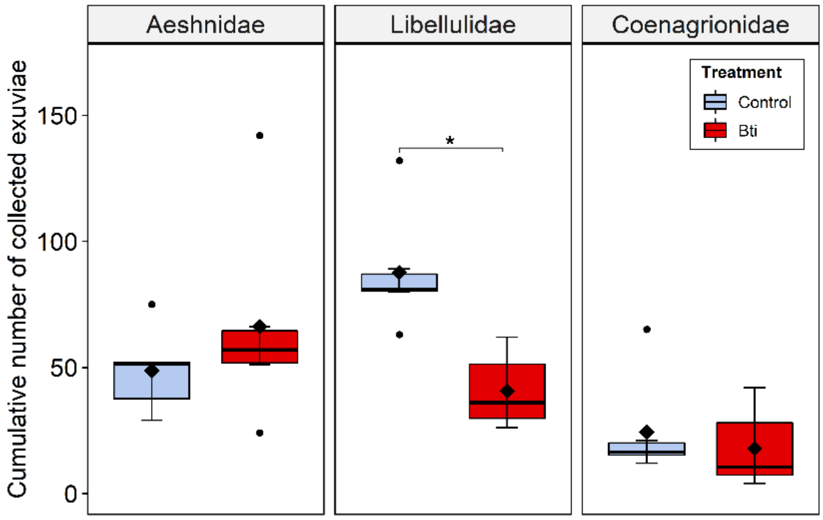


Figure 9: Cumulative number of exuviae in control (blue; n=6) and Bti-treated FPMs (red; n=6) for Aeshnidae (A), Libellulidae (B), and Coenagrionidae (C), collected from June to September 2021. Lower and upper box boundaries show 25th and 75th percentiles, respectively, line inside box show medians and black diamonds show arithmetic mean density. Whiskers and black dots show the variability outside the lower and upper quartiles. Statistically significant differences (p<0.05) are denoted with an asterisk (*). (Figure taken from **Appendix A-1**)

4.2 Discussion of RQ-1

Multiple Bti treatments significantly altered benthic macroinvertebrate community compositions in replicated, but naturally established ecosystems. Expectedly, this was mainly driven by the effect on chironomid larvae, whose susceptibility to Bti has been observed by multiple laboratory and field studies in the past, as reviewed in Boisvert & Boisvert (2000) and Brühl et al. (2020). Our results revealed that Bti-induced effect sizes differed between chironomid subfamilies (Table 1), with Chironominae and Orthocladiinae being reduced by 47% and 62%, respectively, while the effect was lower on Tanypodinae (18% fewer individuals in Bti-FPMs). In fact, many species belonging to the subfamily Tanypodinae are carnivorous, suggesting a relation between susceptibility to Bti and foraging behavior, e.g., deposit-feeders or predators (Vallenduuk & Moller Pillot, 2007). While benthic deposit-feeding chironomid subfamilies like Chironominae and Orthocladiinae presumably ingested Bti after its sedimentation, predatory chironomids presumably ingested fewer amounts of Bti. Our results are comparable to observations from a mesocosm study by Allgeier et al. (2019a), in which Bti had little to no effect on larvae of the subfamily of Tanypodinae, while Chironominae and Orthocladiinae were reduced by 44% and 58%, respectively. Taxa-specific sensitivity of chironomids to Bti was also found in studies by Liber et al (1998) and Lundström et al. (2010). Although Tanypodinae seemed to be less affected due to their feeding strategy, they can still be affected indirectly via reduced prey availability, as they feed on early instar chironomids which are most sensitive to Bti (Kästel et al., 2017), but also by ingesting Bti-contaminated prey, consequently taking up Bti toxins through their prey. As chironomids often dominate wetland invertebrate communities by constituting up to 93% of the total insect emergence (Leeper & Taylor, 1998), a reduction in chironomid abundance can result in cascading effects on the entire invertebrate community compositions. This was also observed in a long-term field study in Minnesotan wetlands (Hershey et al., 1998), which was the first study revealing adverse effects of realistic Bti field rates on benthic macroinvertebrate communities, mainly due to the reduction of chironomid abundance by 60-80%. Allgeier et al. (2019a) and Bordalo et al. (2021) reported significant alterations of benthic community compositions in mesocosms repeatedly exposed to field-relevant Bti application rates due to reduced chironomid abundance, which is in line with our results. In a companion study by Kolbenschlag et al. (2023a) conducted in parallel in the FPM system, chironomid emergence was altered, with a ~10 days earlier and ~26% reduced emergence peak in Bti-treated FPMs, which is comparable to our observations (when considering the time points of sampling). A temporal shift in emergence pattern can have implications for terrestrial predators, like spiders and birds, relying on aquatic subsidy (Kolbenschlag et al., 2023b; Poulin et al., 2010).

Despite the effect on chironomids, Odonata larvae, namely the families Libellulidae (Anisoptera) and Coenagrionidae (Zygoptera), also contributed to the observed dissimilarities between control and Bti-treated macroinvertebrate communities (Table 1), with both 39% fewer individuals in Bti-FPMs. This effect was not observed for Aeshnidae (Anisoptera) which were considerably larger at the time of sampling. In contrast to chironomid larvae, Odonata larvae are not directly susceptible to Bti (Boisvert & Boisvert, 2000; Painter et al., 1996). The lack of effect on larger Aeshnidae indicates an increase of size-dependent intraguild predation as a consequence of reduced prey availability (van Buskirk, 1989, 1992). The results suggested that Aeshnidae might not be as dependent on larval chironomids as a main food source than smaller predators like Libellulidae and Coenagrionidae.

We observed a similar effect on emerging Odonata (estimated by exuviae) in the following year, with fewer number of Libellulidae and Coenagrionidae but higher number of Aeshnidae emerging from Bti-treated FPMs (Figure 9). In our study, Libellulidae were predominantly species of the genus *Sympetrum*, which are univoltine (completion of aquatic life phase in one year) and Aeshnidae, predominantly species of the genus *Anax*, which are mainly semivoltine (larval development over two years) (Wildermuth & Martens, 2019). The observed effect difference for Aeshnidae and Libellulidae implied that the timing of larval development and Bti applications in spring may play a role for the emergence. Larvae of *Sympetrum* usually emerge in late summer. Therefore, early larval development can coincide with Bti treatment of wetlands in spring and summer which, in our study, led to a reduced chironomid availability. At this time, early instar larvae of *Sympetrum* are small and could have served as prey, for example for late instars of semivoltine *Anax*. We suggested that the observed reduction of late emerging Libellulidae can be mainly due to size-dependent intraguild predation by early emerging Aeshnidae, as a result of significantly reduced chironomids as bottom-up effect on aquatic predators. Underlying effects on the aquatic food web will be addressed in the following chapter (RQ-2).

4.3 RQ-2: Indirect effects on food webs

The results of this chapter are based on unpublished data presented in **Appendix A-2**.

We used bulk stable isotopes of C and N to investigate Bti-induced effects on the diet of two top predators in our FPMs, larvae of the palmate newt (*Lissotriton helveticus*) and Aeshnidae (predominantly *Anax imperator*). Structures of stable isotopes of C and N are comparable between control and Bti-treated FPMs (Figure 10 a, b) with similar sizes of resource polygons. Chironomid larvae showed a higher $\delta^{15}N$ in Bti-treated FPMs compared to control FPMs. To reduce the number of resources in the models, we grouped prey organisms with similar isotopic composition to three different resource clusters consisting of: Aeshnidae/newt/damselfly, Chironomid/mayfly, and Libellulidae/zooplankton. The term damselfly and mayfly are used here, but it refers to Coenagrionidae and Ephemeroptera, respectively (as used in the previous chapter). According to the stable isotope mixing model estimates, the diet of newts was dominated by the cluster Libellulidae/zooplankton (> 60%; Figure 10 c), while the resource clusters Aeshnidae/newt/damselfly and Chironomid/mayfly each contributed proportions of approximately 15–20% to the diet of newt larvae, with no effects of Bti treatment. In contrast, in control FPMs, the diet of Aeshnidae was more balanced and consisted of all three resource clusters in equal parts (Figure 10 d), while in Bti-treated FPMs, the diet was dominated by Aeshnidae/newt/damselfly (> 40%), with Chironomid/mayfly and Libellulidae/zooplankton each contributing approximately 30%. The relative niches sizes did not differ between control and Bti-treated FPMs, neither for newts nor for Aeshnidae (Figure 10 e, g). Similar results were obtained for the trophic levels (Figure 10 f, h). Generally, Aeshnidae were one trophic level higher than newts.

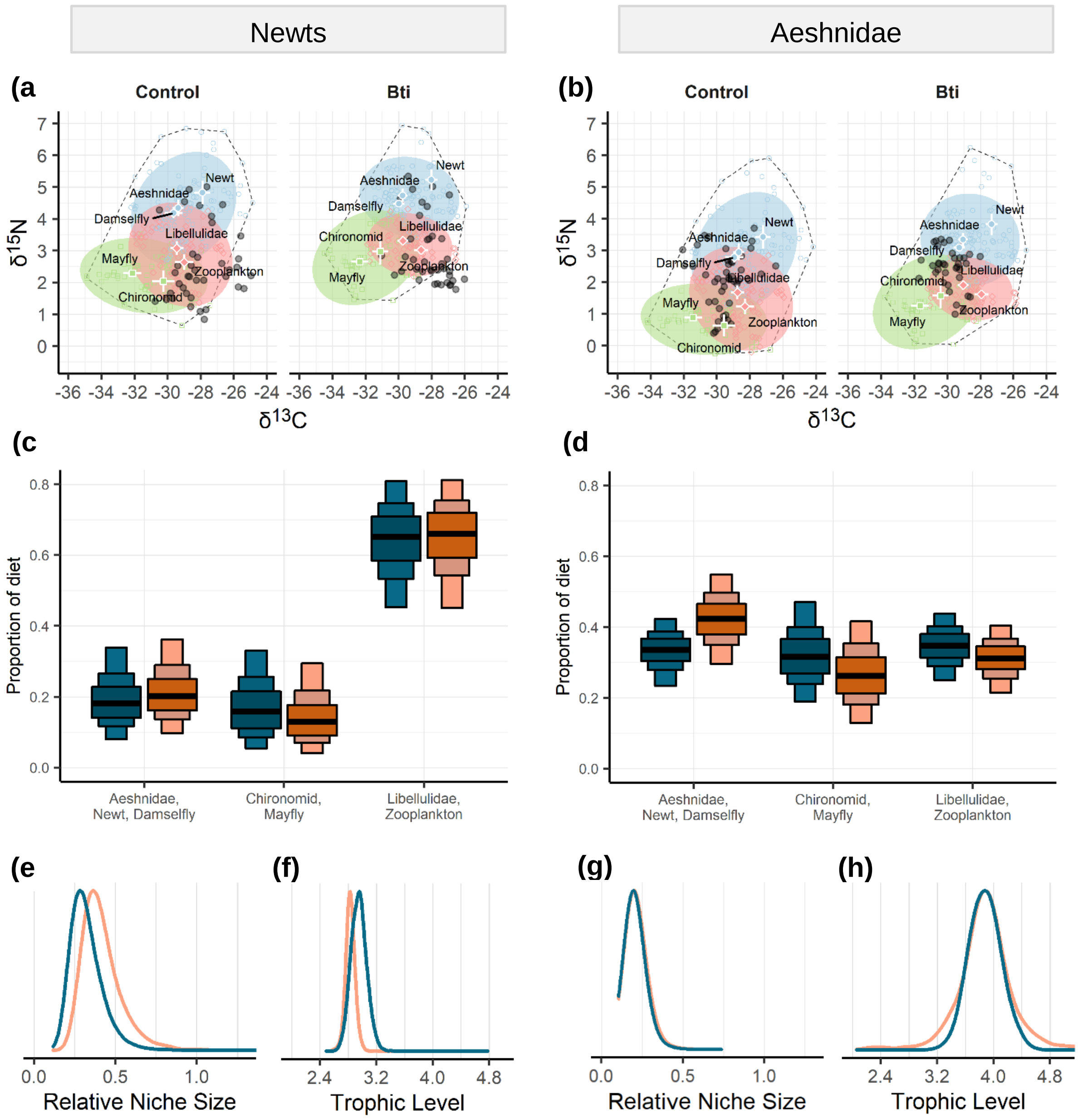


Figure 10: Stable isotope ratios of C and N of newt (a) and Aeshnidae larvae (b) as consumers shown as black dots, means ± SD, and 95 %-confidence ellipses of prey organisms (adjusted for trophic enrichment) for each cluster: Chironomid and mayfly larvae (green squares and ellipse), Aeshnidae, newt and damselfly larvae (blue circles and ellipse), Libellulidae larvae and Zooplankton (red diamonds and ellipse). Dotted polygon delineates outer borders of resource polygon. Proportion of diet of newt larvae (c) and Aeshnidae larvae (d) for each prey cluster, where black lines indicate the medians, and darkest to lightest color of boxes show Bayesian 50%, 75% and 95% equal tail credible intervals, respectively. Niche sizes of newts (e) and Aeshnidae (g) determined from consumer SEA relative to FPM-specific resource SEA, and the trophic position of newts (f) and Aeshnidae (h) in the food web, in control (dark blue) and Bti-treated (orange) FPMs (n = 5). **Figure taken from Appendix A-2.**

We determined a total of 15 different fatty acids in newt larvae from control and Bti-treated FPMs (Table 2). The most abundant FAs were the saturated FAs palmitic acid (16:0) and octadecanoic acid (18:0). Control newts showed approximately 10% higher content of total NLFA than Bti-treated newts. Although not significant, PUFA content was approximately 20% lower in newts from Bti-FPMs, from which ~30% less omega-6 PUFA content showed the highest relative difference to control newts.

Table 2: Mean (± 95% confidence interval; CI) NLFA contents (mg per g dry weight) per fatty acid (FA) and FA group[a] in newt larvae from control (n=7) and Bti-treated (n=10) FPMs.

	Control		**Bti**		Percent difference	$F_{1,2}$	*p*
Fatty acids [a]	Mean	95% CI	Mean	95% CI			
Σ SFA	3.249	0.730	3.010	0.441	-7.94	0.6910	0.493
16:0	1.851	0.401	1.779	0.301	-4.09		
18:0	1.366	0.365	1.207	0.223	-13.14		
22:0	0.032	0.007	0.024	0.007	-31.97		
Σ MUFA	0.670	0.401	0.704	0.311	4.85	0.025	0.888
16:1n-7	0.258	0.240	0.284	0.175	9.09		
18:1	0.387	0.263	0.401	0.146	3.54		
22:1n-9	0.025	0.006	0.019	0.004	-30.63		
Σ PUFA	1.832	0.605	1.526	0.410	-20.03	1.046	0.414
omega-3 PUFA	0.935	0.344	0.822	0.226	-13.75	0.4559	0.569
18:3n-3 (ALA)	0.407	0.209	0.347	0.142	-17.38		
20:3n-3	0.035	0.019	0.036	0.017	3.18		
22:5n-3 (EPA)	0.436	0.155	0.383	0.098	-13.83		
22:6n-3 (DHA)	0.092	0.048	0.092	0.043	0.14		
omega-6 PUFA	0.767	0.225	0.576	0.162	-33.16	2.134	0.282
18:2n-6 (LIN)	0.258	0.071	0.192	0.087	-34.41		
20:2n-6	0.041	0.032	0.047	0.019	12.54		
20:3n-6	0.044	0.025	0.039	0.016	-14.65		
20:4n-6 (ARA)	0.510	0.170	0.384	0.091	-32.72		
22:2n-6	0.009	0.008	0.006	0.006	-35.52		
Σ Total NLFA	5.751	1.460	5.240	1.022	-9.75	0.4832	0.559

[a] Sum (Σ) of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), omega-3 and omega-6 PUFAs, and total neutral lipid fatty acids (Total NLFAs). Physiologically important PUFAs eicosapentaeonic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid (ARA), and their precursors linoleic acid (LIN), and alpha-linolenic acid (ALA).

4.4 Discussion of RQ-2

Considering the 41% reduction of chironomid density in Bti-treated FPMs (**Appendix A-1**), we expected a shift in the diet of newt larvae towards other prey, not affected by Bti, such as mayflies or zooplankton. However, stable isotope mixing models suggest no clear effects of

Bti treatment on the newts' diet. The structure of the resource polygon made it difficult to clearly separate the source of interest, i.e., chironomids, from other sources. A study by Allgeier et al. (2019b) reported that chironomids were the dominant food source for newt larvae over zooplankton, snails and *Asellus aquaticus* based on stable isotope analyses. Additionally, they observed no differences in the proportion of diet in control and Bti-treated mesocosms, despite observing a reduced availability of chironomids (53-87%) in Bti-treated mesocosms compared to controls. Similar to our results, the effect of Bti treatment on the abundance of chironomids (41% reduction; **Appendix A-1**) is not reflected in the newts' diets. However, in our study, it is also possible that newts fed on a source high in δ^{13}C and low in δ^{15}N such as periphyton-feeders, e.g., oligochaete worms (Tubificina) or molluscs, which we could not include in our modelling due to the few and uneven sample sizes between FPMs. Interestingly, chironomids in Bti-treated FPMs were enriched in δ^{15}N compared to chironomids in control FPMs (Figure 10 a, b) supporting the conclusion from our previous study (**Appendix A-1**) that carnivorous chironomids, characterized by higher δ^{15}N (i.e., Tanypodinae), are less affected by Bti than herbivorous chironomid species. The same pattern in enriched δ^{15}N adult chironomids emerged from the Bti-treated FPMs was observed in a companion study investigating riparian spider diets (Kolbenschlag et al., 2023b).

For Aeshnidae, we observed a trend of fewer proportions of Chironomid/mayfly in Bti-treated FPMs suggesting a shift towards large prey, i.e., Aeshnidae/newt/damselfly, likely due to a reduced chironomid density (**Appendix A-1**). Similarly, Allgeier et al. (2019b) recorded 27% lower newt survival in mesocosms exposed to Bti, suggesting intraguild predation by Aeshnidae feeding on newts. Odonata and newts are also known to be cannibalistic under limited prey availability (Vaissi & Sharifi, 2016; van Buskirk, 1989), which might have been the case for our experiment. As discussed above, in our analyses, there was no clear separation of resource clusters which made it difficult to differentiate prey taxa of interest. Still, there was a trend of Aeshnidae consuming larger prey, such as Aeshnidae and newts, indicating cannibalism and intraguild predation. However, in **Appendix A-1**, we investigated Odonata emergence (one year after the sampling for this study) and observed a non-significantly increased emergence of Aeshnidae from Bti-FPMs while emergence of Libellulidae and damselflies were reduced. We concluded that Aeshnidae in Bti-FPMs may have fed to a larger extent on Libellulidae and damselflies larvae, reducing their emergence. Although this was partly confirmed for Aeshnidae, we could not confirm this hypothesis for newt larvae, likely due to the position of newts in the resource polygon and the overlaps of resource clusters. These shortcomings can affect the precision of dietary estimates, but relative differences between control and Bti-FPMs could theoretically still be detected. Since no differences were detected and both the dietary niche sizes calculated by the SEA (consumer SEA relative to source SEA; Figure 1e) as well as the trophic level (Figure 1f) did not differ decisively, our results suggest no considerable differences in the diet of newts from control and Bti-FPMs.

We included analyses of polyunsaturated fatty acids (PUFAs) to obtain another point of view on lipid resource use in newt larvae, which also reveals information on nutritional quality of the ingested prey. NLFAs analyses revealed lower content of total NLFAs (~10%) in newts from Bti-treated FPMs compared to controls. Although not significant, this trend was mainly driven by omega-6 PUFAs (approximately 30% lower content in larvae from Bti-FPMs). This finding suggests that there may be slight differences in newts' diets that have not been detected by stable isotope analyses. While consumers generally have some capacity to modify dietary fatty acids (FAs), the FA composition still strongly depends on the nutritional quality of the consumers' diet (Pietz et al., 2023; Twining et al., 2021). Therefore, the overall lower content of total NLFAs and PUFAs in Bti-treated newts indicates that they likely consumed different food sources with less nutritional quality. At the same time, newts in Bti-FPMs may have experienced a higher energy cost for foraging or escape activities (Pérez-Tris et al., 2004), e.g. as a result of reduced prey availability and higher risk of predation, for instance by Aeshnidae, which may have resulted in increased use of storage lipids. These observations can potentially be relevant for energy and nutrient transfer to terrestrial systems upon metamorphosis.

4.5 RQ-3: Direct sublethal effects on tadpoles

This chapter is based on a peer-reviewed scientific publication in *Bulletin of Environmental Contamination and Toxicology* (**Appendix A-3**).

Due to contrasting results of studies investigating sublethal effects on common frog (*R. temporaria*) tadpoles (Allgeier et al., 2018; Schweizer et al., 2019) under laboratory conditions, we assessed the biochemical stress response (i.e., GST activity and protein carbonyl content) of tadpoles under natural climatic conditions. We measured lower (52%) mean GST activity levels in Bti-treated tadpoles after the first Bti application compared to the control, while results (although not significant) suggest a pattern of higher levels in Bti-treated tadpoles after applications 2 and 3, 17% and 38%, respectively (Figure 11). In this study, the factor application was mainly representative for the temporal aspect, i.e. tadpole development (Gosner, 1960) and water temperature. We recorded an increase of mean GST activity levels in tadpoles over the course of the experiment after each with increasing water temperatures, which were on average 7, 13.5 and 20 °C for application 1, 2 and 3, respectively (Figure 11; calculated from the 48-h time period between application and sampling). For protein carbonyl content, there was no significant Bti-induced effect on protein carbonyl contents due to oxidative stress, and no influence of water temperature or Gosner stage (Figure 11).

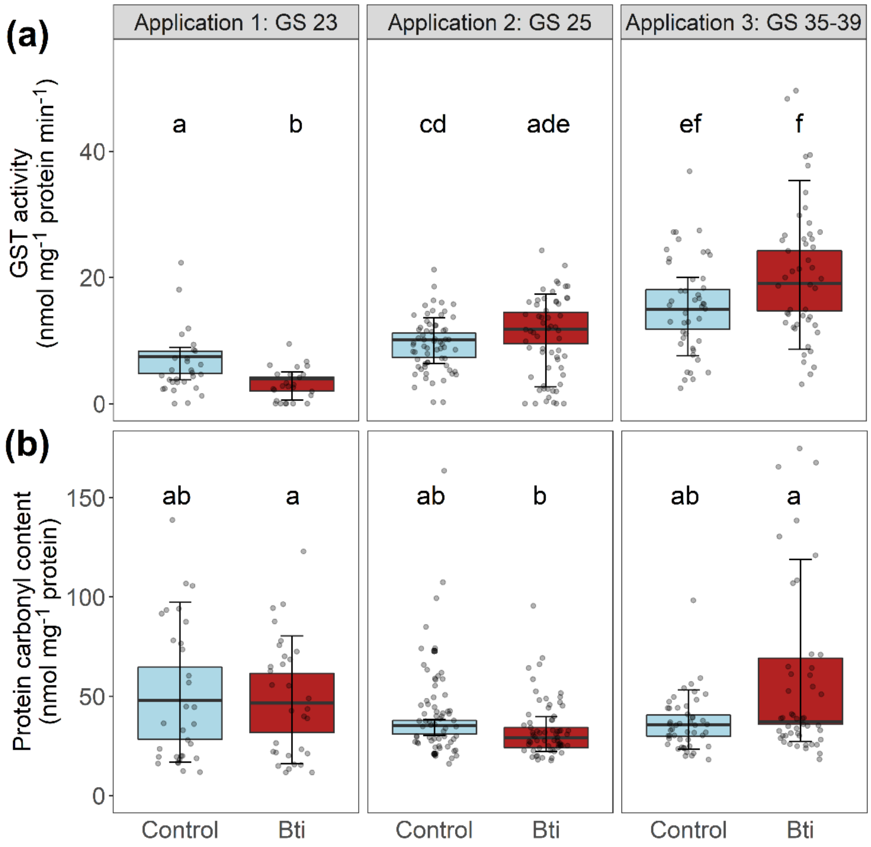


Figure 11: GST activity (a) and protein carbonyl content (b) in *R. temporaria* tadpoles from control and Bti-treated FPMs at Gosner stages (GS) 23, 25 and 35-39. Boxplots are based on mean values within the FPMs (n=6), grey dots represent individual samples (N=288 for each biomarker). Lower and upper box boundaries show 25th and 75th percentiles, respectively, line inside the box show the median. Whiskers and black dots show the variability outside the lower and upper quartiles. Groups that do not share a common letter differ statistically significant (based on least-squares means). (Figure taken from **Appendix A-3**)

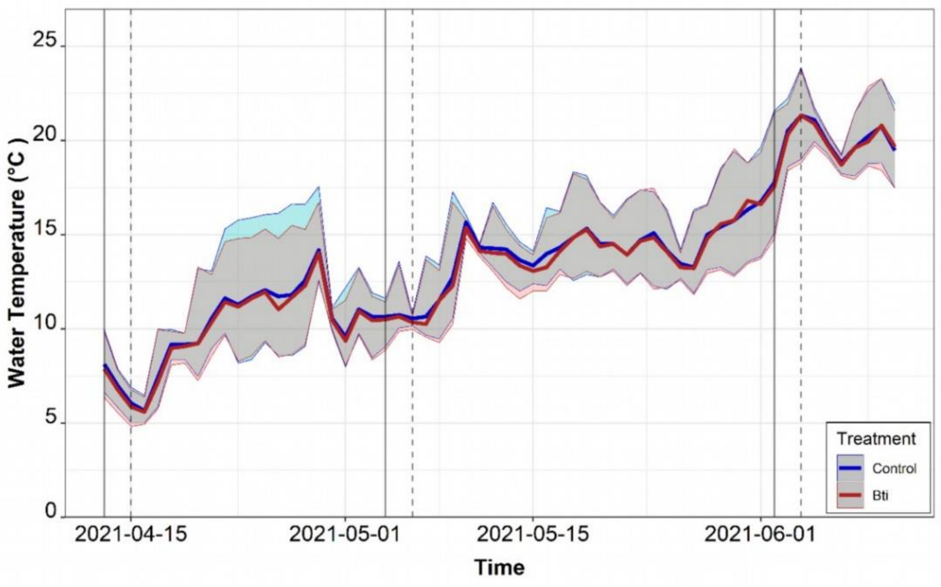


Figure 11: Water temperature in control (blue; n=6) and Bti-treated FPMs (red; n=6) over the course of the experiment. Shown are the median temperatures with lower and upper quartiles and the day of the Bti applications (solid line) and tadpole samplings (dashed line). (Figure taken from **Appendix A-3**)

4.6 Discussion of RQ-3

In aquatic organisms, GST activity levels have been reported to react sensitively to thermal stress as shown in saltwater fish (Madeira et al., 2013) and neotropical tadpoles (Freitas et al., 2017; Freitas & Almeida, 2016). It has been reported that abiotic environmental variables like high water temperature can amplify toxic effects of Bti in chironomid larvae (Charbonneau et al., 1994), therefore we also expected an increase in GST activity in Bti-exposed tadpoles, especially with increasing temperature. Although we observed an effect of application (i.e, temperature), an increased effect of Bti treatment was not confirmed in our experiment. We only observed a slightly increased GST activity in Bti-treated late stage tadpoles. However, a possible effect of warmer exposure conditions could be reduced due to higher developmental stages of tadpoles which are expected to be less sensitive. Amphibians are poikilothermic organisms, therefore the ambient temperature is the most important factor influencing their metabolic activity. Studies investigating mortality and biochemical stress responses of tadpoles exposed to pesticides reported contradictory effects of temperature-dependent toxicity, most likely also related to the specific properties of the pesticide studied (Baier et al., 2016; Freitas et al., 2017; Freitas & Almeida, 2016; Leeb et al., 2022; Madeira et al., 2013). Our results from the field are comparable to the findings of Schweizer et al. (2019), who did not record any Bti-induced difference in stress-related biomarker levels in *R. temporaria* tadpoles at 15 °C. In contrast, Gutierrez-Villagomez et al. (2021) exposed tadpoles of two North American frog species (*Lithobates sylvaticus* and *Anaxyrus americanus*) to two different Bti formulations in a chronic exposure laboratory experiment. Authors did not record a significant change in stress response in a dose-dependent pattern, but they observed modifications in the intestine microbiota as well as an overexpression of the genes *cyp1a* and *sod* in tails of *L. sylvaticus*, indicating detoxification processes and oxidative stress. The *sod* gene expresses an enzyme which is responsible for destroying radicals, such as ROS. In our experiment, we assumed ROS formation in Bti-exposed tadpoles resulting in protein damage, i.e., increased protein carbonylation. However, in our scenario, Bti did not have an effect on protein carbonyl content. Our findings suggest insignificant levels of ROS formation; thus, we assume that Bti does not affect proteins in *R. temporaria* tadpoles under the tested conditions. However, if applications of Bti in early spring coincide with peak temperatures of 20 °C, oxidative stress in early stage *R. temporaria* tadpoles is more likely to happen, as reported in Allgeier et al. (2018). Consequently, an increase of cellular responses to xenobiotics is linked to a higher cost of energy (Steinberg, 2012). This may impair tadpoles' behavior and development, possibly affecting amphibian populations at a later stage (Monaghan et al., 2009). However, this was not observed under the comparably cold temperatures experienced during our experiment.

5 CONCLUSION AND OUTLOOK

This thesis provides evidence that Bti levels, as commonly used in the Upper Rhine Valley, have a negative ecological impact on natural macroinvertebrate communities; not only on limited to the aquatic habitat but also influencing terrestrial ecosystems. Reductions of aquatic chironomid populations, in our study caused by Bti applications, resulted in indirect effects on Odonata by affecting their dietary composition, shifting towards larger prey (**Appendix A-2**), and quantitively altering their emergence (**Appendix A-1**). Although no diet shift was observed in other predators, such as newt larvae, based on stable isotope analyses, quantification of NLFAs provided insights in lipid resource use of newts, in which we observed a slightly reduced content of omega-3 fatty acids in Bti-treated newts. In fact, many aquatic organisms contain physiologically important fatty acids, which are typically not synthesized in terrestrial vertebrates (Kainz et al., 2004; Twining et al., 2016), emphasizing the importance of aquatic subsidy for terrestrial predators. The question of to which extent our observations affect terrestrial predators still remains open for future research.

In our cold-water scenario in the FPM system, adverse subcellular responses of common frog tadpoles to Bti as observed by Allgeier et al. (2018) were not confirmed (**Appendix A-3**). However, we are careful to not generalize our findings to other amphibian species, stages or environmental conditions. As amphibians are the worldwide most threatened vertebrate group, and contribute to temporarily high biomass fluxes from aquatic to terrestrial habitats, long-term research of Bti effects on amphibians is necessary. Also, changes of the intestine microbiota in tadpoles after Bti exposure (Gutierrez-Villagomez et al., 2021) may influence the susceptibility of individuals towards Bti or other stressors (Broderick et al., 2009) and the fitness of individuals, e.g., by facing a higher susceptibility to parasitic infections. Furthermore, some Bt toxins can modify soil microbial communities due to their antimicrobial effects (as reviewed in Belousova et al., 2021). In turn, alterations in microbial communities, e.g., detritus-processing organisms, such as protozoans and nematodes, can have further effects on the food web. For instance, by including of an extra trophic step at the base of the food web due to higher decomposition of dead mosquito larvae as observed by McKie et al. (2023), which possibly also affects detritus-feeding tadpoles.

In view of the global climate change suggesting increased water temperatures, it is likely that direct and indirect effects of Bti on non-target organisms will increase in the future, as higher temperatures have been found to amplify toxic effects of Bti (Charbonneau et al., 1994). Simultaneously, warmer climatic conditions will also promote invasion and breeding of mosquito species with vectorial capacity, such as the Asian tiger mosquito (*Aedes albopictus*) (Bellini et al., 2020), in temperate regions. This will presumably increase the demand of mosquito control programs by the human population. However, disease-transmitting mosquito species usually utilize catch basins and other water-filled containers as breeding sites which can be treated locally. Small-scale application of Bti is one of the most effective and environmentally friendly methods to control urban mosquito populations. In fact, public surveys in the Upper Rhine Valley showed that reduced Bti spraying would be accepted by the majority of the population as long as effective mosquito control is ensured in domestic and urban areas (Allgeier, 2019). Therefore, mosquito control in the Upper Rhine Valley could be implemented more sustainably, for instance, by increasingly using alternative methods in domestic areas where most nuisance arises. This includes raising awareness in the local community on how to prevent and control mosquito populations in urban areas, e.g., by reducing breeding sites (removing/closing containers that hold rainwater) or installing mosquito traps. By reducing nuisance in residential areas, large-scale treatment of wetlands can possibly be limited to smaller areas.

In fact, to preserve biodiversity, large areas of wetlands in the Upper Rhine Valley have been registered as protected nature reserves and Natura 2000 sites, and yet, regularly treated with Bti. These sites constitute important habitats for many protected amphibian and Odonata species. While in France, Austria and Sweden (Lagadic et al., 2016; Lundström et al., 2010; Poulin, 2012; Wolfram et al., 2018), long-term field monitoring of Bti-treated wetlands was implemented, no comparable data is available for wetlands in the Upper Rhine Valley. In view of the presented results and the recognized wide-ranging insect and amphibian decline (Stuart et al., 2004; Hallmann et al., 2017), adverse ecological effects of large-scale Bti applications to (partially protected) wetlands should be minimized to conserve natural ecosystems in the future.

6 REFERENCES

Adams, E., Gerstle, V., & Brühl, C. A. (2021). Dermal Fungicide Exposure at Realistic Field Rates Induces Lethal and Sublethal Effects on Juvenile European Common Frogs (*Rana temporaria*). *Environmental Toxicology and Chemistry*, *40*(5), 1289–1297. https://doi.org/10.1002/etc.4972

Ali, A. (1981). *Bacillus thuringiensis* serovar. *israelensis* (ABG-6108) against chironomids and some nontarget aquatic invertebrates. *Journal of Invertebrate Pathology*, *38*(2), 264–272.

Allgeier, S., Frombold, B., Mingo, V., & Brühl, C. A. (2018). European common frog *Rana temporaria* (Anura: Ranidae) larvae show subcellular responses under field-relevant *Bacillus thuringiensis* var. *israelensis* (Bti) exposure levels. *Environmental Research*, *162*, 271–279. https://doi.org/10.1016/j.envres.2018.01.010

Allgeier, S. (2019). *Mosquito control based on* Bacillus thuringiensis israelensis *(Bti) - Ecological effects on wetland food chains and public acceptance of control alternatives*. University of Koblenz-Landau.

Allgeier, S., Kästel, A., & Brühl, C. A. (2019a). Adverse effects of mosquito control using *Bacillus thuringiensis* var. *israelensis*: Reduced chironomid abundances in mesocosm, semi-field and field studies. *Ecotoxicology and Environmental Safety*, *169*, 786–796. https://doi.org/10.1016/j.ecoenv.2018.11.050

Allgeier, S., Friedrich, A., & Brühl, C. A. (2019b). Mosquito control based on *Bacillus thuringiensis israelensis* (Bti) interrupts artificial wetland food chains. *Science of the Total Environment*, *686*, 1173–1184. https://doi.org/10.1016/j.scitotenv.2019.05.358

Anderson, M. J. (2006). Distance-based tests for homogeneity of multivariate dispersions. *Biometrics*, *62*(1), 245–253.

Anderson, M. J. (2014). Permutational multivariate analysis of variance (PERMANOVA). *Wiley Statsref: Statistics Reference Online*, 1–15.

Arbeiter, S., Schnepel, H., Uhlenhaut, K., Bloege, Y., Schijlze, M., & Hahn, S. (2014). Seasonal shift in the diet composition of european bee-eaters merops apiaster at the northern edge of distribution. *Ardeola*, *61*(1), 161–170. https://doi.org/10.13157/arla.61.1.2014.161

Armitage, P. D., Pinder, L. C., & Cranston, P. S. (1995). The Chironomidae: biology and ecology of non-biting midges. In *The Chironomidae - The biology and ecology of non-biting midges*. Springer Science & Business Media.

Baier, F., Gruber, E., Hein, T., Bondar-Kunze, E., Ivanković, M., Mentler, A., Brühl, C. A., Spangl, B., & Zaller, J. G. (2016). Non-target effects of a glyphosate-based herbicide on Common toad larvae (*Bufo bufo*, Amphibia) and associated algae are altered by temperature. *PeerJ*, *2016*(11). https://doi.org/10.7717/peerj.2641

Barnosky, A. D., Matzke, N., Tomiya, S., Wogan, G. O. U., Swartz, B., Quental, T. B., Marshall, C., McGuire, J. L., Lindsey, E. L., & Maguire, K. C. (2011). Has the Earth's sixth mass extinction already arrived? *Nature*, *471*(7336), 51–57.

Barton, K., & Barton, M. K. (2015). Package 'mumin.' *Version*, *1*(18), 439.

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2014). Fitting linear mixed-effects models using lme4. *ArXiv Preprint ArXiv:1406.5823*.

Becker, N. (1997). Microbial Control of Mosquitoes: Management of the Upper Rhine Mosquito Population as a Model Programme. *Parasitology Today*, *13*(12), 485–487. https://doi.org/https://doi.org/10.1016/S0169-4758(97)01154-X

Becker, N. (2002). Sterilization of *Bacillus thuringiensis israelensis* products by gamma radiation. *Journal of the American Mosquito Control Association*, *18*(1), 57–62.

Becker, N. (2003). Ice granules containing endotoxins of microbial agents for the control of mosquito larvae - a new application technique. *Journal of the American Mosquito Control Association*, *19*(l), 63–66.

Becker, N. (2006). Biological control of mosquitoes: management of the upper Rhine mosquito population as a model programme. In *An ecological and societal approach to biological control* (pp. 227–245). Springer.

Becker, N., Ludwig, M., & Su, T. (2018). Lack of resistance in *Aedes vexans* field populations after 36 years of *Bacillus thuringiensis* subsp. *israelensis* applications in the Upper Rhine valley, Germany. *Journal of the American Mosquito Control Association*, *34*(2), 154–157.

Becker, N., & Lüthy, P. (2017). Mosquito control with entomopathogenic bacteria in Europe. In *Microbial control of insect and mite pests* (pp. 379–392). Elsevier.

Becker, N., Petric, D., Zgomba, M., Boase, C., Madon, M., Dahl, C., & Kaiser, A. (2010). *Mosquitoes and their control*. Springer Science & Business Media.

Bell, A. M. (1975). The diet and dentition of Smooth newt larvae (*Triturus vulguris*). In *J. Zool* (Vol. 176).

Bellini, R., Michaelakis, A., Petrić, D., Schaffner, F., Alten, B., Angelini, P., Aranda, C., Becker, N., Carrieri, M., di Luca, M., Fălcuţă, E., Flacio, E., Klobučar, A., Lagneau, C., Merdić, E., Mikov, O., Pajovic, I., Papachristos, D., Sousa, C. A., … Zgomba, M. (2020). Practical management plan for invasive mosquito species in Europe: I. Asian tiger mosquito (Aedes albopictus). In *Travel Medicine and Infectious Disease* (Vol. 35). Elsevier USA. https://doi.org/10.1016/j.tmaid.2020.101691

Bellmann, H. (1993). *Libellen beobachten - bestimmen*. Naturbuch Verlag.

Belousova, M. E., Malovichko, Y. v., Shikov, A. E., Nizhnikov, A. A., & Antonets, K. S. (2021). Dissecting the environmental consequences of *Bacillus thuringiensis* application for natural ecosystems. In *Toxins* (Vol. 13, Issue 5). MDPI AG. https://doi.org/10.3390/toxins13050355

Ben-Dov, E. (2014). *Bacillus thuringiensis* subsp. *israelensis* and its dipteran-specific toxins. *Toxins*, *6*(4), 1222–1243. https://doi.org/10.3390/toxins6041222

Bereswill, R., Golla, B., Streloke, M., & Schulz, R. (2012). Entry and toxicity of organic pesticides and copper in vineyard streams: Erosion rills jeopardise the efficiency of riparian buffer strips. *Agriculture, Ecosystems & Environment*, *146*(1), 81–92. https://doi.org/https://doi.org/10.1016/j.agee.2011.10.010

Boisvert, M., & Boisvert, J. (2000). Effects of *Bacillus thuringiensis* var. *israelensis* on target and nontarget organisms: A review of laboratory and field experiments. In *Biocontrol Science and Technology* (Vol. 10, Issue 5, pp. 517–561). Carfax Publishing Company. https://doi.org/10.1080/095831500750016361

Bordalo, M. D., Gravato, C., Beleza, S., Campos, D., Lopes, I., & Pestana, J. L. T. (2020). Lethal and sublethal toxicity assessment of *Bacillus thuringiensis* var. *israelensis* and *Beauveria bassiana* based bioinsecticides to the aquatic insect *Chironomus riparius*. *Science of the Total Environment*, *698*. https://doi.org/10.1016/j.scitotenv.2019.134155

Bordalo, M. D., Machado, A. L., Campos, D., Coelho, S. D., Rodrigues, A. C. M., Lopes, I., & Pestana, J. L. T. (2021). Responses of benthic macroinvertebrate communities to a Bti-based insecticide in artificial microcosm streams. *Environmental Pollution*, *282*. https://doi.org/10.1016/j.envpol.2021.117030

Bravo, A., Gill, S. S., & Soberón, M. (2007). Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon*, *49*(4), 423–435. https://doi.org/https://doi.org/10.1016/j.toxicon.2006.11.022

Brochard, C., Croenendijk, D., van der Ploeg, E., & Termaat, T. (2012). *Fotogids larvenhuidjes van libellen*. KNNV Uitgeverij.

Broderick, N. A., Robinson, C. J., McMahon, M. D., Holt, J., Handelsman, J., & Raffa, K. F. (2009). Contributions of gut bacteria to Bacillus thuringiensis-induced mortality vary across a range of Lepidoptera. *BMC Biology*, *7*. https://doi.org/10.1186/1741-7007-7-11

Brühl, C. A., Després, L., Frör, O., Patil, C. D., Poulin, B., Tetreau, G., & Allgeier, S. (2020). Environmental and socioeconomic effects of mosquito control in Europe using the biocide *Bacillus thuringiensis* subsp. *israelensis* (Bti). In *Science of the Total Environment* (Vol. 724). Elsevier B.V. https://doi.org/10.1016/j.scitotenv.2020.137800

Calba, C., Guerbois-Galla, M., Franke, F., Jeannin, C., Auzet-Caillaud, M., Grard, G., Pigaglio, L., Decoppet, A., Weicherding, J., & Savaill, M.-C. (2017). Preliminary report of an autochthonous chikungunya outbreak in France, July to September 2017. *Eurosurveillance*, *22*(39), 17–647.

Cardinale, B. J., Duffy, J. E., Gonzalez, A., Hooper, D. U., Perrings, C., Venail, P., Narwani, A., Mace, G. M., Tilman, D., & Wardle, D. A. (2012). Biodiversity loss and its impact on humanity. *Nature*, *486*(7401), 59–67.

Carter, A. (2000). How pesticides get into water-and proposed reduction measures. *Pesticide Outlook*, *11*(4), 149–156.

Ceballos, G., Ehrlich, P. R., Barnosky, A. D., García, A., Pringle, R. M., & Palmer, T. M. (2015). Accelerated modern human–induced species losses: Entering the sixth mass extinction. *Science Advances*, *1*(5), e1400253.

Chapin III, F. S., Zavaleta, E. S., Eviner, V. T., Naylor, R. L., Vitousek, P. M., Reynolds, H. L., Hooper, D. U., Lavorel, S., Sala, O. E., & Hobbie, S. E. (2000). Consequences of changing biodiversity. *Nature*, *405*(6783), 234–242.

Charbonneau, C. S., Drobney, R. D., & Rabeni, C. F. (1994). Effects of *Bacillus thuringiensis* var. *israelensis* on nontarget benthic organisms in a lentic habitat and factors affecting the efficiacy of the larvicide. In *Environmental Toxicology and Chemistry* (Vol. 13, Issue 2).

Corbet, P. S. (1980). Biology of Odonata. *Annual Review of Entomology*, *25*(1), 189–217. www.annualreviews.org

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., & Colombo, R. (2003). Protein carbonyl groups as biomarkers of oxidative stress. *Clinica Chimica Acta*, *329*(1–2), 23–38. https://doi.org/10.1016/S0009-8981(03)00003-2

de Melo-Santos, M. A. V., de Araújo, A. P., Rios, E. M. M., & Regis, L. (2009). Long lasting persistence of Bacillus thuringiensis serovar. israelensis larvicidal activity in Aedes aegypti (Diptera: Culicidae) breeding places is associated to bacteria recycling. *Biological Control*, *49*(2), 186–191.

Deutscher Wetterdienst. (2023, January 8). *Klimaüberwachung Deutschland, Monats- und Jahreszeitenbericht*. https://www.dwd.de/DE/Home/home_node.html

Duchet, C., Franquet, E., Lagadic, L., & Lagneau, C. (2015). Effects of *Bacillus thuringiensis israelensis* and spinosad on adult emergence of the non-biting midges *Polypedilum nubifer* (Skuse) and *Tanytarsus curticornis* Kieffer (Diptera: Chironomidae) in coastal wetlands. *Ecotoxicology and Environmental Safety*, *115*, 272–278. https://doi.org/10.1016/j.ecoenv.2015.02.029

Duchet, C., Tetreau, G., Marie, A., Rey, D., Besnard, G., Perrin, Y., Paris, M., David, J. P., Lagneau, C., & Després, L. (2014). Persistence and recycling of bioinsecticidal *Bacillus thuringiensis* subsp. *israelensis* spores in contrasting environments: evidence from field monitoring and laboratory experiments. *Microbial Ecology*, *67*(3), 576–586. https://doi.org/10.1007/s00248-013-0360-7

Dupont, C., & Boisvert, J. (1986). Persistence of *Bacillus thuringiensis* serovar. *israelensis* toxic activity in the environment and interaction with natural substrates. *Water, Air and Soil Pollution*, *29*, 425–438.

Empey, M. A., Lefebvre-Raine, M., Gutierrez-Villagomez, J. M., Langlois, V. S., & Trudeau, V. L. (2021). A Review of the Effects of the Biopesticides *Bacillus thuringiensis* Serotypes *israelensis* (Bti) and *kurstaki* (Btk) in Amphibians. In *Archives of Environmental Contamination and Toxicology* (Vol. 80, Issue 4, pp. 789–800). Springer. https://doi.org/10.1007/s00244-021-00842-2

Freitas, J. S., & Almeida, E. A. (2016). Antioxidant defense system of tadpoles (*Eupemphix nattereri*) exposed to changes in temperature and pH. *Zoological Science*, *33*(2), 186–194. https://doi.org/10.2108/zs150075

Freitas, J. S., Felício, A. A., Teresa, F. B., & Alves de Almeida, E. (2017). Combined effects of temperature and clomazone (Gamit®) on oxidative stress responses and B-esterase activity of *Physalaemus nattereri* (Leiuperidae) and *Rhinella schneideri* (Bufonidae) tadpoles. *Chemosphere*, *185*, 548–562. https://doi.org/10.1016/j.chemosphere.2017.07.061

Gergs, R., Koester, M., Schulz, R. S., & Schulz, R. (2014). Potential alteration of cross-ecosystem resource subsidies by an invasive aquatic macroinvertebrate: implications for the terrestrial food web. *Freshwater Biology*, *59*(12), 2645–2655.

Gerstle, V., Manfrin, A., Kolbenschlag, S., Gerken, M., Islam, A. S. M. M. U., Entling, M. H., Bundschuh, M., & Brühl, C. A. (2023a). Benthic macroinvertebrate community shifts based on Bti-induced chironomid reduction also decrease Odonata emergence. *Environmental Pollution*, *316*. https://doi.org/10.1016/j.envpol.2022.120488

Gerstle, V., Solanki, P., Manfrin, A., Kolbenschlag, S., & Brühl, C. A. (2023b). Stress response of common frog (*Rana temporaria*) tadpoles to Bti exposure in an outdoor pond mesocosm. *Bulletin of Environmental Contamination and Toxicology.* (in press)

Goldberg, L. J., & Margalit, J. (1977). A bacterial spore demonstrating rapid larvicidal activity against Anopheles sergentii, Uranotaenia unguiculata, Culex univitattus, Aedes aegypti and Culex pipiens. *Mosquito News*, *37*(3), 355–358.

Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*, *16*(3), 183–190.

Goulson, D., Nicholls, E., Botías, C., & Rotheray, E. L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, *347*(6229), 1255957.

Griffiths, R. A. (1985). A simple funnel trap for studying newt populations and an evaluation of trap behaviour in smooth and palmate newts, *Triturus vulgaris* and *T. helveticus*. *Herpetological Journal*, *1*, 5–10.

Günther, R. (1996). *Die Amphibien und Reptilien Deutschlands*. Springer.

Gutierrez-Villagomez, J. M., Patey, G., To, T. A., Lefebvre-Raine, M., Lara-Jacobo, L. R., Comte, J., Klein, B., & Langlois, V. S. (2021). Frogs Respond to Commercial Formulations of the Biopesticide *Bacillus thuringiensis* var. *israelensis*, Especially Their Intestine Microbiota. *Environmental Science and Technology*, *55*(18), 12504–12516. https://doi.org/10.1021/acs.est.1c02322

Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974). Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry*, *249*(22), 7130–7139. https://doi.org/https://doi.org/10.1016/S0021-9258(19)42083-8

Halasa, Y. A., Shepard, D. S., Fonseca, D. M., Farajollahi, A., Healy, S., Gaugler, R., Bartlett-Healy, K., Strickman, D. A., & Clark, G. G. (2014). Quantifying the impact of mosquitoes on quality of life and enjoyment of yard and porch activities in New Jersey. *PloS One*, *9*(3), e89221.

Hallmann, C. A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., Stenmans, W., Müller, A., Sumser, H., Hörren, T., Goulson, D., & de Kroon, H. (2017). More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLoS ONE*, *12*(10). https://doi.org/10.1371/journal.pone.0185809

Hayes, T. B., Case, P., Chui, S., Chung, D., Haeffele, C., Haston, K., Lee, M., Mai, V. P., Marjuoa, Y., & Parker, J. (2006). Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact? *Environmental Health Perspectives*, *114*(Suppl 1), 40–50.

Heidemann, H., & Seidenbusch, R. (2002). *Libellenlarven Deutschlands*. Goecke & Evers.

Hershey, A. E., Lima, A. R., Niemi, G. J., & Regal, R. R. (1998). Effects of *Bacillus thuringiensis israelensis* (Bti) and methoprene on nontarget macroinvertebrates in Minnesota wetlands. *Ecological Applications*, *8*(1), 41–60.

Holt, R. D., & Polis, G. A. (1997). A theoretical framework for intraguild predation. *The American Naturalist*, *149*(4), 745–764.

IUCN. (2022). *The IUCN Red List of Threatened Species. Version 2022-2.* https://www.iucnredlist.org

Jakob, C., & Poulin, B. (2016). Indirect effects of mosquito control using Bti on dragonflies and damselflies (Odonata) in the Camargue. *Insect Conservation and Diversity*, *9*(2), 161–169. https://doi.org/10.1111/icad.12155

Johnson, D. M. (1991). Behavioral ecology of larval dragonflies and damselflies. *Trends in Ecology & Evolution*, *6*(1), 8–13. https://doi.org/10.1016/0169-5347(91)90140-S

Kainz, M., Arts, M. T., & Mazumder, A. (2004). Essential fatty acids in the planktonic food web and their ecological role for higher trophic levels. In *Limnol. Oceanogr* (Vol. 49, Issue 5).

Kästel, A., Allgeier, S., & Brühl, C. A. (2017). Decreasing *Bacillus thuringiensis israelensis* sensitivity of Chironomus riparius larvae with age indicates potential environmental risk for mosquito control. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-14019-2

Kolbenschlag, S., Gerstle, V., Eberhardt, J., Bollinger, E., Schulz, R., Brühl, C. A., & Bundschuh, M. (2023a). A temporal perspective on aquatic subsidy: Bti affects emergence of Chironomidae. *Ecotoxicology and Environmental Safety*, *250*, 114503. https://doi.org/10.1016/j.ecoenv.2023.114503

Kolbenschlag, S., Bollinger, E., Gerstle, V., Brühl, C. A., Entling, M. H., Schulz, R., & Bundschuh, M. (2023b). *Impact across ecosystem boundaries – does Bti application change quality and composition of the diet of riparian spiders?* https://doi.org/https://doi.org/10.1016/j.scitotenv.2023.162351

Kriska, G. (2013). *Freshwater invertebrates in Central Europe*. Springer Science & Business Media.

Lacey, L. A., & Merritt, R. W. (2003). The safety of bacterial microbial agents used for black fly and mosquito control in aquatic environments. In *Environmental impacts of microbial insecticides* (pp. 151–168). Springer.

Lagadic, L., Roucaute, M., & Caquet, T. (2014). Bti sprays do not adversely affect non-target aquatic invertebrates in French Atlantic coastal wetlands. *Journal of Applied Ecology*, *51*(1), 102–113. https://doi.org/10.1111/1365-2664.12165

Lagadic, L., Schäfer, R. B., Roucaute, M., Szöcs, E., Chouin, S., de Maupeou, J., Duchet, C., Franquet, E., le Hunsec, B., Bertrand, C., Fayolle, S., Francés, B., Rozier, Y., Foussadier, R., Santoni, J. B., & Lagneau, C. (2016). No association between the use of Bti for mosquito control and the dynamics of non-target aquatic invertebrates in French coastal and continental wetlands. *Science of the Total Environment*, *553*, 486–494. https://doi.org/10.1016/j.scitotenv.2016.02.096

Lajmanovich, R. C., Junges, C. M., Cabagna-Zenklusen, M. C., Attademo, A. M., Peltzer, P. M., Maglianese, M., Márquez, V. E., & Beccaria, A. J. (2015). Toxicity of *Bacillus thuringiensis* var. *israelensis* in aqueous suspension on the South American common frog *Leptodactylus latrans* (Anura: Leptodactylidae) tadpoles. *Environmental Research*, *136*, 205–212. https://doi.org/10.1016/j.envres.2014.10.022

Lamborn, R. H. (1890). *Dragon Flies Vs. Mosquitoes: Can the Mosquito Pest be Mitigated? Studies in the Life History of Irritating Insects, Their Natural Enemies, and Artificial Checks*. D. Appleton.

Larson, D., & House, N. (1990). Insect communities of Newfoundland bog pools with emphasis on the Odonata. *The Canadian Entomologist*, *122*, 469–501.

Leeb, C., Schuler, L., Brühl, C. A., & Theissinger, K. (2022). Low temperatures lead to higher toxicity of the fungicide folpet to larval stages of *Rana temporaria* and *Bufotes viridis*. *PLoS ONE*, *17*(8 August). https://doi.org/10.1371/journal.pone.0258631

Leeper, D. A., & Taylor, B. E. (1998). Insect Emergence from a South Carolina (USA) Temporary Wetland Pond, with Emphasis on the Chironomidae (Diptera). *Source: Journal of the North American Benthological Society*, *17*(1), 54–72. https://www.jstor.org/stable/1468051

Lenth, R. v. (2016). Least-squares means: the R package lsmeans. *Journal of Statistical Software*, *69*, 1–33.

Liber, K., Schmude, K. L., & Rau, D. M. (1998). Toxicity of *Bacillus thuringiensis* var. *israelensis* to chironomids in pond mesocosms. *Ecotoxicology*, *7*, 343–354.

Lundström, J. O., Schäfer, M. L., Petersson, E., Persson Vinnersten, T. Z., Landin, J., & Brodin, Y. (2010). Production of wetland Chironomidae (Diptera) and the effects of using *Bacillus thuringiensis israelensis* for mosquito control. *Bulletin of Entomological Research*, *100*(1), 117–125. https://doi.org/10.1017/S0007485309990137

Madeira, D., Narciso, L., Cabral, H. N., Vinagre, C., & Diniz, M. S. (2013). Influence of temperature in thermal and oxidative stress responses in estuarine fish. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, *166*(2), 237–243. https://doi.org/10.1016/j.cbpa.2013.06.008

Margalith, Y., & Ben-Dov, E. (2000). Biological Control by *Bacillus thuringiensis* subsp. *israelensis*. In J. E. Rechcigl & N. A. Rechcigl (Eds.), *Insect pest management: techniques for environmental protection* (pp. 243–281). CRC Press.

McKie, B. G., Taylor, A., Nilsson, T., Frainer, A., & Goedkoop, W. (2023). Ecological effects of mosquito control with Bti: evidence for shifts in the trophic structure of soil- and ground-based food webs. *Aquatic Sciences*, *85*(2). https://doi.org/10.1007/s00027-023-00944-0

Mingo, V., Lötters, S., & Wagner, N. (2017). The use of buccal swabs as a minimal-invasive method for detecting effects of pesticide exposure on enzymatic activity in common wall lizards. *Environmental Pollution*, *220*, 53–62.

Monaghan, P., Metcalfe, N. B., & Torres, R. (2009). Oxidative stress as a mediator of life history trade-offs: Mechanisms, measurements and interpretation. In *Ecology Letters* (Vol. 12, Issue 1, pp. 75–92). https://doi.org/10.1111/j.1461-0248.2008.01258.x

Munstermann, M. J., Heim, N. A., McCauley, D. J., Payne, J. L., Upham, N. S., Wang, S. C., & Knope, M. L. (2022). A global ecological signal of extinction risk in terrestrial vertebrates. *Conservation Biology*, *36*(3). https://doi.org/10.1111/cobi.13852

Noyes, P. D., McElwee, M. K., Miller, H. D., Clark, B. W., van Tiem, L. A., Walcott, K. C., Erwin, K. N., & Levin, E. D. (2009). The toxicology of climate change: Environmental contaminants in a warming world. In *Environment International* (Vol. 35, Issue 6, pp. 971–986). Elsevier Ltd. https://doi.org/10.1016/j.envint.2009.02.006

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., & Wagner, H. (2013). Package 'vegan.' *Community Ecology Package, Version*, *2*(9), 1–295.

Ott, J. (2016). *Citizen Science in Naturschutz und Landesforschung - Informationen zur Gruppe der Libellen (Odoanta)*.

Painter, M. K., Tennessen, K. J., & Richardson, T. D. (1996). Effects of repeated application of *Bacillus thuringiensis israelensis* on the mosquito predator Erythemis simplicicollis (Odonata: Libellulidae) from hatching to final instar. *Environmental Entomology*, *25*(1), 184–191.

Pietz, S., Kainz, M. J., Schröder, H., Manfrin, A., Schäfer, R. B., Zubrod, J. P., & Bundschuh, M. (2023). Metal Exposure and Sex Shape the Fatty Acid Profile of Midges and Reduce the Aquatic Subsidy to Terrestrial Food Webs. *Environmental Science and Technology*, *57*(2), 951–962. https://doi.org/10.1021/acs.est.2c05495

Pérez-Tris, J., Díaz, J. A., & Tellería, J. L. (2004). Loss of body mass under predation risk: cost of antipredatory behaviour or adaptive fit-for-escape? *Animal Behaviour, 67*(3), 511–521.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Heisterkamp, S., van Willigen, B., & Maintainer, R. (2017). Package 'nlme.' *Linear and Nonlinear Mixed Effects Models, Version*, *3*(1).

Poulin, B. (2012). Indirect effects of bioinsecticides on the nontarget fauna: The Camargue experiment calls for future research. *Acta Oecologica*, *44*, 28–32.

Poulin, B., Lefebvre, G., Hilaire, S., & Després, L. (2022). Long-term persistence and recycling of Bacillus thuringiensis israelensis spores in wetlands sprayed for mosquito control. *Ecotoxicology and Environmental Safety*, *243*. https://doi.org/10.1016/j.ecoenv.2022.114004

Poulin, B., Lefebvre, G., & Paz, L. (2010). Red flag for green spray: Adverse trophic effects of Bti on breeding birds. *Journal of Applied Ecology*, *47*(4), 884–889. https://doi.org/10.1111/j.1365-2664.2010.01821.x

Raebel, E. M., Merckx, T., Riordan, P., Macdonald, D. W., & Thompson, D. J. (2010). The dragonfly delusion: Why it is essential to sample exuviae to avoid biased surveys. *Journal of Insect Conservation*, *14*(5), 523–533. https://doi.org/10.1007/s10841-010-9281-7

R Core Team. (2013). *R: A language and environment for statistical computing*.

Relyea, R. A., & Edwards, K. (2010). What doesn't kill you makes you sluggish: how sublethal pesticides alter predator–prey interactions. *Copeia*, *2010*(4), 558–567.

Rezza, G., Nicoletti, L., Angelini, R., Romi, R., Finarelli, A. C., Panning, M., Cordioli, P., Fortuna, C., Boros, S., & Magurano, F. (2007). Infection with chikungunya virus in Italy: an outbreak in a temperate region. *The Lancet*, *370*(9602), 1840–1846.

Sánchez-Hernández, J. (2020). Reciprocal role of salamanders in aquatic energy flow pathways. In *Diversity* (Vol. 12, Issue 1). MDPI AG. https://doi.org/10.3390/d12010032

Schulz, R., Bundschuh, M., Gergs, R., Brühl, C. A., Diehl, D., Entling, M. H., Fahse, L., Frör, O., Jungkunst, H. F., Lorke, A., Schäfer, R. B., Schaumann, G. E., & Schwenk, K. (2015). Review on environmental alterations propagating from aquatic to terrestrial ecosystems. In *Science of the Total Environment* (Vol. 538, pp. 246–261). Elsevier B.V. https://doi.org/10.1016/j.scitotenv.2015.08.038

Schweizer, M., Miksch, L., Köhler, H. R., & Triebskorn, R. (2019). Does Bti (*Bacillus thuringiensis* var. *israelensis*) affect *Rana temporaria* tadpoles? *Ecotoxicology and Environmental Safety*, *181*, 121–129. https://doi.org/10.1016/j.ecoenv.2019.05.080

Skovmand, O., & Becker, N. (2000). Bioassays of *Bacillus thuringiensis* subsp. *israelensis*. In *Bioassays of entomopathogenic microbes and nematodes* (pp. 41–47). CABI Pub.

Soberón, M., López-Díaz, J. A., & Bravo, A. (2013). Cyt toxins produced by Bacillus thuringiensis: a protein fold conserved in several pathogenic microorganisms. *Peptides*, *41*, 87–93.

Soininen, J., Bartels, P. I. A., Heino, J., Luoto, M., & Hillebrand, H. (2015). Toward more integrated ecosystem research in aquatic and terrestrial environments. *BioScience*, *65*(2), 174–182.

Sparling, D. W., Fellers, G. M., & McConnell, L. L. (2001). Pesticides and amphibian population declines in California, USA. *Environmental Toxicology and Chemistry: An International Journal*, *20*(7), 1591–1595.

Stehle, S., Manfrin, A., Feckler, A., Graf, T., Joschko, T. J., Jupke, J., Noss, C., Rösch, V., Schirmel, J., Schmidt, T., Zubrod, J. P., & Schulz, R. (2022). Structural and functional development of twelve newly established floodplain pond mesocosms. *Ecology and Evolution*, *12*, 8674. https://doi.org/10.1002/ece3.8674

Steinberg, C. E. W. (2012). Multiple stressors as environmental realism: synergism or antagonism. In *Stress Ecology* (pp. 295–309). Springer.

Stevens, M. M., Helliwell, S., & Hughes, P. A. (2005). Toxicity of Bacillus thuringiensis var. israelensis formulations, spinosad, and selected synthetic insecticides to Chironomus tepperi larvae. *Journal of the American Mosquito Control Association*, *21*(4), 446–450.

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S. L., Fischman, D. L., & Waller, R. W. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science*, *306*(5702), 1783–1786.

Succo, T., Leparc-Goffart, I., Ferré, J.-B., Roiz, D., Broche, B., Maquart, M., Noel, H., Catelinois, O., Entezam, F., & Caire, D. (2016). Autochthonous dengue outbreak in Nîmes, south of France, July to September 2015. *Eurosurveillance*, *21*(21), 30240.

Tachet, H., Richoux, P., Bournaud, M., & Usseglio-Polatera; Philippe. (2010). *Invertébrés d'eau douce: systématique, biologie, écologie* (Vol. 15). CNRS éditions.

Tetreau, G., Alessi, M., Veyrenc, S., Périgon, S., David, J. P., Reynaud, S., & Després, L. (2012). Fate of Bacillus thuringiensis subsp. Israelensis in the field: Evidence for spore recycling and differential persistence of toxins in leaf litter. *Applied and Environmental Microbiology*, *78*(23), 8362–8367. https://doi.org/10.1128/AEM.02088-12

Theissinger, K., Röder, N., Allgeier, S., Beermann, A. J., Brühl, C. A., Friedrich, A., Michiels, S., & Schwenk, K. (2019). Mosquito control actions affect chironomid diversity in temporary wetlands of the Upper Rhine Valley. *Molecular Ecology*, *28*(18), 4300–4316. https://doi.org/10.1111/mec.15214

Tudoran, A., Nordlander, G., Karlberg, A., & Puentes, A. (2021). A major forest insect pest, the pine weevil *Hylobius abietis*, is more susceptible to Diptera- than Coleoptera-targeted *Bacillus thuringiensis* strains. *Pest Management Science*, *77*(3), 1303–1315. https://doi.org/10.1002/ps.6144

Twining, C. W., Brenna, J. T., Hairston, N. G., & Flecker, A. S. (2016). Highly unsaturated fatty acids in nature: What we know and what we need to learn. *Oikos*, *125*(6), 749–760. https://doi.org/10.1111/oik.02910

Twining, C. W., Parmar, T. P., Mathieu-Resuge, M., Kainz, M. J., Shipley, J. R., & Martin-Creuzburg, D. (2021). Use of Fatty Acids From Aquatic Prey Varies With Foraging Strategy. *Frontiers in Ecology and Evolution, 9*. https://doi.org/10.3389/fevo.2021.735350

Vaissi, S., & Sharifi, M. (2016). Variation in food availability mediate the impact of density on cannibalism, growth, and survival in larval yellow spotted mountain newts (*Neurergus microspilotus*): Implications for captive breeding programs. *Zoo Biology*, *35*(6), 513–521. https://doi.org/10.1002/zoo.21327

Vallenduuk, H. J., & Moller Pillot, H. K. M. (2007). *Chironomidae Larvae: General Ecology and Tanypodinae* (Vol. 1). Brill.

van Buskirk, J. (1989). Density-dependent cannibalism in larval dragonflies. *Ecology*, *70*(5), 1442–1449.

van Buskirk, J. (1992). Competition, cannibalism, and size class dominance in a dragonfly. *Oikos*, 455–464.

Vaughan, I. P., Newberry, C., Hall, D. J., Liggett, J. S., & Ormerod, S. J. (2008). Evaluating large-scale effects of *Bacillus thuringiensis* var. *israelensis* on non-biting midges (Chironomidae) in a eutrophic urban lake. *Freshwater Biology*, *53*(10), 2117–2128. https://doi.org/10.1111/j.1365-2427.2008.02043.x

Venturino, A., & D'Angelo, A. M. P. de. (2005). Biochemical targets of xenobiotics: Biomarkers in amphibian ecotoxicology. *Applied Herpetology*, *2*(3), 335–353.

von Hirsch, H., & Becker, B. (2009). Cost-benefit analysis of mosquito control operations based on microbial control agents in the upper Rhine valley (Germany). *J Euro Mosq Control Assoc*, *27*, 47–55.

Vray, S., Rollin, O., Rasmont, P., Dufrêne, M., Michez, D., & Dendoncker, N. (2019). A century of local changes in bumblebee communities and landscape composition in Belgium. *Journal of Insect Conservation*, *23*, 489–501.

WHO. (2021). *World Health Organization - World malaria report 2022*. https://www.who.int/teams/global-malaria-programme

Wickham, H., Chang, W., & Wickham, M. H. (2016). Package 'ggplot2.' *Create Elegant Data Visualisations Using the Grammar of Graphics. Version*, *2*(1), 1–189.

Wildermuth, H., & Martens, A. (2019). *Die Libellen Europas: alle Arten von den Azoren bis zum Ural im Porträt*. Quelle & Meyer.

Willigalla, C., Schlotmann, F., & Ott, J. (2022). *Rote Liste und Gesamtartenliste der Libellen in Rheinland-Pfalz*.

Wirth, M. C., Park, H.-W., Walton, W. E., & Federici, B. A. (2005). Cyt1A of Bacillus thuringiensis delays evolution of resistance to Cry11A in the mosquito Culex quinquefasciatus. *Applied and Environmental Microbiology*, *71*(1), 185–189.

Wolfram, G., Wenzl, P., & Jerrentrup, H. (2018). A multi-year study following BACI design reveals no short-term impact of Bti on chironomids (Diptera) in a floodplain in Eastern Austria. *Environmental Monitoring and Assessment*, *190*(12). https://doi.org/10.1007/s10661-018-7084-6

Zuur, A. F., Ieno, E. N., Walker, N. J., Saveliev, A. A., & Smith, G. M. (2009). *Mixed effects models and extensions in ecology with R* (Vol. 574). Springer.

7 APPENDICES

Appendix A-1: Scientific publication 1

Benthic macroinvertebrate community shifts based on Bti-induced chironomid reduction also decrease Odonata emergence

in *Environmental Pollution*

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Key words: *Bacillus thuringiensis israelensis*; mosquito control; dragonflies; mesocosms; food webs; non-target organisms

Abbreviations: Bti = *Bacillus thuringiensis* var. *israelensis*
FPM = floodplain pond mesocosms

Abstract

Chironomid larvae often dominate aquatic macroinvertebrate communities and are a key food source for many aquatic predators, such as dragonfly and damselfly larvae (Odonata). Changes in aquatic macroinvertebrate communities may propagate through terrestrial food webs via altered insect emergence. *Bacillus thuringiensis israelensis* (Bti)-based larvicides are widely used in mosquito control but can also reduce the abundance of non-biting chironomid larvae (Diptera: Chironomidae). We applied the maximum field rate of Bti used in mosquito control three times to six mesocosms in a replicated floodplain pond mesocosm (FPM) system in spring for two consecutive years, while the remaining six FPMs were untreated. Three weeks after the third Bti application in the first year, we recorded on average a 41% reduction of chironomid larvae in Bti-treated FPMs compared to untreated FPMs and a shift in benthic macroinvertebrate community composition driven by the reduced number of chironomid, Libellulidae and Coenagrionidae larvae (Odonata). Additionally, the number of emerging Libellulidae (estimated by sampling of exuviae in the second year) was reduced by 54% in Bti-treated FPMs. Since Odonata larvae are not directly susceptible to Bti, our results suggest indirect effects due to reduced prey availability (i.e., chironomid larvae) or increased intraguild predation. As Libellulidae include species of conservation concern, the necessity of Bti applications to their habitats, e.g. floodplains, should be carefully evaluated.

1 Introduction

In aquatic ecosystems, larvae of non-biting chironomid midges (Diptera: Chironomidae) represent a major component of macroinvertebrate communities (Armitage et al., 1995) and serve as a key food source for many aquatic predators such as larvae of dragonflies and damselflies (Odonata), beetles and newts (Allgeier et al., 2019a; Armitage et al., 2012). Reduced prey availability for predators leads to increased competition for resources and consequently also predation between predators (Allgeier, et al., 2019a; Larson & House, 1990; van Buskirk, 1992), also known as intraguild predation (Holt & Polis, 1997). Thus, decreased chironomid densities may lead to bottom-up effects on aquatic predators like Odonata larvae (Allgeier et al., 2019a). Although considered to be of low risk for non-target organisms, laboratory studies indicated that the mosquito control biocide *Bacillus thuringiensis* var. *israelensis* (Bti) shows adverse effects on chironomid larvae (e.g., Bordalo et al., 2020; Charbonneau et al., 1994; Kästel et al., 2017). In addition to reduced larval survival and emergence, sublethal effects on chironomid larvae may include detoxifications processes, i.e., increased catalase activity after Bti exposure (Bordalo et al., 2020). In aquatic ecosystems, a reduction of non-target chironomids can alter the benthic invertebrate community compositions as recently shown in indoor stream mesocosms (Bordalo et al., 2021). In contrast to laboratory mesocosms, Bti-related changes in macroinvertebrate communities observed in field studies, characterized by differences in habitat structure, revealed contrasting results regarding effects on the abundance of chironomids (e.g., Allgeier et al., 2019b; Hershey et al., 1998; Lagadic et al., 2014, 2016; Lundström et al., 2010; Wolfram et al., 2018).

Bti is a bacterium widely used as biocide to control populations of biting insects, such as mosquitoes (Diptera: Culicidae) and blackflies (Diptera: Simuliidae), in wetlands of temperate regions in Europe and North America (Belousova et al., 2021; Brühl et al., 2020). While disease-transmitting, container-breeding mosquitoes (Culicidae: *Culex, Aedes, Anopheles*) are combatted locally, nuisance by floodwater mosquitoes (*Aedes vexans*) is controlled by large-scale Bti applications (Becker, 1997). Large-scale treatment of floodwater mosquito breeding habitats, i.e., Upper Rhine Valley in Germany, usually takes place between April and September, when females of *A. vexans* lay eggs into wet soil of regularly flooded areas (Becker, 1997). Treatment of breeding sites begins during flooding periods when mosquito larvae hatch, either by a combination of spraying a Bti liquid solution and applying sand granule formulation, or with large-scale aerial treatment by helicopters using Bti ice pellets (Becker, 2003). Conventional larvicides used in the last century were mostly broad-spectrum insecticides like organochlorides (e.g., DDT), organophosphates, carbamates and pyrethroids which have a high toxicity on many vertebrates and invertebrates (Becker et al., 2010a). In contrast, Bti has the advantage of being highly selective towards larvae of the Dipteran suborder Nematocera, i.e., targeted mosquitoes and blackflies. The larvicidal activity of Bti relies on the production of δ-endotoxins (Cry and Cyt proteins) during sporulation of the bacterium (Becker, 1997; Ben-Dov, 2014; Bravo et al., 2007) which are activated in the midgut epithelium of target species after ingestion. Site-specific proteases and receptors as well as alkaline conditions in the midgut are required for activation of the toxins (Becker, 2006; Bravo et al., 2007). Bti spores are often applied in their viable form which are more likely to proliferate rather than being biodegraded, thus making the viable Bti product more persistent in the environment (Poulin et al., 2022). However, in Germany, Bti products are sterilized with gamma-radiation prior to application which prevents spores from disseminating (Becker, 2002). Because of its high selectivity and relatively low persistency (in sterilized form), Bti has been rated as a more environmentally acceptable alternative to conventional larvicides (Becker et al., 2010a; Lacey & Merritt, 2003). Currently, Bti is the only larvicide homologated for controlling mosquitoes in the European Union.

Many aquatic insects, including chironomids and Odonata, have a biphasic life cycle with aquatic larvae and terrestrial/aerial adult stages (Armitage et al., 1995; Corbet, 1980; Vallenduuk & Moller Pillot, 2007). Hence, Bti-mediated indirect effects on aquatic predators, such as Odonata, may propagate to terrestrial ecosystems via delayed or reduced emergence. Bti-related studies on Odonata larvae are scarce, as they are not directly susceptible to Bti (Painter et al., 1996). However, in the French Camargue region where Bti has been regularly applied since 2006, surveys revealed a reduced number of adult Odonata in Bti-treated wetlands likely due to reductions in chironomid abundance (Jakob & Poulin, 2016). Since adult Odonata do not only play a role as terrestrial predators, but also serve as important prey for insectivorous birds (Arbeiter et al., 2014; Poulin et al., 2010), the potential propagation of Bti-mediated effects across ecosystem boundaries raises concerns and new questions about the impact of large-scale Bti treatment on linked aquatic-terrestrial ecosystems in natural wetlands.

Until now, experimental data on Bti-mediated effects on benthic invertebrate communities were obtained from laboratory mesocosm, semi-field enclosures or field studies. On the one hand, the realism of laboratory mesocosm or enclosure studies is limited by their small size and corresponding lack of comparability with field sites. In field studies, on the other hand, it is often difficult to disentangle treatment effects from natural diversity among field sites due to potential confounding environmental factors influencing invertebrate communities. To directly address effects of a frequently used biocide like Bti on realistic benthic communities and possible propagation through food webs and to terrestrial ecosystems, replicated field experiments are required. In this study, we used twelve constructed floodplain pond mesocosms (FPMs; area $\approx 104\ m^2$) that allowed natural community establishment and interactions, but also controlled flooding events and repeated Bti application, similar to those used in control programs in the Upper Rhine valley.

Due to the Bti-related reduction of chironomid larvae observed in field and lab mesocosm studies, we expected a lower density of chironomid larvae in Bti-treated FPMs compared to control FPMs. To test this hypothesis, we sampled benthic macroinvertebrates in the first year of the experiment. Since chironomids account for large parts of lentic benthic communities (Armitage et al., 1995; Hershey et al., 1998), we assumed that a Bti-mediated reduction of chironomids induces a shift in benthic community composition, while also adversely affecting predatory Odonata larvae due to limited prey availability. To analyse long-term effects on emerging Odonata, we collected exuviae (cast-off moult) during the second year of Bti treatment. We expected a lower number of emerging Odonata from Bti-treated FPMs due to possible effects of Bti on the food web, e.g., intraguild predation and competition, resulting from reduced prey availability.

2 Material & Methods

2.1 Study site

The field experiment was conducted at the Eußerthal Ecosystem Research Station (49°15'14"N, 7°57'42"E) in the Palatinate forest in south-west Germany. The study site is located in the forest with only minor anthropogenic influences in the upstream area (Stehle et al., 2022). Neither Bti nor any other mosquito control agents have ever been applied to the test system or any nearby water bodies. Twelve FPMs of 23.5 m × 7.5 m were constructed adjacent to each other in 2017, three years prior to the start of the experiment. The FPMs are open to natural colonization by organisms from nearby freshwater habitats. The water depth is 30 cm at the deepest point from where it gradually decreases into a shallow floodplain and terrestrial part at one shore (Figure 1). FPMs are characterized by an aquatic part consisting of coarse pebble gravel (microlithal; grain size ~1-3 cm), partly overgrown by submerged macrophytes (i.e., waterweeds and coontails) as well as emerged plants (i.e., cattails) in the littoral zone. Between the aquatic part and the terrestrial part (mostly covered by rush grass), there is a shallow floodplain area which is completely covered by submerged macrophytes and emerged plants. The FPMs can be flooded using stream water until the terrestrial part is completely under water. The study site and FPMs are described in detail by Stehle and colleagues (2022).

The study site is a breeding habitat for many amphibians, i.e., common frogs (*Rana temporaria*), common toads (*Bufo bufo*), palmate newts (*Lissotriton helveticus*) and alpine newts (*Ichthyosaura alpestris*). Amphibian larvae constitute an important part of aquatic food webs, especially as prey for Odonata larvae, and temporarily high densities can strongly influence food web structures. For this reason, we installed an amphibian fence around the FPMs to avoid uncontrolled immigration. Additionally, we placed three bottle traps in each pond (Griffiths, 1985) for three weeks to remove adult newts residing in the ponds. However, to obtain a homogenous abundance of amphibians in the ponds while not completely excluding them from the food web, we introduced 2-3 toad egg strings and five frog egg clutches of similar weight as well as 20 adult palmate newts (male and female) to each pond one week before the start of the experiment.

2.2 Bti application and flooding

In the Upper Rhine Valley in Germany, floodplains are extensively treated with Bti products since the 1980s (Becker, 2006), predominantly in form of icy pearls spread from helicopters or distributed by hand using a liquid solution or sand granule formulation. Bti products used in Germany must be sterilized by gamma radiation prior to usage to kill spores and avoid sporulation in nature, which causes a toxicity reduction of the formulation by approximately 20% (Becker, 2002). Generally, the toxicity of Bti products is expressed in international toxic units (ITU; Skovmand & Becker, 2000).

To mimic a field-relevant Bti application scenario, we applied Bti three times during controlled flooding periods between April and May 2020 and April and June 2021, for a total of six applications in two years (see application scheme in Table S1). Bti treatments were linked to a 20-day cycle (exact dates varied depending on weather conditions; see Table S1) with an alternating 10-day shallow (30 cm water depth) and 10-day flooding period (50 cm water depth). On the third day of the flood, six out of twelve FPMs (not adjacent; control ponds in between) were treated with the maximum field rate (FR = 2.88×10^9 ITU/ha) of a VectoBac® WDG suspension (Valent BioSciences Corporation, Illinois, USA). The suspension was evenly applied to the water surface using a knapsack sprayer (Prima 5, Gloria, Germany). According to Becker (1997), the maximum field rate of Bti is applied when the water is deeper than 10 cm and/or older instar larvae are targeted.

Currently, no method exists to quantify sterilized Bti-toxins in the environment. We therefore used a biotest for each application to verify that the product and application rate had the expected effect on mosquito larvae. Therefore, we filled 10-L plastic buckets with filtered pond water (mesh size: 55 µm) and introduced ~20 mosquito larvae (*Culex sp.*; mixture of 2nd and 3rd instar larvae) obtained from local rainwater tanks. Buckets were put inside the FPMs before application and larval mortality was monitored daily for one week. For comparison, buckets were also set up in control FPMs to ensure the effect in treated FPMs was induced by the Bti application. In the treatment buckets, mosquito larvae were reduced by at least 90% (calculated relative to control mortality) within the first week after application, thus the Bti treatment was considered successful (Becker, 1997).

2.3 Sampling and identification of benthic macroinvertebrates

The sampling of benthic macroinvertebrates took place two weeks after the third and last Bti application in the first year of the experiment (Table S1) in two habitats within the FPMs. Sampled habitats included macrophytes at the sides of the FPM and in the shallow floodplain area, and in gravel in the aquatic part (Figure 1). In order to collect samples representative for the benthic invertebrate community and cover FPM habitat variability, we sampled three one-meter transects for each habitat in each FPM (see Figure 1) using a kicknet sampler (25 x 25 cm, mesh size: 500 µm; Bioform, Germany). The kicknet was dragged along the bottom of the FPM while disturbing the sediment by foot. This invasive sampling technique allowed the collection of sediment-dwelling chironomid larvae from the FPM bottom. The content in the net was transferred into a tray using a sieve (mesh size: 500 µm), where coarse substrate (i.e., plant material) was carefully sorted out. Samples were stored in 85% Ethanol (Carl Roth, Deutschland). For identification of benthic macroinvertebrates (see taxonomic resolution in Table 1), we used a stereomicroscope (SZX-ZB9, Olympus, Japan) and identification keys from Kriska (2013) and Tachet et al. (2010). Determination of Odonata larvae was done according to Brochard et al. (2012) and Heidemann & Seidenbusch (2002).

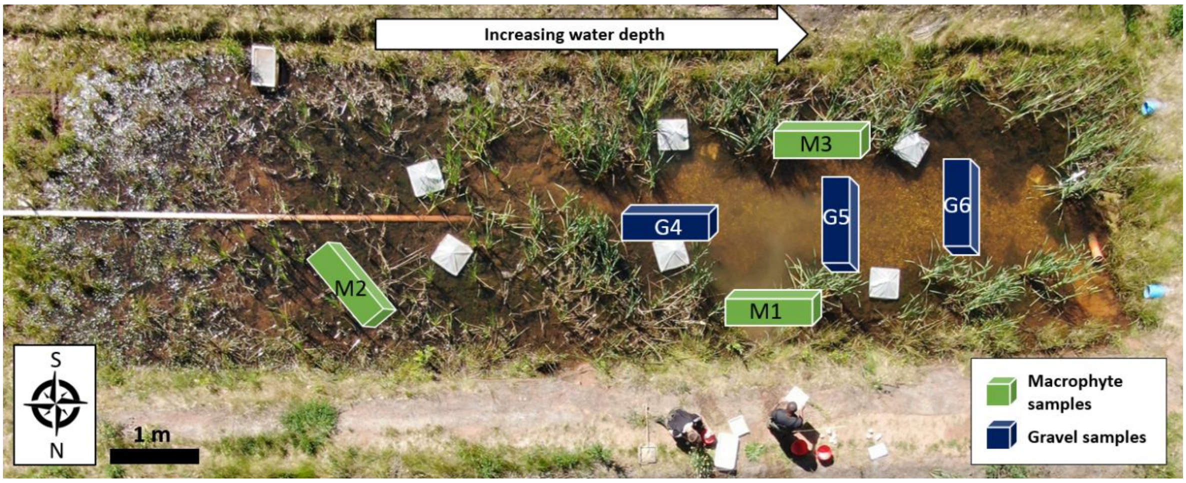

Figure 1: Top view of one floodplain pond mesocosm (A ≈ 104 m^2) with three sampling transects (1m) for each habitat (macrophytes as green polygons and gravel as blue polygons) using a standard kicknet sampler (25 x 25 cm; mesh size = 500 µm). The kicknet was dragged along the bottom of the mesocosm while disturbing the sediment by foot.

Table 1: Taxonomic resolution of sampled benthic macroinvertebrates as used for statistical analyses.

Order	Suborder	Family	Level of identification	Taxa
Odonata	Anisoptera	Aeshnidae	Family	Aeshnidae
		Libellulidae	Family	Libellulidae
	Zygoptera	Coenagrionidae	Family	Coenagrionidae
Diptera	Nematocera	Chironomidae	Subfamily	Chironominae Orthocladiinae Tanypodinae Chironomidae sp.
		Culicidae	Family	Culicidae
		Ceratopogonidae	Family	Ceratopogonidae
		Chaoboridae	Family	Chaoboridae
	Brachycera	Rhagionidae	Family	Rhagionidae
		Tabanidae	Family	Tabanidae
Megaloptera		Sialidae	Family	Sialidae
Ephemeroptera		Baetidae	Family	Baetidae
		Caenidae	Family	Caenidae
Coleoptera	Adephaga	Haliplidae	Family	Haliplidae
		Dytiscidae	Family	Dytiscidae
	Polyphaga	Elmidae	Family	Elmidae
		Hydrophilidae	Family	Hydrophilidae
Hemiptera	Heteroptera	Pleidae	Family	Pleidae
		Notonectidae	Family	Notonectidae
Trichoptera		Leptoceridae	Family	Leptopceridae

2.4 Sampling and identification of Odonata exuviae

We collected exuviae as proxy for Odonata emergence in the second year of the experiment weekly or bi-weekly (sampling was not done during heavy rain or wind) from June to September 2021 resulting in ten data points (Table S1). Sampling of exuviae is the only method that accounts for successful completion of the aquatic life cycle compared to sampling of aquatic larvae (Raebel et al., 2010), while surveys of flying adults are not suitable for experimental ponds in close proximity, like our pond system. In each FPM, one person collected exuviae on emerged and riparian plants for 20 minutes. Exuviae were identified on species level using a stereomicroscope (S9i, Leica, Germany) and identification keys from Bellmann (1993) and Heidemann & Seidenbusch (2002).

2.5 Statistical analysis

Prior to any calculations, we transformed taxa abundance (collected individuals per 0.25 m^2) to taxa density per m^2. Mixed effect models (*lme*) were performed to determine treatment-induced differences in total macroinvertebrate density and chironomid density using the *lme4* package (Pinheiro et al., 2017) for R (version 4.1.2; R Core Team, 2013) with habitat (macrophytes and gravel), treatment (Bti and control) and their interaction as fixed effects. Pond identity was used as random effect to account for multiple collections within each pond. Transect identity (position of the sample) as nested factor in the pond was removed from the final model due to decrease of the model fit (determined by Akaike's information criterion; AIC). Response variables were $\log_{10}$-transformed to meet the model assumptions. Residual normality of the final model was checked graphically with quantile-quantile plots and heterogeneity with residuals versus fits plots (Zuur et al., 2009). Marginal R^2 values were calculated using *r.squaredGLMM* from the *MuMIn* package (Barton & Barton, 2015).

For community composition analyses, we treated the three samples from one habitat in each pond as pseudo-replicates and therefore used the arithmetic mean densities, resulting in a total of six replicates per habitat for each treatment. We used the *vegan* package (Oksanen et al., 2013) in R. Prior to statistical analyses of benthic communities, we used square root transformation to reduce the influence of highly dominant taxa and heterogeneity of variances (Zuur et al., 2009). Subsequently, homogeneity of group dispersions was confirmed using the *betadisper* function (Table S3; Anderson, 2006). Differences in community composition among treatments and habitats were investigated using computation of Bray-Curtis dissimilarity indices using *vegdist* and permutational analysis of variances (perMANOVA, *adonis* function; Anderson, 2014) using treatment, habitat and their interaction as factors and restricting permutations within the ponds. The Bray-Curtis dissimilarity matrix was plotted in a reduced ordination space using non-metric multidimensional scaling (nMDS; *metaMDS* function). The optimal number of dimensions was determined using stress values (badness of fit). Groups (treatment × habitat) were delineated by 95% confidence ellipses. Additionally, we used analysis of similarity percentages (*simper* function) to detect taxa that contribute most (up to 70%) to dissimilarities between control and treatment FPMs.

For sake of comparability between Odonata larvae and exuviae, we used the same taxonomic resolution, i.e., Aeshnidae, Libellulidae and Coenagrionidae. For exuviae, we summed up all sampling dates and calculated arithmetic mean values between control and Bti for each FPM. We used non-metric Wilcoxon rank sum test to test for significant differences between control and treatment FPMs. The significance level for all analyses was set to $p < 0.05$. Figures were generated using ggplot2 (Wickham et al., 2016) for R.

3 Results

3.1 Total macroinvertebrate density

Chironomid larvae accounted for ~72% of all 13,688 collected macroinvertebrates in total (Table S2), with mayflies (Ephemeroptera) being the second most (~12%) and Odonata the third most abundant taxon (~11%). The treatment (control or Bti) had a significant effect on the total macroinvertebrate density with approximately 36% fewer macroinvertebrates found in Bti-treated FPMs compared to control FPMs ($F_{1,10} = 5.90$, $p \approx 0.04$; Figure 2a). Overall, the habitat type (gravel or macrophytes) had a significant effect on the total macroinvertebrate density with ~70% fewer individuals in the habitat gravel compared to macrophytes ($F_{1,58} = 104.67$, $p < 0.0001$; Figure 2a). There was no significant interaction in the total benthic macroinvertebrate density between the factors treatment and habitat (no interaction effect; $F_{1,58} = 2.38$, $p \approx 0.13$).

Compared to the control, the total number of chironomids was significantly reduced by ~41% in Bti-treated FPMs ($F_{1,10} = 6.81$, $p \approx 0.03$) independent of the habitat. In particular, we found significantly fewer larvae in Bti-treated FPMs in the Chironominae subfamily (~47%; $F_{1,10} = 8.00$; $p \approx 0.02$), and a similar pattern, although not statistically significant, for the other subfamilies of Orthocladiinae (~62%; $F_{1,10} = 2.92$; $p \approx 0.12$) and Tanypodinae (~18%; $F_{1,10} = 0.17$; $p \approx 0.69$; Figure 2b).

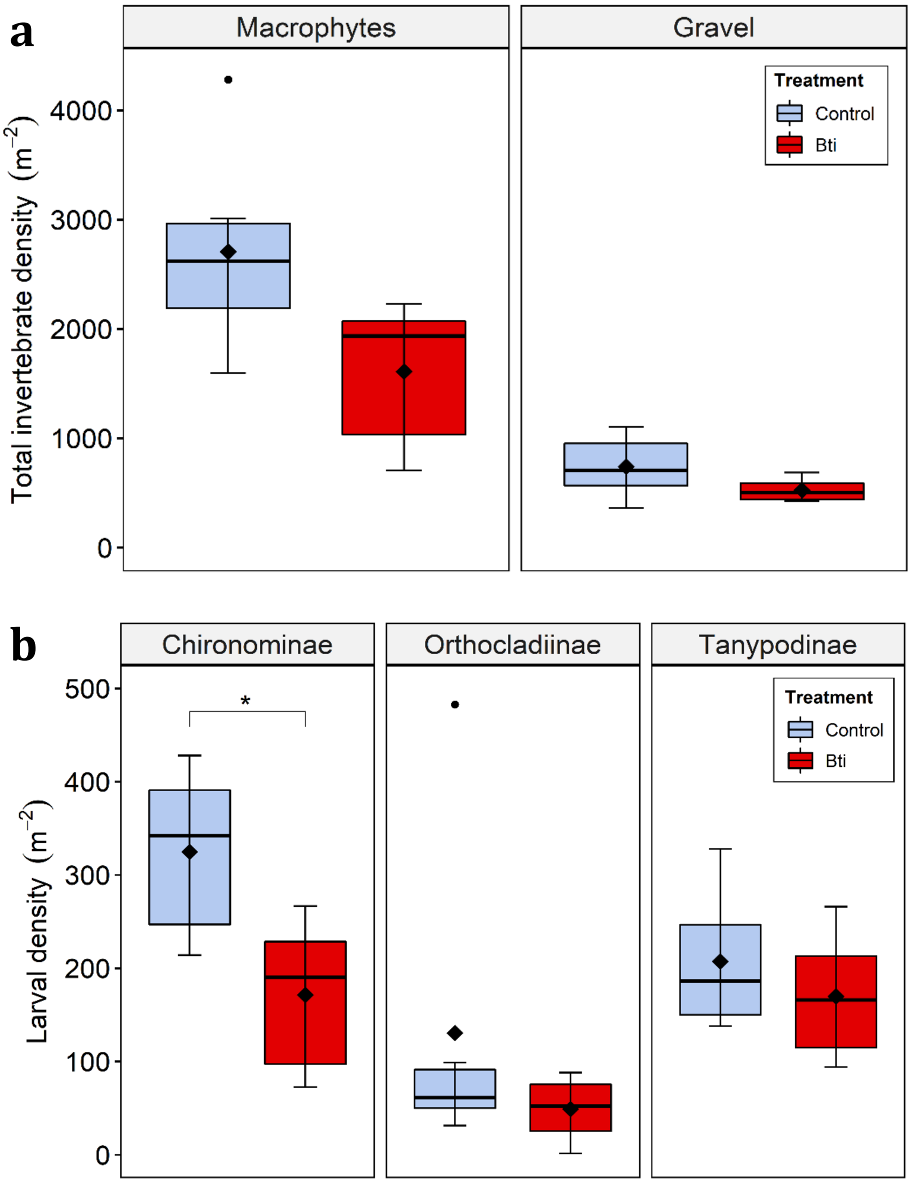

Figure 2: (a) Total invertebrate density (number of individuals per m^2) in macrophytes and gravel habitats and in control (blue; n=6) and Bti-treated FPMs (red; n=6), based on habitat means (n=3) within each FPM. (b) Chironomid larval density of the three identified chironomid subfamilies in control and Bti-treated FPMs. Based on FPM mean density in macrophyte (n=3) and gravel habitats (n=3). Lower and upper box boundaries show 25th and 75th percentiles, respectively, line inside box show medians, and black diamonds show arithmetic mean density. Whiskers and black dots show the variability outside the lower and upper quartiles. Statistically significant difference ($p < 0.05$, after *lme*) between control and treatment FPMs is denoted with an asterisk (*).

3.2 Macroinvertebrate community composition

Both treatment and habitat had a significant effect on the benthic invertebrate community composition with treatment explaining 6% and habitat explaining 46% of the observed dissimilarities between groups (Table 2). Graphically, in the nMDS plot (Figure 3), ellipses showed a separation in benthic invertebrate composition between gravel and macrophyte habitats (no overlap of 95% confidence ellipses) and partially between control and Bti treatment.

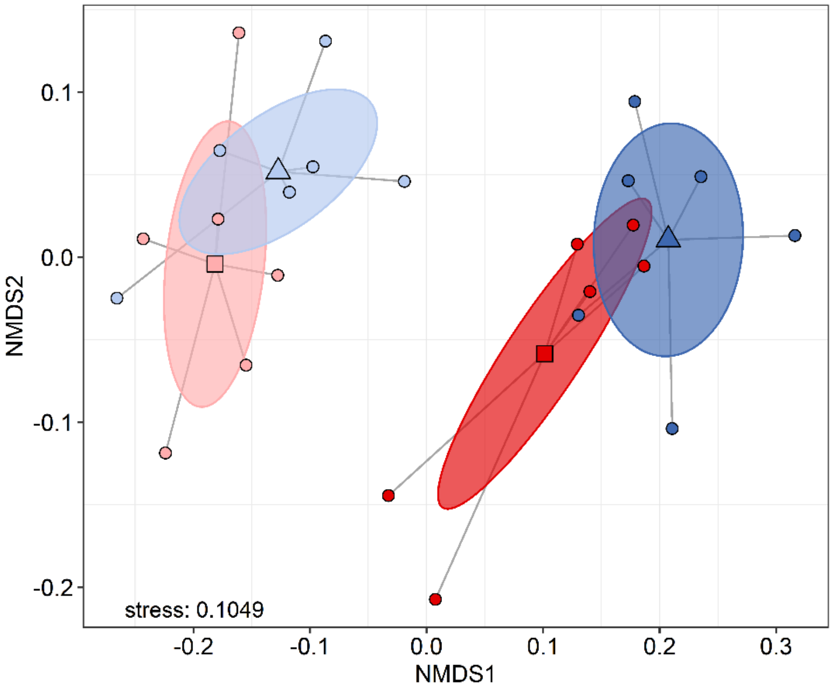

Figure 3: Non-metric multidimensional scaling (nMDS) ordination plot illustrating differences in benthic macroinvertebrate community composition between treatment (red squares; n=6) and control FPMs (blue triangles; n=6), and habitats gravel (light color; n=6) and macrophytes (dark color; n=6), including 95% confidence ellipses.

Table 2: Treatment and habitat effects on the community composition using perMANOVA. Significant p-values are printed in bold. Number of permutations = 999; degrees of freedom (DF), sums of squares (SS), and mean squares (MS).

Effects	DF	SS	MS	F Model	R^2	p-value
Treatment	1	0.083	0.083	2.424	0.057	**0.001**
Habitat	1	0.671	0.671	19.703	0.463	**0.001**
Treatment × Habitat	1	0.014	0.014	0.410	0.010	0.737
Residuals	20	0.681	0.034		0.478	
Total	23	1.450			1.000	

According to SIMPER analysis, up to 70% of dissimilarities in community composition between control and treatment FPMs were explained by seven taxa (Table 3). Differences in benthic invertebrate community composition were particularly driven by the three chironomid subfamilies Chironominae, Tanypodinae and Orthocladiinae, each contributing more than 10% to the explained dissimilarity between control and treatment communities, followed by two Odonata families (Libellulidae and Coenagrionidae).

Table 3: Taxa driving the dissimilarity (cumulative contribution ~70%) between control (n=6) and treatment FPMs (n=6) after SIMPER analysis on square root transformed densities. Average density (individuals per m^2) and respective square root transformed average densities (in brackets) are shown for control and treatment for each taxon, and the percent difference in Bti-treated FPMs compared to control FPMs.

	Average density (m^{-2})		Contributing	Percent
Taxa	Control	Treatment	percent	difference
Chironominae	324.8 (17.2)	173.1 (12.5)	13.5	↓ 47%
Tanypodinae	207.2 (13.2)	170.9 (12.0)	12.8	↓ 18%
Orthocladiinae	130.7 (8.8)	49.1 (5.5)	12.2	↓ 62%
Libellulidae	63.3 (6.7)	39.0 (5.3)	8.8	↓ 39%
Coenagrionidae	28.8 (4.2)	17.4 (3.6)	6.7	↓ 39%
Chironomidae sp.	30.2 (4.7)	14.3 (3.0)	6.5	↓ 53%
Ceratopogonidae	18.0 (3.1)	1.4 (0.6)	5.2	↓ 93%

3.3 Emerging Odonata

A total of 1,717 Odonata exuviae were collected and identified (Table 4). Libellulidae were the most common family contributing 45% to the total number of exuviae, followed by Aeshnidae (40%) and Coenagrionidae (15%). We found the number of Libellulidae exuviae was significantly reduced by 54% (Wilcox test: $p < 0.01$; Figure 4) in Bti-treated FPMs. Aeshnidae and Libellulidae were largely dominated by two genera, *Anax* and *Sympetrum*, respectively.

Table 4: Cumulative number of Odonata emerging from control (n=6) and treatment FPMs (n=6) collected from June to September 2021.

	Number of exuviae		Percent
Family	Control	Treatment	difference
Aeshnidae	292	397	↑ 26%
Libellulidae	530	245	↓ 54%
Coenagrionidae	146	107	↓ 27%

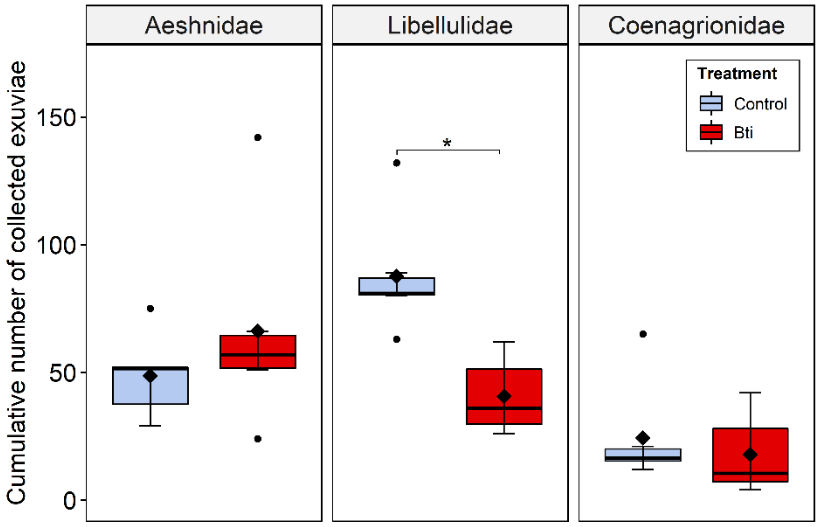

Figure 4: Cumulative number of exuviae in control (blue; n=6) and Bti-treated FPMs (red; n=6) for Aeshnidae (A), Libellulidae (B), and Coenagrionidae (C), collected from June to September 2021. Lower and upper box boundaries show 25th and 75th percentiles, respectively, line inside box show medians and black diamonds show arithmetic mean density. Whiskers and black dots show the variability outside the lower and upper quartiles. Statistically significant differences ($p<0.05$) are denoted with an asterisk (*).

4 Discussion

4.1 Total macroinvertebrate density

Chironomids were the dominant family contributing up to 72% to the benthic macroinvertebrate community. Independent of the habitat, chironomid density declined by 41% in Bti-treated FPMs, which is also reflected in modifications in their emergence pattern covered by a companion study (Kolbenschlag et al., submitted). Since non-target chironomids and target mosquitoes are physiologically similar, chironomids are directly susceptible to Bti once ingested (Ali, 1981; Kästel et al., 2017). The effect varied among chironomid subfamilies, likely depending on their foraging behavior, e.g. deposit-feeding or predatory species, and therefore the amount of Bti ingested by chironomid larvae. Although Bti is applied on the water surface, benthic deposit-feeding chironomid families like Chironominae and Orthocladiinae (Vallenduuk & Moller Pillot, 2007) likely ingest Bti after its sedimentation. Since Bti spores can persist in soil for several months (Duchet et al., 2014) or even up to four years after cessation of spraying (Poulin et al., 2022), the risk of exposure to Bti for sediment-dwelling chironomids can remain high over weeks. Unlike species of Chironominae and Orthocladiinae, many species of the subfamily Tanypodinae are predatory (Vallenduuk & Moller Pillot, 2007), presumably ingesting lower amounts of Bti compared to non-predatory chironomid species. Our results are comparable to observations in a mesocosm study from Allgeier et al. (2019b), in which Bti had little to no effect on larvae of the subfamily of Tanypodinae, while Chironominae and Orthocladiinae were reduced by 44% and 58%, respectively. Taxa-specific sensitivity to Bti was also found in studies by Liber et al (1998) and Lundström et al. (2010). Although the foraging behavior can explain taxa-specific sensitivities to Bti, Theissinger and colleagues (2019) found a similar percentage of predatory and filtering chironomid species being reduced by Bti (both approx. 65%). This result suggests that the feeding strategy might not be the only driver for sensitivity towards Bti. However, it is also possible that Tanypodinae are indirectly affected by Bti via the reduced prey availability, as they also feed on early instar chironomid larvae (Armitage et al., 1995; Kästel et al., 2017) and at the same time ingest Bti-contaminated prey.

In contrast to chironomid larvae, Odonata larvae are not directly susceptible to Bti (Boisvert & Boisvert, 2000; Painter et al., 1996). Despite this, we found fewer larvae of both Libellulidae and Coenagrionidae, in Bti-treated FPMs compared to control FPMs (~ 39%; Table S2). It is worth mentioning that Libellulidae in our benthic samples were predominantly early instar larvae and we did not observe this effect on the abundance of Libellulidae in their late instars or on Aeshnidae. This finding indicates that Bti may have an indirect effect on smaller early instar Odonata larvae. We assume the reduced chironomid availability to be the main reason for this effect, since for many aquatic predators, i.e., Odonata larvae, chironomids are an important food source and contribute to large parts of their diet (Wildermuth & Martens, 2019). Consequently, the density of Odonata is directly proportional to the prey availability, as shown in previous studies (van Buskirk, 1989, 1992). Additionally, depending on prey density and competition, cannibalism and intraguild predation in Odonata are common (Johnson, 1991; van Buskirk, 1989) and late instar larvae feed on early instar larvae (van Buskirk, 1992). Late instar Odonata larvae are strongly opportunistic predators and are, unlike small larvae such as Coenagrionidae or early instar Libellulidae, also able to feed on larger prey, i.e. amphibian larvae. Thus, late instar Odonata larvae may be less dependent on chironomids as food source relative to early instar larvae and at the same time have a lower risk to be preyed on (e.g. cannibalism). These considerations may explain the lack of effect on Aeshnidae which were already larger in size at the sampling period in June.

4.2 Macroinvertebrate community composition

The differences in benthic macroinvertebrate community composition between Bti and control FPMs were mostly based on the densities of Chironominae, Tanypodinae, Orthocladiinae, as well as Libellulidae and Coenagrionidae (Table 3). Since chironomids often dominate wetland invertebrate communities by constituting up to 93% of total insect emergence (Leeper & Taylor, 1998), a reduction of chironomids can result in cascading effects on the entire invertebrate community. A long-term field study in Minnesotan wetlands (Hershey et al., 1998) was the first to show that realistic Bti treatments significantly affect benthic macroinvertebrate communities, mainly due to the reduction of chironomid abundance by 60-80%. Allgeier et al. (2019b) and Bordalo et al. (2021) revealed significant alterations of benthic community compositions in mesocosms repeatedly exposed to field-relevant Bti application rates. The observed effect on the community composition was mainly due to the reduction of chironomids, in accordance with our results.

There are contrasting results with regard to environmental risks of Bti for non-target organisms (Boisvert & Boisvert, 2000; Brühl et al., 2020). Semi-field and field studies published by Lagadic et al. (2014, 2016) and Duchet et al. (2015) suggested no effects on chironomids (benthic and emerging chironomids, respectively) in Bti-treated wetlands in France. However, the authors did not provide information about the successful control of mosquito larvae and hence lack a confirmation of the desired effect of Bti. In general, a possible reason why field studies do not detect effects on chironomid emergence in temporary wetlands is the timing between flooding, Bti treatment and sampling. In temporary floodplains, chironomids need more time to emerge than mosquitoes as they colonize wetlands after flooding compared to floodwater mosquitoes which lay their eggs before flooding. Therefore, the duration of sampling should be several weeks or months to include a possible delay in emergence. A companion study by Kolbenschlag et al. (submitted) determined the number of emerging insects from the FPMs weekly over 3.5 months using emergence traps. Although the cumulative number of collected insects was only ~12% lower in treated FPMs, Bti resulted in an earlier (~10 days) and reduced emergence peak (~26%) of chironomids. Wolfram et al. (2018) conducted a 5-year field monitoring in the floodplains of river Morava and its tributary Dyje in Eastern Austria where Bti has been applied regularly in inundated areas for about 10 to 15 years. The authors followed a Before-After-Control-Impact (BACI) sampling design and found that at Bti-treated sites mosquito larvae were successfully combatted, whereas chironomids were not affected over the 5-year sampling period. However, the authors addressed the issue that it is difficult to identify single stressor effects in rapidly changing and complex environments due to natural and anthropogenic factors, which we can largely exclude in our study based on the replicated FPM test design.

Just like Wolfram et al. (2018), we sampled chironomid larvae using a net with a mesh size of 500 µm most likely excluding the first (and second) instars. A Bti-mediated effect could be even more pronounced in early instar chironomids, since the sensitivity of chironomid larvae decreases from first to fourth instar (Kästel et al., 2017). Early instar chironomid larvae are an important food source for small aquatic predators, such as early instar Odonata, predatory species of Tanypodinae, Coleoptera and newt larvae.

4.3 Emerging Odonata

Comparing Odonata larval densities from the first experimental year to the number of emerging Odonata from the second year, the effect size on the three Odonata families (Aeshnidae, Libellulidae and Coenagrionidae) are similar (Table S2 vs. Table 4). Sampling of exuviae showed that the Libellulidae, predominantly *Sympetrum*, were reduced by 54% in Bti-treated FPMs, whereas Aeshnidae, predominantly *Anax*, were not adversely affected by Bti (with a non-significant 29% higher number of emerging individuals in Bti-treated FPMs; Table 4, Figure S1). Since we started sampling in mid-June, it is possible that we missed the emergence peaks of early emerging species, e.g. *Anax imperator* and Coenagrionidae species that start to emerge in May (Figure S1; Wildermuth & Martens, 2019). Nevertheless, the observed effect difference for Aeshnidae and Libellulidae implies that the timing of larval development and Bti applications in spring may play a role for the emergence, particularly for univoltine (completion of aquatic life phase in one year) Odonata like *Sympetrum*. Larvae of this genus usually emerge in late summer, eggs overwinter in ponds where larvae hatch in spring (Wildermuth & Martens, 2019). Therefore, early larval development can coincide with Bti treatment of wetlands in spring and summer which, in our study, led to a reduced chironomid availability. At this time, early instar larvae of *Sympetrum* are small and could serve as prey, for example for late instars of semivoltine (completion of aquatic life phase in two years) *Anax*. Therefore, we suggest that the observed reduction of late emerging Libellulidae can be mainly due to size-dependent intraguild predation by early emerging Aeshnidae, as a result of significantly reduced chironomids as bottom-up effect on aquatic predators.

5 Conclusion

To the best of our knowledge, this is the first study to assess effects of Bti applications in a replicated field experiment with natural benthic communities. Reductions of aquatic chironomid populations, in our study caused by Bti applications, resulted in indirect effects on Odonata, but can possibly also affect other predators such as beetles, fish and newts inhabiting aquatic ecosystems. In addition, these effects can propagate from aquatic to terrestrial food webs with effects on adult Odonata, riparian spiders, birds, bats and other predators in terrestrial ecosystems via reduced adult insect biomass or altered emergence dynamics (Kolbenschlag et al., submitted). With the recognized wide-ranging insect decline (Hallmann et al., 2017) and the general protection status of Odonata in Germany with one third of the species considered threatened (Ott, 2016; Willigalla et al., 2022), environmental effects of large-scale Bti applications of floodplains and wetlands for mosquito control should be carefully evaluated.

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7 References

Ali, A. (1981). *Bacillus thuringiensis* serovar. *israelensis* (ABG-6108) against chironomids and some nontarget aquatic invertebrates. *Journal of Invertebrate Pathology*, *38*(2), 264–272.

Allgeier, S., Friedrich, A., & Brühl, C. A. (2019a). Mosquito control based on *Bacillus thuringiensis israelensis* (Bti) interrupts artificial wetland food chains. *Science of the Total Environment*, *686*, 1173–1184. https://doi.org/10.1016/j.scitotenv.2019.05.358

Allgeier, S., Kästel, A., & Brühl, C. A. (2019b). Adverse effects of mosquito control using *Bacillus thuringiensis* var. *israelensis*: Reduced chironomid abundances in mesocosm, semi-field and field studies. *Ecotoxicology and Environmental Safety*, *169*, 786–796. https://doi.org/10.1016/j.ecoenv.2018.11.050

Anderson, M. J. (2006). Distance-based tests for homogeneity of multivariate dispersions. *Biometrics*, *62*(1), 245–253.

Anderson, M. J. (2014). Permutational multivariate analysis of variance (PERMANOVA). *Wiley Statsref: Statistics Reference Online*, 1–15.

Arbeiter, S., Schnepel, H., Uhlenhaut, K., Bloege, Y., Schijlze, M., & Hahn, S. (2014). Seasonal shift in the diet composition of european bee-eaters merops apiaster at the northern edge of distribution. *Ardeola*, *61*(1), 161–170. https://doi.org/10.13157/arla.61.1.2014.161

Armitage, P. D., Pinder, L. C., & Cranston, P. S. (1995). The Chironomidae: biology and ecology of non-biting midges. In *The Chironomidae - The biology and ecology of non-biting midges*. Springer Science & Business Media.

Barton, K., & Barton, M. K. (2015). Package 'mumin.' *Version*, *1*(18), 439.

Becker, N. (1997). Microbial Control of Mosquitoes: Management of the Upper Rhine Mosquito Population as a Model Programme. *Parasitology Today*, *13*(12), 485–487.

Becker, N. (2002). Sterilization of *Bacillus thuringiensis israelensis* products by gamma radiation. *Journal of the American Mosquito Control Association*, *18*(1), 57–62.

Becker, N. (2003). Ice granules containing endotoxins of microbial agents for the control of mosquito larvae - a new application technique. *Journal of the American Mosquito Control Association*, *19*(l), 63–66.

Becker, N. (2006). Biological control of mosquitoes: management of the upper Rhine mosquito population as a model programme. In *An ecological and societal approach to biological control* (pp. 227–245). Springer.

Becker, N., Petric, D., Zgomba, M., Boase, C., Madon, M., Dahl, C., & Kaiser, A. (2010). Chemical Control. In *Mosquitoes and their control* (pp. 441–475). Springer Science & Business Media.

Bellmann, H. (1993). *Libellen beobachten - bestimmen*. Naturbuch Verlag.

Belousova, M. E., Malovichko, Y. v., Shikov, A. E., Nizhnikov, A. A., & Antonets, K. S. (2021). Dissecting the environmental consequences of *Bacillus thuringiensis* application for natural ecosystems. In *Toxins* (Vol. 13, Issue 5). MDPI AG. https://doi.org/10.3390/toxins13050355

Ben-Dov, E. (2014). *Bacillus thuringiensis* subsp. *israelensis* and its dipteran-specific toxins. *Toxins*, *6*(4), 1222–1243. https://doi.org/10.3390/toxins6041222

Boisvert, M., & Boisvert, J. (2000). Effects of *Bacillus thuringiensis* var. *israelensis* on target and nontarget organisms: A review of laboratory and field experiments. In *Biocontrol Science and Technology* (Vol. 10, Issue 5, pp. 517–561). Carfax Publishing Company. https://doi.org/10.1080/095831500750016361

Bordalo, M. D., Gravato, C., Beleza, S., Campos, D., Lopes, I., & Pestana, J. L. T. (2020). Lethal and sublethal toxicity assessment of *Bacillus thuringiensis* var. *israelensis* and Beauveria bassiana based bioinsecticides to the aquatic insect Chironomus riparius. *Science of the Total Environment*, *698*. https://doi.org/10.1016/j.scitotenv.2019.134155

Bordalo, M. D., Machado, A. L., Campos, D., Coelho, S. D., Rodrigues, A. C. M., Lopes, I., & Pestana, J. L. T. (2021). Responses of benthic macroinvertebrate communities to a Bti-based insecticide in artificial microcosm streams. *Environmental Pollution*, *282*. https://doi.org/10.1016/j.envpol.2021.117030

Bravo, A., Gill, S. S., & Soberón, M. (2007). Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon*, *49*(4), 423–435.

Brochard, C., Croenendijk, D., van der Ploeg, E., & Termaat, T. (2012). *Fotogids larvenhuidjes van libellen*. KNNV Uitgeverij.

Brühl, C. A., Després, L., Frör, O., Patil, C. D., Poulin, B., Tetreau, G., & Allgeier, S. (2020). Environmental and socioeconomic effects of mosquito control in Europe using the biocide *Bacillus thuringiensis* subsp. *israelensis* (Bti). In *Science of the Total Environment* (Vol. 724). Elsevier B.V. https://doi.org/10.1016/j.scitotenv.2020.137800

Charbonneau, C. S., Drobney, R. D., & Rabeni, C. F. (1994). Effects of *Bacillus thuringiensis* var. *israelensis* on nontarget benthic organisms in a lentic habitat and factors affecting the efficiacy of the larvicide. In *Environmental Toxicology and Chemistry* (Vol. 13, Issue 2).

Corbet, P. S. (1980). Biology of Odonata. *Annual Review of Entomology*, *25*(1), 189–217. www.annualreviews.org

Duchet, C., Franquet, E., Lagadic, L., & Lagneau, C. (2015). Effects of *Bacillus thuringiensis israelensis* and spinosad on adult emergence of the non-biting midges *Polypedilum nubifer* (Skuse) and *Tanytarsus curticornis* Kieffer (Diptera: Chironomidae) in coastal wetlands. *Ecotoxicology and Environmental Safety*, *115*, 272–278. https://doi.org/10.1016/j.ecoenv.2015.02.029

Duchet, C., Tetreau, G., Marie, A., Rey, D., Besnard, G., Perrin, Y., Paris, M., David, J. P., Lagneau, C., & Després, L. (2014). Persistence and recycling of bioinsecticidal *Bacillus thuringiensis* subsp. *israelensis* spores in contrasting environments: evidence from field monitoring and laboratory experiments. *Microbial Ecology*, *67*(3), 576–586. https://doi.org/10.1007/s00248-013-0360-7

Griffiths, R. A. (1985). A simple funnel trap for studying newt populations and an evaluation of trap behaviour in smooth and palmate newts, *Triturus vulgaris* and *T. helveticus*. *Herpetological Journal*, *1*, 5–10.

Hallmann, C. A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., Stenmans, W., Müller, A., Sumser, H., Hörren, T., Goulson, D., & de Kroon, H. (2017). More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLoS ONE*, *12*(10). https://doi.org/10.1371/journal.pone.0185809

Heidemann, H., & Seidenbusch, R. (2002). *Libellenlarven Deutschlands*. Goecke & Evers.

Hershey, A. E., Lima, A. R., Niemi, G. J., & Regal, R. R. (1998). Effects of *Bacillus thuringiensis israelensis* (Bti) and methoprene on nontarget macroinvertebrates in Minnesota wetlands. *Ecological Applications*, *8*(1), 41–60.

Holt, R. D., & Polis, G. A. (1997). A theoretical framework for intraguild predation. *The American Naturalist*, *149*(4), 745–764.

Jakob, C., & Poulin, B. (2016). Indirect effects of mosquito control using Bti on dragonflies and damselflies (Odonata) in the Camargue. *Insect Conservation and Diversity*, *9*(2), 161–169. https://doi.org/10.1111/icad.12155

Johnson, D. M. (1991). Behavioral ecology of larval dragonflies and damselflies. *Trends in Ecology & Evolution*, *6*(1), 8–13. https://doi.org/10.1016/0169-5347(91)90140-S

Kästel, A., Allgeier, S., & Brühl, C. A. (2017). Decreasing *Bacillus thuringiensis israelensis* sensitivity of Chironomus riparius larvae with age indicates potential environmental risk for mosquito control. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-14019-2

Kolbenschlag, S., Gerstle, V., Eberhardt, J., Bollinger, E., Schulz, R., Brühl, C. A., & Bundschuh, M. (2023). A temporal perspective of aquatic subsidy: Bti affects the emergence of Chironomidae. *Ecotoxicology and Environmental Safety*.

Kriska, G. (2013). *Freshwater invertebrates in Central Europe*. Springer Science & Business Media.

Lacey, L. A., & Merritt, R. W. (2003). The safety of bacterial microbial agents used for black fly and mosquito control in aquatic environments. In *Environmental impacts of microbial insecticides* (pp. 151–168). Springer.

Lagadic, L., Roucaute, M., & Caquet, T. (2014). Bti sprays do not adversely affect non-target aquatic invertebrates in French Atlantic coastal wetlands. *Journal of Applied Ecology*, *51*(1), 102–113. https://doi.org/10.1111/1365-2664.12165

Lagadic, L., Schäfer, R. B., Roucaute, M., Szöcs, E., Chouin, S., de Maupeou, J., Duchet, C., Franquet, E., le Hunsec, B., Bertrand, C., Fayolle, S., Francés, B., Rozier, Y., Foussadier, R., Santoni, J. B., & Lagneau, C. (2016). No association between the use of Bti for mosquito control and the dynamics of non-target aquatic invertebrates in French coastal and continental wetlands. *Science of the Total Environment*, *553*, 486–494. https://doi.org/10.1016/j.scitotenv.2016.02.096

Larson, D., & House, N. (1990). Insect communities of Newfoundland bog pools with emphasis on the Odonata. *The Canadian Entomologist*, *122*, 469–501.

Leeper, D. A., & Taylor, B. E. (1998). Insect Emergence from a South Carolina (USA) Temporary Wetland Pond, with Emphasis on the Chironomidae (Diptera). *Source: Journal of the North American Benthological Society*, *17*(1), 54–72. https://www.jstor.org/stable/1468051

Liber, K., Schmude, K. L., & Rau, D. M. (1998). Toxicity of *Bacillus thuringiensis* var. *israelensis* to chironomids in pond mesocosms. *Ecotoxicology*, *7*, 343–354.

Lundström, J. O., Schäfer, M. L., Petersson, E., Persson Vinnersten, T. Z., Landin, J., & Brodin, Y. (2010). Production of wetland Chironomidae (Diptera) and the effects of using *Bacillus thuringiensis israelensis* for mosquito control. *Bulletin of Entomological Research*, *100*(1), 117–125. https://doi.org/10.1017/S0007485309990137

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., & Wagner, H. (2013). Package 'vegan.' *Community Ecology Package, Version*, *2*(9), 1–295.

Ott, J. (2016). *Citizen Science in Naturschutz und Landesforschung - Informationen zur Gruppe der Libellen (Odoanta)*.

Painter, M. K., Tennessen, K. J., & Richardson, T. D. (1996). Effects of repeated application of *Bacillus thuringiensis israelensis* on the mosquito predator Erythemis simplicicollis (Odonata: Libellulidae) from hatching to final instar. *Environmental Entomology*, *25*(1), 184–191.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Heisterkamp, S., van Willigen, B., & Maintainer, R. (2017). Package 'nlme.' *Linear and Nonlinear Mixed Effects Models, Version*, *3*(1).

Poulin, B., Lefebvre, G., Hilaire, S., & Després, L. (2022). Long-term persistence and recycling of Bacillus thuringiensis israelensis spores in wetlands sprayed for mosquito control. *Ecotoxicology and Environmental Safety*, *243*. https://doi.org/10.1016/j.ecoenv.2022.114004

Poulin, B., Lefebvre, G., & Paz, L. (2010). Red flag for green spray: Adverse trophic effects of Bti on breeding birds. *Journal of Applied Ecology*, *47*(4), 884–889. https://doi.org/10.1111/j.1365-2664.2010.01821.x

Raebel, E. M., Merckx, T., Riordan, P., Macdonald, D. W., & Thompson, D. J. (2010). The dragonfly delusion: Why it is essential to sample exuviae to avoid biased surveys. *Journal of Insect Conservation*, *14*(5), 523–533. https://doi.org/10.1007/s10841-010-9281-7

R Core Team. (2013). *R: A language and environment for statistical computing*.

Skovmand, O., & Becker, N. (2000). Bioassays of *Bacillus thuringiensis* subsp. *israelensis*. In *Bioassays of entomopathogenic microbes and nematodes* (pp. 41–47). CABI Pub.

Stehle, S., Manfrin, A., Feckler, A., Graf, T., Joschko, T. J., Jupke, J., Noss, C., Rösch, V., Schirmel, J., Schmidt, T., Zubrod, J. P., & Schulz, R. (2022). Structural and functional development of twelve newly established floodplain pond mesocosms. *Ecology and Evolution*, *12*, 8674. https://doi.org/10.1002/ece3.8674

Tachet, H., Richoux, P., Bournaud, M., & Usseglio-Polatera; Philippe. (2010). *Invertébrés d'eau douce: systématique, biologie, écologie* (Vol. 15). CNRS éditions.

Theissinger, K., Röder, N., Allgeier, S., Beermann, A. J., Brühl, C. A., Friedrich, A., Michiels, S., & Schwenk, K. (2019). Mosquito control actions affect chironomid diversity in temporary wetlands of the Upper Rhine Valley. *Molecular Ecology*, *28*(18), 4300–4316. https://doi.org/10.1111/mec.15214

Vallenduuk, H. J., & Moller Pillot, H. K. M. (2007). *Chironomidae Larvae: General Ecology and Tanypodinae* (Vol. 1). Brill.

van Buskirk, J. (1989). Density-dependent cannibalism in larval dragonflies. *Ecology*, *70*(5), 1442–1449.

van Buskirk, J. (1992). Competition, cannibalism, and size class dominance in a dragonfly. *Oikos*, 455–464.

Wickham, H., Chang, W., & Wickham, M. H. (2016). Package 'ggplot2.' *Create Elegant Data Visualisations Using the Grammar of Graphics. Version*, *2*(1), 1–189.

Wildermuth, H., & Martens, A. (2019). *Die Libellen Europas: alle Arten von den Azoren bis zum Ural im Porträt*. Quelle & Meyer.

Willigalla, C., Schlotmann, F., & Ott, J. (2022). *Rote Liste und Gesamtartenliste der Libellen in Rheinland-Pfalz*.

Wolfram, G., Wenzl, P., & Jerrentrup, H. (2018). A multi-year study following BACI design reveals no short-term impact of Bti on chironomids (Diptera) in a floodplain in Eastern Austria. *Environmental Monitoring and Assessment*, *190*(12). https://doi.org/10.1007/s10661-018-7084-6

Zuur, A. F., Ieno, E. N., Walker, N. J., Saveliev, A. A., & Smith, G. M. (2009). *Mixed effects models and extensions in ecology with R* (Vol. 574). Springer.

Supporting information for A-1

Table S1: Dates of flooding, Bti application and benthos and exuviae sampling in the first and second year of the experiment. Application dates were adjusted to weather conditions.

11 April 2020	Flooding, 30>40cm
12 April 2020	Flooding, 40>50cm
14 April 2020	**1st Bti application**
21 April 2020	Release water, 50>40cm
22 April 2020	Release water, 40>30cm
02 May 2020	Flooding, 30>40cm
03 May 2020	Flooding, 40>50cm
04 May 2020	**2nd Bti application**
12 May 2020	Release water, 50>40cm
13 May 2020	Release water, 40>30cm
23 May 2020	Flooding, 30>40cm
24 May 2020	Flooding, 40>50cm
25 May 2020	**3rd Bti application**
02 June 2020	Release water, 50>40cm
03 June 2020	Release water, 40>30cm
16-19 June 2020	**Benthos sampling**

11 April 2021	Flooding, 30>40cm
12 April 2021	Flooding, 40>50cm
13 April 2021	**1st Bti application**
21 April 2021	Release water, 50>40cm
22 April 2021	Release water, 40>30cm
02 May 2021	Flooding, 30>40cm
03 May 2021	Flooding, 40>50cm
08 May 2021	**2nd Bti application**
16 May 2021	Release water, 50>40cm
17 May 2021	Release water, 40>30cm
31 May 2021	Flooding, 30>40cm
01 June 2021	Flooding, 40>50cm
02 June 2021	**3rd Bti application**
10 June 2021	Release water, 50>40cm
11 June 2021	Release water, 40>30cm
11 June 2021	Exuviae sampling
15 June 2021	Exuviae sampling
01 July 2021	Exuviae sampling
15 July 2021	Exuviae sampling
23 July 2021	Exuviae sampling
30 July 2021	Exuviae sampling
12 August 2021	Exuviae sampling
20 August 2021	Exuviae sampling
26 August 2021	Exuviae sampling
03 September 2021	Exuviae sampling

Table S2: Number of individuals collected in macrophytes and gravel habitat and control (n=6) and Bti-treated FPMs (n=6), per sampled unit (A = 0.25 m^2).

			Macrophytes		Gravel	
Order	Family	Taxa	Control	Treatment	Control	Treatment
Diptera	Chironomidae	Chironomidae	184	59	88	70
		Chironominae	2007	1048	916	510
		Orthocladiinae	1085	401	91	41
		Tanypodinae	1566	1261	299	277
	Culicidae	Culicidae	43	0	0	0
	Ceratopogonidae	Ceratopogonidae	152	12	10	1
	Chaoboridae	Chaoboridae	40	23	84	71
	Rhagionidae	Rhagionidae	0	1	0	0
	Tabanidae	Tabanidae	5	3	1	0
Odonata	Aeshnidae	Aeshnidae	54	88	4	8
	Libellulidae	Libellulidae	523	310	47	35
	Coenagrionidae	Coenagrionidae	248	140	10	17
Ephemeroptera	Baetidae	Baetidae	391	462	290	320
	Caenidae	Caenidae	97	65	14	32
Megaloptera	Sialidae	Sialidae	38	13	11	1
Coleoptera	Haliplidae	Haliplidae	32	11	2	0
	Dytiscidae	Dytiscidae	12	12	0	0
	Elmidae	Elmidae	0	1	0	0
	Hydrophilidae	Hydrophilidae	0	1	3	6
Hemiptera	Pleidae	Pleidae	5	2	1	2
	Notonectidae	Notonectidae	16	8	3	2
Trichoptera	Leptoceridae	Leptoceridae	2	0	0	0
Total			**6500**	**3921**	**1874**	**1393**

Table S3: Permutation test of multivariate homogeneity of groups dispersions (variances) for treatment and habitat effects on the community composition. Statistically significant P values are printed in bold. Number of permutations = 999.

	DF	SS	MS	F Model	P-value
Groups	3	0.009	0.003	0.232	0.873
Residuals	20	0.265	0.013		

DF = degrees of freedom; SS = sums of squares; MS = mean squares

Appendix A-2: Submitted manuscript

Trophic effects of Bti-based mosquito control on two top predators in floodplain pond mesocosms

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Abstract

Chironomid (Diptera: Chironomidae) larvae play a key role in aquatic food webs as prey for predators like amphibian and dragonfly larvae. This trophic link may be disrupted by anthropogenic stressors such as *Bacillus thuringiensis* var*. israelensis* (Bti), a biocide widely used in mosquito control. In a companion study, we recorded a 41% reduction of non-target larval chironomids abundance in outdoor floodplain pond mesocosms (FPMs) treated with Bti. Therefore, we examined the diet of two top predators in the FPMs, larvae of the palmate newt (Salamandridae: *Lissotriton helveticus*) and dragonfly (Aeshnidae: predominantly *Anax imperator*), using bulk stable isotope analyses of carbon and nitrogen. Additionally, we determined neutral lipid fatty acids in newt larvae to assess diet-related effects on their physiological condition. We hypothesized a dietary shift of both predators towards prey that is not affected by Bti, such as conspecifics, mayflies or zooplankton. We did not find any effects of Bti on the diet proportions of newt larvae. Although not statistically significant, we observed ~30% lower content of omega-6 polyunsaturated fatty acids in newts from Bti-FPMs, pointing to an altered dietary quality and/or energy cost. In Bti-FPMs, Aeshnidae larvae consumed slightly less chironomids and mayflies and a higher proportion of predators (newts, damselflies and conspecifics) compared to controls likely due to a reduction of prey. Our findings point to bottom-up effects of Bti on aquatic food webs, but should be further evaluated, for instance, by using compound-specific stable isotope analyses of fatty acids or GC-metabarcoding approaches.

1 Introduction

Chironomids are a dipteran family of non-biting midges, whose larvae play a crucial role in food webs of aquatic ecosystems. Their high abundance in wetlands make them an important food source for aquatic and terrestrial predators (Armitage et al., 1995). Despite them being non-target organisms, the use of the mosquito control biocide *Bacillus thuringiensis* var*. israelensis* (Bti) in wetlands has been shown to reduce the abundance of aquatic chironomid larvae (Allgeier et al., 2019a; Gerstle et al., 2023). Bti is a naturally occurring soil bacterium that produces crystal proteins toxic to larvae of the dipteran suborder Nematocera, which includes families of biting Culicidae, Simuliidae, and Ceratopogonidae, as well as non-biting Chironomidae. Once ingested by the organism, the crystal proteins bind to receptors in the midgut, causing cell lysis and ultimately the death of the larvae (Becker, 1997; Ben-Dov, 2014).

Due to its specific mode of action, direct lethal effects of Bti on non-target organisms, apart from chironomids, are unlikely (as reviewed by Boisvert & Boisvert, 2000, and Brühl et al., 2020), which leads to a labelling of Bti as an environmentally friendly biocide. However, the impact of Bti on non-target chironomids can potentially shift food webs, inducing bottom-up effects on aquatic predators (Allgeier et al., 2019b; Gerstle et al., 2023). In an outdoor mesocosm experiment involving macroinvertebrate communities and two predators, namely dragonfly (Odonata: Aeshnidae) and newt (Urodela: Salamandridae) larvae, stable isotope analyses of carbon (C) and nitrogen (N) revealed that chironomids were the preferred food source of newts, constituting over 56% of their diet (Allgeier et al., 2019b). Additionally, mortality of newts was 27% higher in mesocosms treated with Bti, suggesting increased intraguild predation (competition and predation of species with similar trophic niches; Holt & Polis, 1997). Similarly, a recent study by Gerstle et al. (2023) reported a 41% abundance reduction of chironomid larvae in Bti-treated floodplain pond mesocosms (FPMs), as well as a 54% reduced emergence of the Odonata family Libellulidae. These findings further strengthened the hypothesis of intraguild predation in dragonflies due to decreased chironomid availability. In general, predator coexistence strongly depends on prey density (Preston et al., 2018; Takatsu, 2022). Therefore, changes in their diet are of considerable interest when assessing food webs.

A different effect pathway of Bti for the aquatic food web is by altering soil microbial communities (as reviewed in Belousova et al., 2021). These alterations can affect detritus-processing organisms, such as protozoans and nematodes (McKie et al., 2023). McKie et al. (2023) used stable isotope analysis of C and N and suggested an increased food chain length in Bti-treated floodplains due to potential feeding by protozoans on decaying mosquito larvae. These findings further highlight the complexity of food webs and consequently the need for field experiments with natural communities but yet in a replicated comparable test design.

To address this, we investigated the diets of two top predators, larval palmate newts (*Lissotriton helveticus*) and Aeshnidae (mostly *Anax imperator*), in twelve outdoor FPMs, half of them exposed three times to the maximum field rate of Bti between April and May 2020. We used stable isotope analyses of C and N to understand how the diet of the two top predators responds to a reduction of larval chironomid availability as prey. Due to the 41% reduction of chironomid larvae in Bti-treated FPMs recorded by Gerstle et al. (2023) in the same experiment, we hypothesized that larval newts and Aeshnidae switch to other prey than chironomids, such as mayflies, zooplankton or other predators and smaller conspecifics (intraguild predation). Therefore, we hypothesized a higher trophic position of newts and Aeshnidae in Bti-FPMs. We also hypothesized a larger niche size in Bti-FPMs because of a more generalist feeding activity and consumption of alternative prey. To obtain information on physiological consequences of an altered diet, we included the analyses of neutral lipid fatty acids (NLFAs) in newt larvae as vertebrate top predators. NLFAs are mostly utilized as storage lipids in tissues (Turkish & Sturley, 2009), which gives further insight in the utilization of the ingested prey and the newts' diet, i.e., retention of fatty acids (FAs) (Kainz et al., 2004; Twining et al., 2016, 2021) and, in turn, also the nutritional value for aquatic and terrestrial higher-level carnivores, e.g. birds, large ground beetles, predatory fish.

2 Material and methods

2.1 Study site

The experiment was conducted in twelve FPMs at the Eußerthal Ecosystem Research Station (49°15′14″N, 7°57′42″E; RPTU Kaiserslautern-Landau) in the Palatinate Forest in Southwest Germany. The FPMs were constructed in 2017 and were allowed for natural colonization by organisms from nearby freshwater habitats (for details see Stehle et al., 2022). Each FPM (23.5 m × 7.5 m) is equipped with an inlet connected to the adjacent small stream Sulzbach and an adjustable outlet, with which the water depth can be regulated. In its initial state, the water level is 30 cm at the deepest point and gradually fades into a shallow floodplain area as well as a terrestrial shore at one side. The vegetation in the aquatic part predominantly consists of waterweeds, coontails and filamentous green algae, while the shores are mostly covered by emergent plants such as bulrushes and soft rush.

The FPMs are a breeding habitat for various amphibians, predominantly European common frogs (*Rana temporaria*), European common toads (*Bufo bufo*), and palmate newts (*Lissotriton helveticus*). Since temporarily high densities of amphibian larvae strongly influence aquatic food web structures, we aimed for an even distribution of amphibian larvae across the twelve FPMs. Therefore, we installed an amphibian fence around the FPMs to control immigration of adult frogs and toads and placed bottle traps inside the FPMs to capture adult newts (Griffiths, 1985; for more information see Gerstle et al., 2023). To ensure that amphibian larvae are not completely excluded from the food web, we introduced two to three toad egg strings, five frog egg clutches of similar weight and 20 adult palmate newts (ten male and ten female) to each FPM one week prior to the start of the experiment.

2.2 Bti application and flooding

In floodplains of the Upper Rhine Valley in southwest Germany, inundated wetlands are treated multiple times per year on a large-scale to control nuisance by mass emergence of the floodwater mosquito (*Aedes vexans*; Becker, 2003). Since inundation triggers the hatching of the larvae, Bti applications in these wetlands are linked to temporary floodings. To mimic a realistic Bti application scenario, we flooded the FPMs three times by increasing the water level from 30 cm to 50 cm from mid-April to the end of May 2020. On the third day of flooding (for dates see Table S 1), we applied the maximum field rate of VectoBac WDG (2.88×10^9 ITU/ha; Valent BioSciences, Illinois, USA) to every other FPM using a knapsack sprayer (Prima 5, Gloria, Germany). The maximum field rate of Bti is applied when the water is deeper than 10 cm and/or late instar mosquito larvae are targeted (Becker, 1997).

As there is currently no analytical method available to quantify sterilized Bti in the environment, we implemented a biotest using mosquito larvae (*Culex sp.*) to validate the efficacy of the Bti treatment (i.e., at least 90% mortality of mosquito larvae within a week; for more information on the biotest see Gerstle et al., 2023).

2.3 Sampling

For stable isotope analyses, we sampled newt larvae (*L. helveticus*) and Aeshnidae larvae (predominantly *A. imperator*) as well as their potential resources three weeks after the last Bti application (13-16 June 2020, Table S 1). Resources included larval Libellulidae, chironomids, mayflies and zooplankton. Depending on the number of organisms present in the FPM (for sample size see Table S 2), we aimed to sample ten individuals of similar sizes for each consumer and five subsamples of each resource per FPM (total of 10 FPMs used for statistical analyses due to limited availability of consumers; Table S 2). Sampling of similar sizes of consumers was done to reduce possible effects of size-selective prey selection for diet analyses and did not represent the mean body size of consumers in the FPMs. To collect newt larvae and macroinvertebrates, we used a 500-µm-mesh dip net. Zooplankton was sampled by dragging a 55-µm-mesh dip net through the water column of the entire FPM. We applied a non-quantitatively sampling until all relevant resources were collected. All organisms were kept in filtered pond water overnight (mesh size: 55 µm) to allow the release of gut contents.

Thereafter, samples were rinsed with distilled water and shock-frozen in liquid nitrogen. All samples were stored at -20 °C until further processing.

For FA analyses, we collected, if possible, five *L. helveticus* larvae from four FPMs using a dip-net (two control and two Bti-treated FPMs). We sampled larvae of similar sizes and developmental stages from four instead of 12 FPMs, since we were not able to sample enough larvae of comparable body size in each FPM to allow for statistical investigation. Sampling was not done This procedure was further motivated by, firstly, newts' diet-dependency on their body size (size-selective predation) and, secondly, energy reserves changing during development and metamorphosis (Crump, 1981; Pfab et al., 2020). Sampling of larvae for FA analyses was conducted simultaneously to sampling of larvae for stable isotope analyses and samples were treated as described above, but stored at -80 °C.

2.4 Stable isotope analysis

We freeze-dried all samples until complete dryness (at least 48 hours). For consumers, we dissected the body part with the highest content of muscle tissue, namely the tail for newt larvae and the thorax for Aeshnidae (Allgeier et al., 2019b; Seifert & Scheu, 2012). Dried samples were homogenized in 1.5-mL Eppendorf tubes using metal beads and a tissue-lyzer (Retsch, MM 301, Germany). For each sample, a dry mass of 0.6 ± 0.1 mg homogenized sample was packed into a tin capsule (5 × 9 mm, IVA, Meerbusch, Germany) using an ultrafine balance (precision: 0.1 µg; Sartorius, Germany). We pooled several individuals to one sample if an individual organism did not yield enough biomass (i.e., chironomids, mayflies and zooplankton). Stable isotope ratios of C and N were determined using an elemental analyzer (EA, Flash 2000 HT, Thermo Scientific, Bremen, Germany) coupled to an isotope ratio mass spectrometer (IRMS, Delta V Advantage, Thermo Scientific, Bremen, Germany). The values are reported in delta notation:

$$\delta X = \left(\frac{R_{sample}}{R_{standard}} - 1\right) * 1000\ ‰ \qquad \text{[Eq. 1]}$$

with δX being $\delta^{13}C$ or $\delta^{15}N$, and R_{sample} and $R_{standard}$ the abundance ratios of the heavy to the light isotope of the sample and the international standard (i.e., Vienna Pee Dee Belemnite for C and atmospheric air for N, respectively). As a working standard, casein was measured in duplicate every ten samples with a precision of <0.06‰.

2.4 NLFAs analyses

We examined whole-body neutral lipid fatty acid (NLFA) contents of newt larvae from control-FPMs (n=2; 7 larvae in total) and Bti-FPMs (n=2; 10 larvae in total). Samples were freeze-dried for 48 h and weighed (13.49 ± 4.80 mg (mean ± SD); Fig. S2). As described in Konschak et al. (2020) and Pietz et al. (2023), we extracted NLFAs from whole-body samples (manually crushed using glass Pasteur pipettes) using a chloroform:methanol:Milli-Q water mixture (1:2:0.8; v:v:v). In a second step, chloroform and Milli-Q water were added to obtain a final mixture of 2:2:1.8 (v:v:v). As an internal standard, we added a triacylglycerol with 3 deuterated 18:0 FAs (Tristearin-D105; Larodan). Lipids were extracted from the samples by overnight storage at 4 °C. The following day, neutral lipids were separated from phospholipids and glycolipids via solid-phase extraction by eluting in 4 mL of chloroform and pre-conditioned (4 mL of chloroform) polar modified polystyrene/divinylbenzene copolymer cartridges (Chromabond® easy polypropylene columns; Machery-Nagel). Samples were dried at 40 °C under a stream of nitrogen gas and afterwards re-dissolved in 100 µL of dichloromethane. To hydrolyze lipids and methylate fatty acids to fatty acid methyl esters (FAMEs), we added trimethylsulfonium hydroxide (Sigma-Aldrich).

FAMEs were analyzed using gas chromatography with flame-ionization detection (GC-FID; Trace GC Ultra; Thermo Fisher Scientific) and a Restek FAMEWAX capillary GC column (30 m x 0.25 mm x 0.25 µm film thickness), and helium (1.4 mL/min) as carrier gas. Based on different retention times, FAMEs were determined using FAME standards (37-component FAME Mix; Supelco CRM47885) and quantified as µg FA per mL using an external standard calibration. The FAME of the internal standard (Methyl D-35 Octadecanoate; Larodan) was added directly to the standards of the calibration series in the same concentration as the lipid internal standard (Tristearin-D105) in the samples. Using the recovery rate of the internal standard and blank correction, concentrations of FAs were adjusted for inaccuracies of the instrument. To obtain FA concentration in µg FA per mg dry weight, we normalized the corrected FA concentration by the total volume and dry weight of the samples.

2.5 Statistical analyses

Stable isotope analyses

To test for a possible influence of the body size on the isotopic composition of the consumers, differences in body mass of Aeshnidae and newts were investigated using generalized linear mixed models (GLMM) from the package “lme4” (version 1.1.27.1, Bates et al., 2014) for R (version 4.1.1, R Core Team, 2013). Prior to GLMM, we checked for normality and homoscedasticity using visual inspection of quantile-quantile plots and Levene's test ("car" package, version 3.0-11; Fox, 2015), respectively. We then applied Bti treatment as a fixed effect to the GLMM and FPM identity as random effect to account for pseudo-replication within the FPMs. Post-hoc comparisons were conducted using the *emmeans* (estimated marginal means) function from the package "emmeans" (version 1.7.0; Lenth (2016) for R.

Before analyses of diet proportions, we calculated the trophic levels (TL) for Aeshnidae and newts [Equation 2]:

$$TL_{consumer} = TL_{base} + \frac{d^{15}N_{consumer} - mean\ (d^{15}N_{base})}{TEF_{d15N}} \qquad \text{[Eq. 2]}$$

with a primary consumer, i.e., zooplankton as baseline organism (TL = 2) and consumer-specific trophic enrichment factors (TEF). For newt-specific TEFs, we used 0.1 ± 0.4‰ for $\delta^{13}C$ and 2.3 ± 0.4‰ for $\delta^{15}N$ (Cloyed et al., 2015) and for Aeshnidae, we used 0.8 ± 0.2‰ and 0.9 ± 0.2‰ (Brauns et al., 2018). Since there are no third level carnivorous consumers (TL 5) in the FPM system, we removed samples with TL higher than 4, as this may be the result of a methodological artefact (6 newts removed) and would most likely confound diet analyses.

We grouped sources by k-means clustering with the optimal number of clusters being determined based on within sum of squares and the structure of the polygon. The reduction of number of sources (i.e., clusters) in the model (Phillips et al., 2014) lowers its underdetermination. The diet proportions of newt and Aeshnidae larvae were determined using Bayesian mixing models for each FPM (chain length = 50,000; burn-in = 5,000; thinning = 100; chains = 4; resid_err = TRUE; process_err = TRUE) using the package "MixSIAR" (version 3.1.12; Stock et al., 2018) for R. We applied the above-mentioned consumer-specific TEFs to the resources to account for trophic enrichment, that is systematical increase in stable isotopes throughout the food web. To estimate the relative niche sizes (i.e., the resources an organism can utilize) of newt and Aeshnidae larvae, we calculated Bayesian standard ellipse areas (SEA) using the package "SIBER" (version 2.1.6; Jackson et al., 2011) for R and normalized to the SEA of resources to allow comparison between FPMs. We determined the trophic position of newts and Aeshnidae in the food web using the package "tRophicPosition" (version 0.8.0; Quezada-Romegialli et al., 2018) for R and the same TEFs applied before [see Equation 2].

Fatty acid analyses

We grouped single FAs into FA groups of saturated FAs (SFA), monounsaturated FA (MUFA), and polyunsaturated FA (PUFA), and physiologically relevant omega-3 and omega-6 PUFAs (for grouping see Table 1). We removed one outlier (one newt larvae from control; above the 1.5 interquartile range) and checked data for normality and homogeneity of variance using visual inspection (quantile-quantile plot) and Levene's test, respectively. We performed a linear mixed effect model (LME; package "nlme" version 3.1.152; Pinheiro et al., 2017) with Bti treatment as fixed effect and FPM identity as random effect, followed by an analysis of variance (ANOVA).

All data visualizations were conducted in R (version 4.1.1) using the packages "ggplot2" (version 3.3.5; Wickham et al., 2016) and "ggpubr" (version 0.4.0; Kassambra, 2020).

3 Results

3.1 Stable isotopes

The body mass of consumers was similar among control and Bti-treated FPMs (Figure S1), minimizing potential effects of size-related prey selection (GLMM for newts: degrees of freedom (df) = 7.99, p = 0.190; Aeshnidae: df = 8.94, p = 0.736).

We determined the following resource clusters used for stable isotope mixing models: Aeshnidae/newt/damselfly, chironomid/mayfly, and Libellulidae/zooplankton. Structures of stable isotopes of the resource clusters were comparable between control and Bti-treated FPMs (Figure 1a, b) with similar areas of resource polygons (Bayesian median (95% equal tail credible interval): $SEA_{Control}$ = 3.80 ‰^{2} (3.09-4.47 ‰^{2}) and SEA_{Bti} = 3.50 ‰^{2} (2.96-5.17 ‰^{2})). In chironomid larvae, we observed approximately 1‰ higher $\delta^{15}N$ in Bti-treated FPMs compared to control FPMs. According to the mixing model estimates, the diet of newts was dominated by Libellulidae larvae and zooplankton (in both treatments approximately 65%; for values see Table S 3; Figure 1c). The two other resource clusters Aeshnidae/newt/damselfly and chironomid/mayfly contributed smaller but similar proportions to the diet of newt larvae in control and Bti-FPMs (Figure 1c). In contrast, the diet of Aeshnidae was balanced between the three resource clusters in control FPMs (~33% each; Figure 1d). In Bti-FPMs, diet of Aeshnidae consisted mostly of Aeshnidae/newt/damselfly (~42%), and similar parts of chironomid/mayfly and Libellulidae/zooplankton. The relative niche size calculated by the SEA of the consumer relative to the SEA of the sources, was both for control and Bti-treated FPMs similar for newts ($SEA_{Control}$ = 0.301 (0.184-0.562), SEA_{Bti} =

0.393 (0.244-0.711); Figure 1e) and for Aeshnidae ($SEA_{Control}$ = 0.198 (0.136-0.321); SEA_{Bti} = 0.204 (0.136-0.341); Figure 1g). The same pattern was observed for the trophic level, where there was no difference between control and Bti-treated FPMs for newts ($TP_{Control}$ = 3.0 (2.8-3.2); TP_{Bti} = 2.8 (2.7-2.9; Figure 1f) and for Aeshnidae ($TP_{Control}$ = 3.9 (3.5-4.4); TP_{Bti} = 3.8 (2.9-5.1; Figure 1h). Independent of the treatment, Aeshnidae were approximately one trophic level higher than newts.

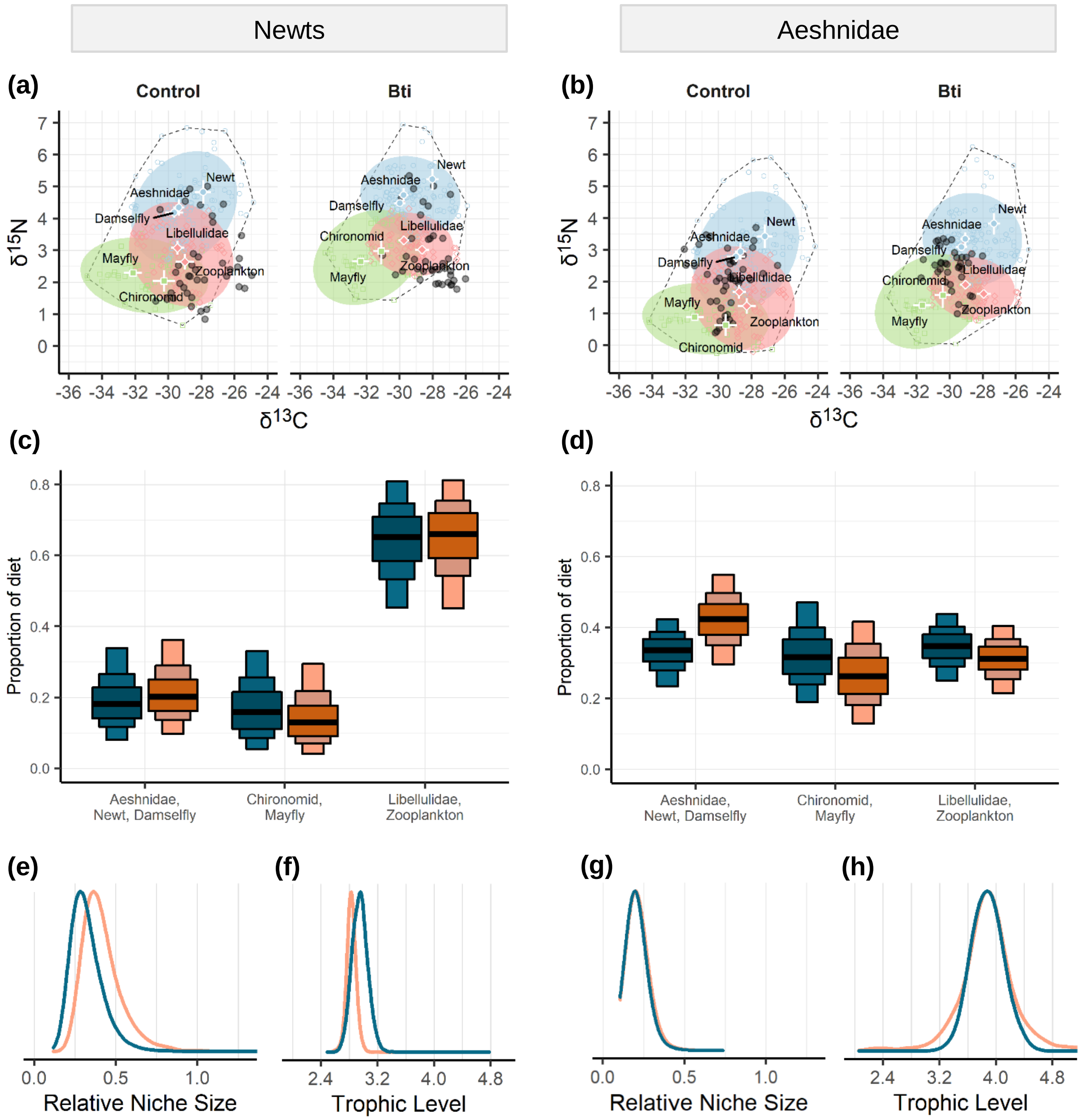

Figure 12: Stable isotope ratios of C and N of newt (a) and Aeshnidae larvae (b) as consumers shown as black dots, means ± SD, and 95 %-confidence ellipses of prey organisms (adjusted for trophic enrichment) for each cluster: Chironomid and mayfly larvae (green squares and ellipse), Aeshnidae, newt and damselfly larvae (blue circles and ellipse), Libellulidae larvae and Zooplankton (red diamonds and ellipse). Dotted polygon delineates outer borders of resource polygon. Proportion of diet of newt larvae (c) and Aeshnidae larvae (d) for each prey cluster, where black lines indicate the medians, and darkest to lightest color of boxes show Bayesian 50%, 75% and 95% equal tail credible intervals, respectively. Niche sizes of newts (e) and Aeshnidae (g) determined from consumer SEA relative to FPM-specific resource SEA, and the trophic position of newts (f) and Aeshnidae (h) in the food web, in control (dark blue) and Bti-treated (orange) FPMs (n = 5).

3.2 Fatty acids

Neither the body mass ($t_{15.593}$ = -0.846, p = 0.4102) nor the FA content ($F_{1,2}$ = 0.4832, p = 0.559) differed significantly between newts from control and Bti-treated FPMs (Figure S 2).

We determined a total of 15 different FAs in newt larvae (Table 1). The two most abundant FAs were the saturated FAs palmitic acid (16:0) and octadecanoic acid (18:0). Newts from control-FPMs showed approximately 10% higher mean content of total NLFA than Bti-treated newts (Table 1). Although not significant, mean PUFA content was approximately 20% lower in newts from Bti-FPMs compared to controls. This was mainly driven by omega-6 PUFA (Table 1).

Table 1: Mean (± 95% confidence interval; CI) NLFA contents (µg per mg dry weight) per fatty acid (FA) and FA group[a] in newt larvae from control (n=7) and Bti-treated (n=10) FPMs, and results from ANOVA for FA groups.

	Control		**Bti**		Percent difference in Bti-FPMs compared to control	$F_{1,2}$	p
Fatty acids [a]	Mean	95% CI	Mean	95% CI			
Σ SFA	3.249	0.730	3.010	0.441	-7.94	0.6910	0.493
16:0	1.851	0.401	1.779	0.301	-4.09		
18:0	1.366	0.365	1.207	0.223	-13.14		
22:0	0.032	0.007	0.024	0.007	-31.97		
Σ MUFA	0.670	0.401	0.704	0.311	4.85	0.025	0.888
16:1n-7	0.258	0.240	0.284	0.175	9.09		
18:1	0.387	0.263	0.401	0.146	3.54		
22:1n-9	0.025	0.006	0.019	0.004	-30.63		
Σ PUFA	1.832	0.605	1.526	0.410	-20.03	1.046	0.414
omega-3 PUFA	0.935	0.344	0.822	0.226	-13.75	0.4559	0.569
18:3n-3 (ALA)	0.407	0.209	0.347	0.142	-17.38		
20:3n-3	0.035	0.019	0.036	0.017	3.18		
22:5n-3 (EPA)	0.436	0.155	0.383	0.098	-13.83		
22:6n-3 (DHA)	0.092	0.048	0.092	0.043	0.14		
omega-6 PUFA	0.767	0.225	0.576	0.162	-33.16	2.134	0.282
18:2n-6 (LIN)	0.258	0.071	0.192	0.087	-34.41		
20:2n-6	0.041	0.032	0.047	0.019	12.54		
20:3n-6	0.044	0.025	0.039	0.016	-14.65		
20:4n-6 (ARA)	0.510	0.170	0.384	0.091	-32.72		
22:2n-6	0.009	0.008	0.006	0.006	-35.52		
Σ Total NLFA	5.751	1.460	5.240	1.022	-9.75	0.4832	0.559

[a] Sum (Σ) of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), omega-3 and omega-6 PUFAs, and total neutral lipid fatty acids (Total NLFAs). Physiologically relevant PUFAs eicosapentaeonic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid (ARA), and their precursors linoleic acid (LIN), and alpha-linolenic acid (ALA).

4 Discussion

4.1 Effects on newts

Contrary to our hypothesis, the results of stable isotope mixing models showed that the Bti treatment had no effect on the diet of newt larvae, despite the observed 41% reduction in chironomid abundance in Bti-FPMs (Gerstle et al., 2023). We hypothesized a diet shift of newts from chironomids towards prey not affected by Bti, such as mayflies (Gerstle et al., 2023; Kolbenschlag et al., 2023a) or zooplankton. Since a clear separation of the isotopic signature of chironomids from other sources was not possible, chironomids and mayflies were grouped into one cluster based on the structure of the resource polygon. Hence, there is the possibility that slight diet shifts between the sources chironomids and mayflies could not be detected (Figure 1a, c). Allgeier et al. (2019b) reported that in a mesocosm experiment, chironomid larvae were the preferred food source for palmate newt larvae over zooplankton, freshwater molluscs and *Asellus aquaticus*. Despite them recording a reduced abundance of chironomids (53-87%) in Bti-mesocosms compared to controls, these authors also did not observe any differences in estimated diet proportions using stable isotope analyses of C and N. These observations are in accordance with our results, showing that the effect of Bti on the abundance of chironomids (Gerstle et al., 2023) was not reflected in the estimated diet proportions. Interestingly, we determined a higher $\delta^{15}N$ in larval chironomids from Bti-treated FPMs compared to controls. A similar pattern of higher $\delta^{15}N$ in emerging chironomids from Bti-treated FPMs observed in the companion study by Kolbenschlag et al. (2023b). As found by Gerstle et al. (2023), the chironomid subfamily Tanypodinae, which includes predatory species, was less affected by Bti treatment than the predominantly detritivorous Chironominae and Orthocladiinae. This would explain the higher $\delta^{15}N$ in chironomids from Bti-FPMs since heavier ^{15}N isotopes enrich with every trophic level. However, recent results by Röder et al. (2024), who investigated phylogenetic affiliations and ecological traits of chironomid species emerged from the FPMs in the same year of our study, suggests that feeding type only plays a minor role in the sensitivity towards Bti. Therefore, another possible explanation for the $\delta^{15}N$ enriched chironomid larvae in Bti-FPMs, not related to the feeding strategy, is that due to increased organic matter from dead chironomids an additional level at the bottom of the food web is introduced (by an increase in $\delta^{15}N$ in detritus), similar to observations by McKie et al. (2023).

Approximately 15% and 30% of newt larvae in control and Bti-treated FPMs, respectively, were outside of the resource polygon (Figure 1a) which indicates that newts could have fed on sources high in δ^{13}C and low in δ^{15}N. This could be, for instance, periphyton-feeders such as oligochaete worms (Tubificina) or molluscs, which we could not include in our modelling due to the low and uneven sample sizes between FPMs. Additionally, we used TEF values from literature (i.e., skin tissue of adult frogs; Cloyed et al., 2015), whose application has been reviewed in Stephens et al. (2023). These authors pointed out that TEFs are strongly diet- and tissue-specific and there is still a lack of TEFs determined specifically for amphibians and insects. To the best of our knowledge, there is no TEF value that was specifically determined for newt larvae or muscle tissue of amphibians. Therefore, it is possible that the actual newt TEFs deviate from the TEFs applied in our study which would change the position of newts in the resource polygon (Figure 1a). Although these shortcomings can affect the precision of dietary estimates, relative differences between control and Bti-FPMs can theoretically still be detected. Since both the dietary niche sizes calculated by the SEA (consumer SEA relative to source SEA; Figure 1e) as well as the trophic level (Figure 1f) did not notably differ, our results suggest no differences in the diet of newts from control and Bti-FPMs according to stable isotope analyses.

Despite no detected differences in newt's diet using stable isotope analyses, FA analyses revealed lower content of total NLFAs (~10%) in newts from Bti-treated FPMs compared to controls. Although not significant, we observed the highest difference in omega-6 PUFAs with approximately 30% lower content in larvae from Bti-FPMs (Table 1). This finding suggests that there may be slight differences in newts' diets that have not been detected by stable isotope analyses and are more likely based on differences in the quality of the consumed prey. FA composition strongly depends on the nutritional quality of the consumers' diet (e.g., Pietz et al., 2023), except for a few exceptions in which consumers have the capacity to modify dietary FA from precursors (Twining et al., 2016, 2021). The observed lower content of total NLFAs and PUFAs in Bti-treated newts may suggest that when exposed to Bti, newts either consumed prey with lower PUFA content and/or may have experienced a higher energy cost and thus increased usage of storage lipids (i.e., NLFAs). In Bti-treated FPMs, the reduced chironomid availability might have also increased the predatory avoidance (Pérez-Tris et al., 2004), e.g., escaping from Aeshnidae, for which the reduction of chironomids likely caused an increased intake of newts in their diet (see section 4.2).

4.2 Effects on Aeshnidae

Aeshnidae consumed a higher proportion of large prey items, like Aeshnidae, newts or damselflies in Bti-FPMs compared to control-FPMs. As hypothesized, the decreased chironomid abundance has led to increased intraguild predation and/or cannibalism (Gerstle et al., 2023). The more even proportions of different resources in the diet of Aeshnidae (Figure 1d) suggests a more divers feeding behavior than newts, however, the analysis of the relative niche size (Figure 1g) points to a more specialized feeding of Aeshnidae and no effect of Bti on the niche size. Compared to newts, Aeshnidae are approximately one trophic level higher (Figure 1h), which could also be influenced by the application of different consumer-specific TEFs, as discussed above. Still, Aeshnidae are considered top predators in fishless ponds, while newts and salamander larvae are intermediate predators, likely also due to the smaller body size compared to Aeshnidae (Stemp et al., 2021; Wilbur, 1997). Depending on the larval stage and body size of consumers, Aeshnidae function both as predator and prey (Wilbur, 1997) and are also known to be cannibalistic under limited prey availability (Vaissi & Sharifi, 2016; Van Buskirk, 1989), possibly leading to increased intraguild predation and a higher trophic level in the food web compared to other predators like newts. Results from Allgeier et al. (2019b), who recorded 27% lower newt survival in mesocosms exposed to Bti, suggesting intraguild predation of newts by Aeshnidae. Also, in a companion study, Gerstle et al. (2023) observed a reduced emergence of Libellulidae and damselflies from Bti-treated FPMs, but no difference in Aeshnidae. This result would be compatible with a diet shift of Aeshnidae in Bti-treated FPMs away from chironomids and towards other prey, for instance, newt larvae.

5 Conclusion

The results of this study suggest potential bottom-up shifts in aquatic food webs induced by Bti. However, our observations demonstrate the challenges of solely using bulk stable isotope analyses to study aquatic systems and consequently suggest the complementary use with compound-specific stable isotope analysis of fatty acids (Twining et al., 2020) and gut content metabarcoding investigations (Sheppard et al., 2005). This would allow for a more sensitive assessment of predators' diets, trophic interactions and energy flow. In our study, the extension by fatty acid analyses for newt larvae pointed to differences in their diet and/or energy budget. Despite being not statistically significant, the observed effect sizes may potentially be relevant for energy and nutrient transfer along the food web within and across aquatic-terrestrial ecosystem boundaries.

6 Declarations

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.

All experimental procedures in our study were evaluated and approved by the Institutional Animal Care and Use Committee at the University of Koblenz-Landau and the federal investigation office (Landesuntersuchungsamt – LUA Rhineland-Palatinate, Germany, NTP-ID:00035820-2-7). We thank the Struktur- und Genehmigungsdirektion Süd (SGD) in Neustadt, Germany, for sampling permissions.

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8 References

Allgeier, S., Kästel, A., & Brühl, C. A. (2019a). Adverse effects of mosquito control using *Bacillus thuringiensis* var. *israelensis*: Reduced chironomid abundances in mesocosm, semi-field and field studies. *Ecotoxicology and Environmental Safety*, *169*, 786–796. https://doi.org/10.1016/j.ecoenv.2018.11.050

Allgeier, S., Friedrich, A., & Brühl, C. A. (2019b). Mosquito control based on *Bacillus thuringiensis israelensis* (Bti) interrupts artificial wetland food chains. *Science of the Total Environment*, *686*, 1173–1184. https://doi.org/10.1016/j.scitotenv.2019.05.358

Armitage, P. D., Pinder, L. C., & Cranston, P. S. (1995). The Chironomidae: biology and ecology of non-biting midges. In *The Chironomidae - The biology and ecology of non-biting midges*. Springer Science & Business Media.

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2014). Fitting linear mixed-effects models using lme4. *ArXiv Preprint ArXiv:1406.5823*.

Becker, N. (1997). Microbial Control of Mosquitoes: Management of the Upper Rhine Mosquito Population as a Model Programme. *Parasitology Today*, *13*(12), 485–487. https://doi.org/https://doi.org/10.1016/S0169-4758(97)01154-X

Becker, N. (2003). Ice granules containing endotoxins of microbial agents for the control of mosquito larvae - a new application technique. *Journal of the American Mosquito Control Association*, *19*(l), 63–66.

Belousova, M. E., Malovichko, Y. v., Shikov, A. E., Nizhnikov, A. A., & Antonets, K. S. (2021). Dissecting the environmental consequences of *Bacillus thuringiensis* application for natural ecosystems. In *Toxins* (Vol. 13, Issue 5). MDPI AG. https://doi.org/10.3390/toxins13050355

Ben-Dov, E. (2014). *Bacillus thuringiensis* subsp. *israelensis* and its dipteran-specific toxins. *Toxins*, *6*(4), 1222–1243. https://doi.org/10.3390/toxins6041222

Boisvert, M., & Boisvert, J. (2000). Effects of *Bacillus thuringiensis* var. *israelensis* on target and nontarget organisms: A review of laboratory and field experiments. In *Biocontrol Science and Technology* (Vol. 10, Issue 5, pp. 517–561). Carfax Publishing Company. https://doi.org/10.1080/095831500750016361

Brauns, M., Boëchat, I. G., de Carvalho, A. P. C., Graeber, D., Gücker, B., Mehner, T., & von Schiller, D. (2018). Consumer-resource stoichiometry as a predictor of trophic discrimination (Δ13C, Δ15N) in aquatic invertebrates. *Freshwater Biology*, *63*(10), 1240–1249. https://doi.org/10.1111/fwb.13129

Brühl, C. A., Després, L., Frör, O., Patil, C. D., Poulin, B., Tetreau, G., & Allgeier, S. (2020). Environmental and socioeconomic effects of mosquito control in Europe using the biocide *Bacillus thuringiensis* subsp. *israelensis* (Bti). In *Science of the Total Environment* (Vol. 724). Elsevier B.V. https://doi.org/10.1016/j.scitotenv.2020.137800

Cloyed, C. S., Newsome, S. D., & Eason, P. K. (2015). Trophic discrimination factors and incorporation rates of carbon- and nitrogen-stable isotopes in adult green frogs, lithobates clamitans. *Physiological and Biochemical Zoology*, *88*(5), 576–585. https://doi.org/10.1086/682576

Crump, M. L. (1981). Energy accumulation and amphibian metamorphosis. *Oecologia*, *49*, 167–169.

Fox, J. (2015). *Applied regression analysis and generalized linear models*. Sage publications.

Gerstle, V., Manfrin, A., Kolbenschlag, S., Gerken, M., Islam, A. S. M. M. U., Entling, M. H., Bundschuh, M., & Brühl, C. A. (2023). Benthic macroinvertebrate community shifts based on Bti-induced chironomid reduction also decrease Odonata emergence. *Environmental Pollution*, *316*. https://doi.org/10.1016/j.envpol.2022.120488

Griffiths, R. A. (1985). A simple funnel trap for studying newt populations and an evaluation of trap behaviour in smooth and palmate newts, *Triturus vulgaris* and *T. helveticus*. *Herpetological Journal*, *1*, 5–10.

Holt, R. D., & Polis, G. A. (1997). A theoretical framework for intraguild predation. *The American Naturalist*, *149*(4), 745–764.

Jackson, A. L., Inger, R., Parnell, A. C., & Bearhop, S. (2011). Comparing isotopic niche widths among and within communities: SIBER–Stable Isotope Bayesian Ellipses in R. *Journal of Animal Ecology*, *80*(3), 595–602.

Kainz, M., Arts, M. T., & Mazumder, A. (2004). Essential fatty acids in the planktonic food web and their ecological role for higher trophic levels. In *Limnol. Oceanogr* (Vol. 49, Issue 5).

Kassambra, A. (2020). *ggpubr:'ggplot2'Based Publication Ready Plots. R package version 0.3. 0*.

Kolbenschlag, S., Gerstle, V., Eberhardt, J., Bollinger, E., Schulz, R., Brühl, C. A., & Bundschuh, M. (2023a). A temporal perspective of aquatic subsidy: Bti affects the emergence of Chironomidae. *Ecotoxicology and Environmental Safety, 250,* 114503.

Kolbenschlag, S., Bollinger, E., Gerstle, V., Brühl, C. A., Entling, M. H., Schulz, R., & Bundschuh, M. (2023b). *Impact across ecosystem boundaries – does Bti application change quality and composition of the diet of riparian spiders?* https://doi.org/https://doi.org/10.1016/j.scitotenv.2023.162351

Konschak, M., Zubrod, J. P., Baudy, P., Fink, P., Kenngott, K., Lüderwald, S., Englert, K., Jusi, C., Schulz, R., & Bundschuh, M. (2020). The importance of diet-related effects of the antibiotic ciprofloxacin on the leaf-shredding invertebrate Gammarus fossarum (Crustacea; Amphipoda). *Aquatic Toxicology*, *222*, 105461.

Lenth, R. V. (2016). Least-squares means: the R package lsmeans. *Journal of Statistical Software*, *69*, 1–33.

McKie, B. G., Taylor, A., Nilsson, T., Frainer, A., & Goedkoop, W. (2023). Ecological effects of mosquito control with Bti: evidence for shifts in the trophic structure of soil- and ground-based food webs. *Aquatic Sciences*, *85*(2). https://doi.org/10.1007/s00027-023-00944-0

Pérez-Tris, J., Díaz, J. A., & Tellería, J. L. (2004). Loss of body mass under predation risk: cost of antipredatory behaviour or adaptive fit-for-escape? *Animal Behaviour*, *67*(3), 511–521.

Pfab, F., DiRenzo, G. V, Gershman, A., Briggs, C. J., & Nisbet, R. M. (2020). Energy budgets for tadpoles approaching metamorphosis. *Ecological Modelling*, *436*, 109261.

Phillips, D. L., Inger, R., Bearhop, S., Jackson, A. L., Moore, J. W., Parnell, A. C., Semmens, B. X., & Ward, E. J. (2014). Best practices for use of stable isotope mixing models in food-web studies. *Canadian Journal of Zoology*, *92*(10), 823–835.

Pietz, S., Kainz, M. J., Schröder, H., Manfrin, A., Schäfer, R. B., Zubrod, J. P., & Bundschuh, M. (2023). Metal Exposure and Sex Shape the Fatty Acid Profile of Midges and Reduce the Aquatic Subsidy to Terrestrial Food Webs. *Environmental Science and Technology*, *57*(2), 951–962. https://doi.org/10.1021/acs.est.2c05495

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Heisterkamp, S., Van Willigen, B., & Maintainer, R. (2017). Package 'nlme.' *Linear and Nonlinear Mixed Effects Models, Version*, *3*(1).

Preston, D. L., Henderson, J. S., Falke, L. P., Segui, L. M., Layden, T. J., & Novak, M. (2018). What drives interaction strengths in complex food webs? A test with feeding rates of a generalist stream predator. *Ecology*, *99*(7), 1591–1601.

Quezada-Romegialli, C., Jackson, A. L., Hayden, B., Kahilainen, K. K., Lopes, C., & Harrod, C. (2018). tRophicPosition, an R package for the Bayesian estimation of trophic position from consumer stable isotope ratios. *Methods in Ecology and Evolution*, *9*(6), 1592–1599.

R Core Team. (2013). *R: A language and environment for statistical computing*.

Röder, N., Stoll, V. S., Jupke, J. F., Kolbenschlag, S., Bundschuh, M., Theißinger, K., & Schwenk, K. (2024). How non-target chironomid communities respond to mosquito control: Integrating DNA metabarcoding and joint species distribution modelling. *Science of The Total Environment*, *913*, 169735.

Seifert, L. I., & Scheu, S. (2012). Linking aquatic and terrestrial food webs - Odonata in boreal systems. *Freshwater Biology*, *57*(7), 1449–1457. https://doi.org/10.1111/j.1365-2427.2012.02807.x

Sheppard, S. K., Bell, J., Sunderland, K. D., Fenlon, J., Skervin, D., & Symondson, W. O. C. (2005). Detection of secondary predation by PCR analyses of the gut contents of invertebrate generalist predators. *Molecular Ecology*, *14*(14), 4461–4468.

Stehle, S., Manfrin, A., Feckler, A., Graf, T., Joschko, T. J., Jupke, J., Noss, C., Rösch, V., Schirmel, J., Schmidt, T., Zubrod, J. P., & Schulz, R. (2022). Structural and functional development of twelve newly established floodplain pond mesocosms. *Ecology and Evolution*, *12*, 8674. https://doi.org/10.1002/ece3.8674

Stemp, K. M., Anderson, T. L., & Davenport, J. M. (2021). *The effects of intraguild interactions (or lack of) on prey diversity in experimental ponds food webs*.

Stephens, R. B., Shipley, O. N., & Moll, R. J. (2023). Meta-analysis and critical review of trophic discrimination factors (Δ13C and Δ15N): Importance of tissue, trophic level and diet source. In *Functional Ecology* (Vol. 37, Issue 9, pp. 2535–2548). British Ecological Society. https://doi.org/10.1111/1365-2435.14403

Stock, B. C., Jackson, A. L., Ward, E. J., Parnell, A. C., Phillips, D. L., & Semmens, B. X. (2018). Analyzing mixing systems using a new generation of Bayesian tracer mixing models. *PeerJ*, *6*, e5096.

Takatsu, K. (2022). Predator cannibalism can shift prey community composition toward dominance by small prey species. *Ecology and Evolution*, *12*(5), e8894.

Turkish, A. R., & Sturley, S. L. (2009). The genetics of neutral lipid biosynthesis: an evolutionary perspective. *American Journal of Physiology-Endocrinology and Metabolism*, *297*(1), E19–E27.

Twining, C. W., Brenna, J. T., Hairston, N. G., & Flecker, A. S. (2016). Highly unsaturated fatty acids in nature: What we know and what we need to learn. *Oikos*, *125*(6), 749–760. https://doi.org/10.1111/oik.02910

Twining, C. W., Parmar, T. P., Mathieu-Resuge, M., Kainz, M. J., Shipley, J. R., & Martin-Creuzburg, D. (2021). Use of Fatty Acids From Aquatic Prey Varies With Foraging Strategy. *Frontiers in Ecology and Evolution*, *9*. https://doi.org/10.3389/fevo.2021.735350

Twining, C. W., Taipale, S. J., Ruess, L., Bec, A., Martin-Creuzburg, D., & Kainz, M. J. (2020). Stable isotopes of fatty acids: Current and future perspectives for advancing trophic ecology. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *375*(1804). https://doi.org/10.1098/rstb.2019.0641

Vaissi, S., & Sharifi, M. (2016). Variation in food availability mediate the impact of density on cannibalism, growth, and survival in larval yellow spotted mountain newts (*Neurergus microspilotus*): Implications for captive breeding programs. *Zoo Biology*, *35*(6), 513–521. https://doi.org/10.1002/zoo.21327

Van Buskirk, J. (1989). Density-dependent cannibalism in larval dragonflies. *Ecology*, *70*(5), 1442–1449.

Wilbur, H. M. (1997). Experimental ecology of food webs: Complex systems in temporary ponds. *Ecology*, *78*(8), 2279–2302. https://doi.org/10.1890/0012-9658(1997)078[2279:EEOFWC]2.0.CO;2

Supporting information for A-2

Dates of flooding and Bti application

Table S 1: Dates of flooding, Bti application and sampling. Application dates were adjusted to weather conditions.

11 April 2020	Flooding, 30>40cm
12 April 2020	Flooding, 40>50cm
14 April 2020	**1st Bti application**
21 April 2020	Release water, 50>40cm
22 April 2020	Release water, 40>30cm
02 May 2020	Flooding, 30>40cm
03 May 2020	Flooding, 40>50cm
04 May 2020	**2nd Bti application**
12 May 2020	Release water, 50>40cm
13 May 2020	Release water, 40>30cm
23 May 2020	Flooding, 30>40cm
24 May 2020	Flooding, 40>50cm
25 May 2020	**3rd Bti application**
02 June 2020	Release water, 50>40cm
03 June 2020	Release water, 40>30cm
16-19 June 2020	**Sampling**

Dry mass of newts and Aeshnidae used for stable isotope analyses

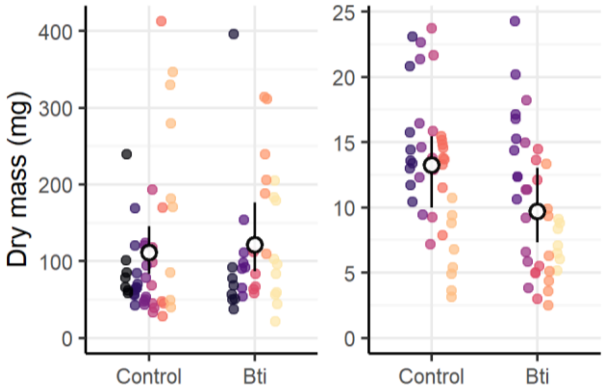

Figure S 1: Dry body mass (mg) of Aeshnidae (left; $F_{1,8.51}$ = 0.122; p = 0.74) and newts (right; $F_{1,8.18}$ = 1.78; p = 0.22). No significant differences between control and Bti-FPMs according to GLMM. Different colors indicate the FPM.

Sample size used for stable isotope analyses

Table S 2: Sample size (n) per taxa and FPM (even numbers indicate Bti-treated FPMs) for stable isotope analyses, taxa which are not available in some FPMs are denoted with n.a.

FPM	Aeshnidae	chironomids	Mayfly	Libellulidae	newts	Zooplankton	Damselfly
1	7	3	5	10	n.a.	5	5
2	8	5	5	10	n.a.	3	5
3	10	1	5	n.a.	9	5	5
4	7	1	5	10	9	5	5
5	8	5	5	10	7	5	5
6	n.a.	5	5	n.a.	8	5	5
7	7	4	5	8	7	5	5
8	5	3	5	10	7	5	5
9	5	5	5	6	10	5	5
10	6	5	5	9	9	5	6
11	8	5	5	10	10	5	6
12	10	5	5	6	8	5	6
n(control)	45	23	30	44	43	30	31
n(Bti)	36	24	30	45	41	28	32
Total	81	47	60	89	84	58	63

Diet proportions of newt and Aeshnidae

Table S 3: Diet proportions of newt and Aeshnidae larvae according to stable isotope mixing models.

Consumer	Resource cluster	Treatment	Bayesian median	95% credible interval
Newt	Aeshnidae/newt/damselfly	Control	18.9	8.0-33.8
		Bti	20.1	9.7-36.2
	Chironomid/mayfly	Control	15.7	5.3-33.1
		Bti	12.9	4.0-29.4
	Libellulidae/zooplankton	Control	65.1	45.1-80.9
		Bti	66.0	45.0-81.8
Aeshnidae	Aeshnidae/newt/damselfly	Control	33.5	23.4-42.3
		Bti	42.4	29.6-54.9
	Chironomid/mayfly	Control	31.6	19.0-47.0
		Bti	26.2	12.9-41.6
	Libellulidae/zooplankton	Control	34.7	25.0-43.8
		Bti	31.2	21.5-40.4

Dry mass of newts used for fatty acid analyses

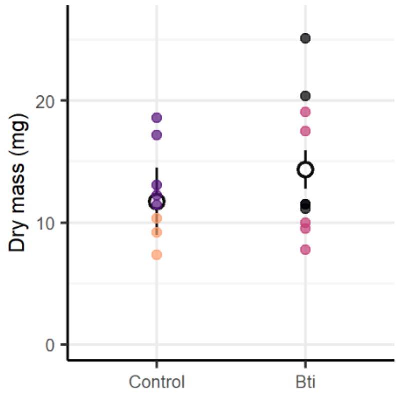

Figure S 2: Dry weight (mg) of newts for NLFA analyses. Different colors indicate different FPMs.

Appendix A-3: Scientific publication 2

Stress response of European common frog (*Rana temporaria*) tadpoles to Bti exposure in an outdoor pond mesocosm

in *Bulletin of Environmental Contamination and Toxicology*

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Key words: *Bacillus thuringiensis israelensis*; mosquito control; amphibians; GST; protein carbonyl

Abbreviations:

Bti = *Bacillus thuringiensis* var. *israelensis*
CAT = catalase
ENAs = Erythrocyte nuclear abnormalities
EERES = Eußerthal Ecosystem Research Station
FPM = floodplain pond mesocosm
FR = Field rate
GST = glutathione-S-transferase
ITU = International toxic unit
LME = linear mixed effect model
ROS = Reactive oxygen species

Abstract

The biocide *Bacillus thuringiensis* var. *israelensis* (Bti) is applied to wetlands to control nuisance by mosquitoes. Amphibians inhabiting these wetlands can be exposed to Bti multiple times, potentially inducing oxidative stress in developing tadpoles. For biochemical stress responses, ambient water temperature plays a key role. Therefore, we exposed tadpoles of the European common frog (*Rana temporaria*) three times to field-relevant doses of Bti in outdoor floodplain pond mesocosms (FPM) under natural environmental conditions. We sampled tadpoles after each Bti application over the course of a 51-day experiment (April to June 2021) and investigated the activity of the glutathione-S-transferase (GST) and protein carbonyl content as a measure for detoxification and oxidative damage. GST activity increased over the course of the experiment likely due to a general increase of water temperature. We did not observe an effect of Bti on either of the investigated biomarkers under natural ambient temperatures. However, Bti-induced effects may be concealed by the generally low water temperatures in our FPMs, particularly at the first application in April, when we expected the highest effect on the most sensitive early stage tadpoles. In light of the global climate change, temperature-related effects of pesticides and biocides on tadpoles should be carefully monitored - in particular since they are known as one of the factors driving the worldwide decline of amphibian populations.

1 Introduction

Insecticides are used in agricultural context for plant protection but also as biocides in vector control, for example against mosquitoes to combat malaria. Bio-insecticides which are assumed to have a lower environmental impact than synthetic insecticides applied in the past are increasingly used (Becker et al., 2018). This is particularly true in regions with mass emergence of floodwater mosquitoes, such as the Upper Rhine Valley in Southwest Germany. For over 40 years, products with the active ingredient *Bacillus thuringiensis* var. *israelensis* (Bti) are applied to these wetlands multiple times per year to control nuisance by mosquito bites (Becker, 1997).

Bti is a bacterium which produces endotoxins during sporulation (Cry proteins; Margalith & Ben-Dov, 2000). After ingestion, the Bti toxins bind to specific receptor sites in the gut of the organism which, under alkaline conditions, activates the toxin leading to a rapid death of the target organism (Bravo et al., 2007; Margalith & Ben-Dov, 2000). Compared to synthetic insecticides, Bti toxins are considered to be environmentally friendly due to their low persistency (half-life 2-4 days or up to three weeks depending on the toxin and the environment; Tetreau et al., 2012) and specific mode of action towards target larvae of the dipteran suborder Nematocera. In Germany, viable Bti spores have to be sterilized using gamma-radiation prior to use in wetlands (Becker, 2002), which prevents recycling of spores and proliferation in the field (Poulin et al., 2022). While effects on target organisms are well understood, recent studies shed light on the fact that Bti can affect non-target organisms in freshwater ecosystems, such as larvae of Chironomidae (e.g., Bordalo et al., 2020; Gerstle et al., 2023; Kästel et al., 2017), Coleoptera (Tudoran et al., 2021) and amphibian tadpoles (Allgeier et al., 2018; Gutierrez-Villagomez et al., 2021; Lajmanovich et al., 2015). For a detailed evaluation of the available environmental studies see also the review by Brühl et al. (2020).

Amphibians are rated the globally most threatened group of vertebrates (Munstermann et al., 2022; Stuart et al., 2004). The local decline of amphibian populations is a result of various impacts such as habitat loss due to climate change, habitat fragmentation and environmental contamination, diseases and invasive species as well as pesticides (Sparling et al., 2001; Stuart et al., 2004). In contrast to pesticides that end up in water bodies unintentionally, Bti is applied directly to the water surface (Becker, 1997). Since many amphibian and mosquito species share breeding habitats in temporarily flooded wetlands, spawning of amphibians spatially and temporally coincides with Bti applications.

Due to their importance for aquatic-terrestrial food webs and possible exposure to Bti, recent studies investigated direct effects of Bti exposure on amphibian tadpoles under laboratory conditions (as reviewed by Empey et al., 2021). A study from Argentina (Lajmanovich et al., 2015) reported reduced survival of Bti-exposed *Leptodactylus latrans* tadpoles, adverse effects on genotoxicity, erythrocyte nuclear abnormalities (ENAs) and an increase of stress biomarkers such as glutathione-S-transferase (GST) and catalase (CAT). The results of this study also raised concerns in Germany where Bti is applied on a large-scale in wetlands for decades and, so far, no evaluation of effects of the main formulations on native amphibians was performed. Hence, in a lab study in 2018, larvae of the European common frog (*Rana temporaria*) were exposed to Bti (Allgeier et al. 2018). *Rana temporaria* is the most common species in Europe belonging to the family Ranidae. In Germany, they breed in February and March in small stagnant (temporary) freshwater ponds and floodplains and their tadpoles develop for 8-10 weeks (Günther, 1996), or faster depending on food resources and temperature (Günther, 1996). Allgeier et al. (2018) recorded no effect on survival of *R.*

temporaria tadpoles, but Bti resulted in increased activity levels of GST and glutathione-reductase (GR) indicating oxidative stress in tadpoles. In a follow-up study by Schweizer et al. (2019), evaluating histopathology and levels of B-esterases and Hsp70, no effects of Bti on stress responses of *R. temporaria* tadpoles were observed. These contrasting results may be attributed to the selection of different biomarkers and experimental conditions, such as the lower water temperature used in Schweizer et al. (2019) compared to Allgeier et al. (2018), i.e., 15 °C vs. 18-24 °C, respectively. Indeed, higher temperature induces thermal stress which can increase GST activity, while promoting oxidative damage to lipids, proteins, DNA and carbohydrates inside cells (Freitas et al., 2017). In both laboratory studies from Allgeier et al. (2018) and Schweizer et al. (2019), the limited capacity to implement environmental parameters and their range might have differently driven the results. In nature, realistic exposure scenarios do not only include diurnal temperature fluctuations and flooding events, but also food supply and high densities of tadpoles. In most laboratory studies, small groups of individuals are held in containers and supplied with highly nutritious fish food (Allgeier et al., 2018; Schweizer et al., 2019). These factors may influence body condition and, consequently, support energy-demanding processes like biochemical stress responses. Therefore, effects of Bti on *R. temporaria* tadpoles under realistic exposure scenarios in the field still remain unclear.

Because of the contrasting laboratory results, we assessed the direct effect of multiple Bti exposures on biochemical responses of *R. temporaria* tadpoles under natural climatic conditions in the field. We introduced early stage tadpoles (classified after Gosner, 1960) to cages placed in twelve outdoor floodplain pond mesocosms (FPMs). Between April and June 2021, six FPMs were treated three times with the maximum recommended Bti field rate, the remaining six FPMs served as untreated controls. We sampled tadpoles 48 h after each of the three Bti applications, corresponding to early stage (GS 23), medium stage (GS 25) and late stage (GS 35-39) tadpoles, respectively. Because of the temperature increase over the course of the experiment from April to June, we expected a higher GST activity level in Bti-exposed tadpoles after the last application in summer, while minor effects would be observed at lower temperatures. On the other hand, as early stage tadpoles are hypothesized to be the most sensitive (Allgeier et al., 2018), we also expected a higher GST activity after the first application when tadpoles are in their early larval stage. As the increase in biomarker activity due to oxidative stress is likely induced by formation of reactive oxygen species (ROS), we analysed the protein carbonyl content as a measure for oxidative damage by ROS (Dalle-

Donne et al., 2003). Assuming ROS formation after Bti exposure, we hypothesized that treated tadpoles show higher protein carbonylation compared to organisms from the control.

2 Methods and Materials

2.1 Study site

The experiment took place in twelve constructed floodplain pond mesocosms (FPMs) at the Eußerthal Ecosystem Research Station (49°15'14"N, 7°57'42"E; EERES, University of Kaiserslautern-Landau) in the Palatinate Forest in Southwest Germany. The FPMs (23.5 x 7.5 m) are open to natural colonization from adjacent aquatic and terrestrial habitats since 2017 and thus considered to be established ecosystems in terms of flora and fauna (Stehle et al., 2022). They are 30 cm deep at the deepest point and gradually fade into a shallow water-land transition zone with a shore at one side. The FPMs can be flooded with stream water from the Sulzbach, an adjacent oligotrophic cold-water stream with minor anthropogenic influences in the upstream area (neither Bti or any mosquito control agent was applied in this region), until the terrestrial shore is completely under water (see Stehle et al. 2022 for further details).

2.2 Tadpole collection and experimental design

In March 2021, six freshly laid egg clutches were collected from a pristine freshwater pond in close proximity to the FPMs (49°15'17.1"N, 7°57'42.6"E). Egg clutches were held separately in 10-L buckets filled with filtered (55 µm) stream water from the Sulzbach at outside temperatures. One week before test start, egg clutches were brought to the lab to hatch (T = 18 ± 2 °C) to ensure we have enough tadpoles in GS 23 before start of the experiment. To avoid fungal growth, stream water in the buckets was renewed every day. Once hatched (two days before the first Bti application), the buckets containing the tadpoles were brought to the FPMs at outside temperatures to let them acclimatize for 24 h before transfer into the FPMs. To ensure high genetic variability among treatments, tadpoles hatched from six egg clutches were transferred into one tray.

One wooden cage (dimensions: 40 × 65 × 30 cm) with a 1-mm mesh was placed into the shallow water-land transition zone of each FPM with at least the bottom half of the cage permanently under water. Each cage was equipped with temperature-loggers (HOBO Pendant data logger, UA-002-64) fixed on the bottom of the cage, recording in 15-min intervals. To calculate the water temperature for each application (Figure S1), we used the mean temperature recorded by the loggers, starting from the day of application to sampling, covering the 48-h exposure period. Additionally, oxygen saturation, pH and water conductivity inside the cages were measured with a hand-held device (MultiLine Multi 3630, WTW Germany) once a week (Table S1). 24 h before the test start (i.e., first Bti application), 150 acclimatized tadpoles (GS 23) were randomly transferred into each cage. We introduced tadpoles instead of egg clutches to have equal numbers of individuals in the cage and to ensure that all introduced individuals were alive. Only freely swimming tadpoles with external gills were introduced (indicating Gosner stage 23). During the study period, we did not provide additional food for the tadpoles. However, due to flooding and water circulation through the mesh, detritus, algae and biofilm were sufficiently available.

2.3 Flooding, Bti application and sampling

General procedure of flooding and Bti application are also described in detail in Gerstle et al. (2023) and Kolbenschlag et al. (2023). Regular Bti applications to the FPM system started in 2020, the year before this experiment was conducted. Since in Germany, only sterilized Bti spores are allowed (Becker, 2002), we do not expect recycling of Bti spores as observed in the Camargue in France (Poulin et al., 2022). The maximum field rate (FR = 2.88 x 10^9 international toxic units (ITU)/ha) of VectoBac WDG suspension (Valent BioSciences Corporation, Illinois, USA) was applied three times during the experiment (Figure 1) to every second of the twelve FPMs using a knapsack sprayer (Prima 5, Gloria, Germany). In the Upper Rhine Valley, the maximum field rate of Bti is applied when late instar mosquito larvae are targeted or the water is deeper than 10 cm (Becker, 1997). In this region, floodwater mosquitoes, whose larvae hatch when floodplains are inundated, are the main target organism. Therefore, the treatment of floodplains is strongly linked to rainfall and snowmelt induced flooding. Depending on the frequency of flooding events, Bti applications can be conducted in a weekly or bi-weekly interval, up to twelve times per year. Regular treatment

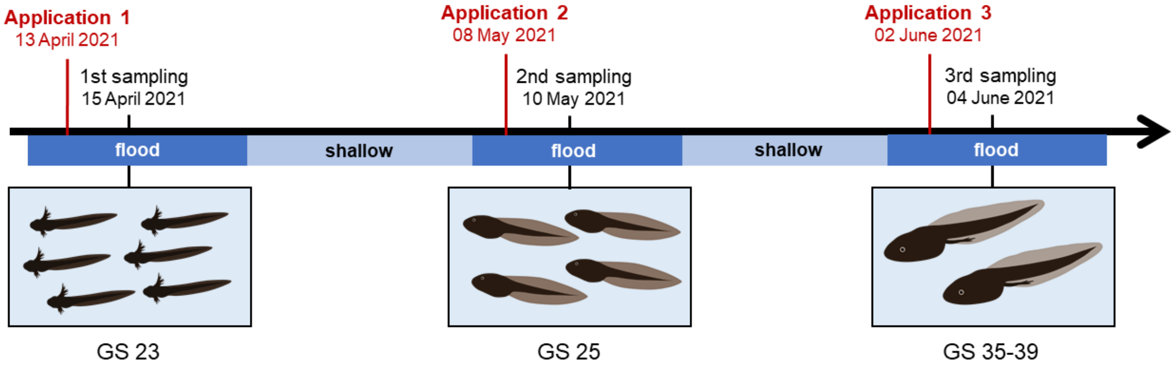

Figure 13: Schematic overview of the flooding, Bti application and samplings of *Rana temporaria* tadpoles from cages in the floodplain pond mesocosms, 48h after the 1st, 2nd and 3rd Bti application at Gosner stages (GS) 23, 25 and 35-39.

of floodplains usually starts in March and last until late summer (Allgeier et al., 2018). Hence, also early stage tadpoles inhabiting these wetlands can be exposed multiple times during their development. To simulate a field-relevant application scenario, we linked Bti applications to controlled flooding periods of alternating 10-d flooding and 10-d shallow periods (see Figure 1; exact dates varied according to weather conditions). 48 h after each Bti application, eight tadpoles of similar size were sampled from each cage by dip-netting, therefore a total of 288 tadpoles were used for biomarker assays. Individuals were euthanized in a buffered 0.1% MS-222 solution, shock frozen in liquid nitrogen and stored separately in 1.5-mL Eppendorf tubes at -80 °C until being used for biomarker analyses. In this study, we did not include body mass as an endpoint, since we focused on sampling tadpoles in the same Gosner stage (as done in Allgeier et al., 2018 and Schweizer et al., 2019), not individuals representing the average size in the cage. The study was terminated in mid-June (two weeks after the last sampling), before metamorphosis of the frogs, since in some FPMs the O_2 saturation dropped under 20% and experimental conditions could not be maintained.

To verify the efficacy of Bti applications, we implemented a biotest using mosquito larvae as a reference organism, since currently there is no method available to quantify sterilized Bti-toxins in the environment. For details on the biotest see Gerstle et al. (2023) and Kolbenschlag et al. (2023).

2.4 Biomarker assays

We analysed GST, a phase II detoxifying enzyme involved in the antioxidant system (Steinberg, 2012; Venturino & D'Angelo, 2005). As a result of insufficient ROS defence by the antioxidant system inside the cells, proteins can be oxidized forming protein carbonyls (Dalle-Donne et al., 2003). Unlike ROS, protein carbonyls are stable making them a convenient biomarker for our study. Both biomarkers were expected to increase under stress (induced by Bti treatment) and were investigated spectrophotometrically using a multiplate reader (Synergy HT-I, BioTek, USA).

For the tissue homogenate, whole individuals were freeze-dried, weighed (Mettler Toledo, XA105 Dual Range, USA) and homogenized in RIPA lysis buffer (Thermo Scientific, USA) using a tissue lyzer (Retsch, MM 301, Germany) and metal beads. The tissue homogenate was used for both GST and protein carbonyl assays. To calculate the GST activity in nmol per mg protein per minute, total protein content was determined using a Micro BCA Protein Assay Kit (Thermo Scientific, USA) with bovine serum albumin (BSA) as standard. GST activity was measured following Habig et al. (1974) and Mingo et al. (2017), adapted for tissue homogenate. We used equine liver GST as an assay positive control. Protein carbonyl content was determined using a Protein Carbonyl Content Assay Kit (Sigma-Aldrich, USA). After derivatization of carbonyl groups with 2,4-dinitrophenyl-hydrazine (DNPH), protein contents in the derivatized samples were measured again using the same BCA Protein Content Assay Kit. Protein carbonyl was expressed in nmol carbonyl per mg protein.

2.5 Statistical analyses

Since negative GST activity levels are biologically impossible, negative values were considered zero (for GST, 9% of samples). Five values have been removed from the data set, due to methodological errors during the biomarker assays (see Table S2, S3). We performed linear mixed effect (LME) models to determine Bti effects on GST activity and protein carbonyl content using the *lme4* package (Bates et al., 2014; Pinheiro et al., 2017) for R (version 4.1.2; R Core Team, 2013). Using F-test-based backwards model selection (Zuur et al., 2009), we implemented treatment (control or Bti; n = 6) and application (1st, 2nd or 3rd application, also representing Gosner stages in course of the experiment) and their interaction as fixed effects in the final LME model. Since the water temperature did not vary significantly between the FPMs (see Figure S1, Table S1), we only used the three sequential applications to also describe temperature differences over the course of the experiment. We used FPM identity as random effect to account for multiple collection within each FPM (eight tadpoles for each FPM and application, resulting in a total of 288 tadpoles analysed). In case of a significant factor or factor interaction term, we used least-squares means (*lsmeans* package; Lenth, 2016) with FDR adjustment as a pairwise contrast post-hoc test to identify significant differences between groups. Response variables were $\log_{10}$-transformed to meet the model assumptions. Residual normality of the final model was checked graphically with quantile-quantile plots and heterogeneity with residuals versus fits plots (Zuur et al., 2009). The significance level for all analyses was set to $p < 0.05$. Plots were created using *ggplot2* (Wickham et al., 2016).

3 Results and Discussion

We measured lower (52%) mean GST activity levels in Bti-treated tadpoles after the first application compared to the control, while results (although not significant) suggest a pattern of higher levels in Bti-treated tadpoles after applications 2 and 3, 17% and 38%, respectively (Figure 2 a, Table 1, Table S2, S4). Also, mean GST activity levels in tadpoles increased over the course of the experiment after each application (here representative for Gosner stage and water temperature), with increasing water temperatures, which were on average 7, 13.5 and

20 °C for application 1, 2 and 3, respectively (Figure 3, Figure S1). In aquatic organisms, GST activity levels have been reported to react sensitively to thermal stress as shown in saltwater fish (Madeira et al., 2013) and neotropical tadpoles (Freitas et al., 2017; Freitas & Almeida, 2016). Since abiotic environmental variables like high water temperature can amplify toxic effects of Bti in chironomid larvae (Charbonneau et al., 1994), we also expected an increase in GST activity in Bti-exposed tadpoles, especially with increasing temperature, which was not recorded in our experiment.

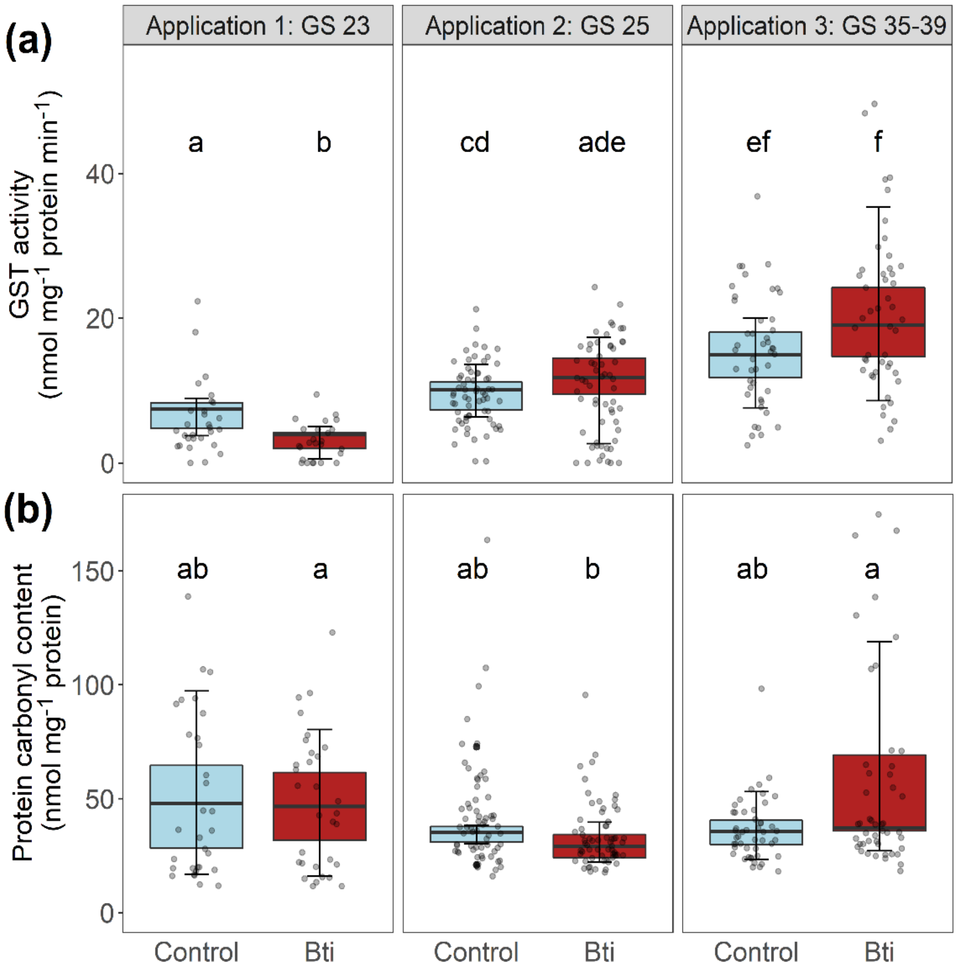

Figure 14: GST activity (a) and protein carbonyl content (b) in *R. temporaria* tadpoles from control and Bti-treated FPMs at Gosner stages (GS) 23, 25 and 35-39. Boxplots are based on mean values within the FPMs (n=6), grey dots represent individual samples (N=288 for each biomarker). Lower and upper box boundaries show 25th and 75th percentiles, respectively, line inside the box show the median. Whiskers and black dots show the variability outside the lower and upper quartiles. Groups that do not share a common letter differ statistically significant (based on least-squares means).

Table 3: Effect of Bti treatment and number of applications (as proxy for the temporal aspect, i.e., increase in water temperature and deveolmental stage of tadpoles) on GST activity and protein carbonyl content in *R. temporaria* tadpoles. Numerator degrees of freedom (numDF), denominator degrees of freedom (denDF), F values and p values are shown, statistically significant p values are printed in bold.

		numDF	denDF	F value	p value
GST activity	Treatment	1	10	0.483	0.503
	Application	2	270	69.218	**<0.001**
	Treatment × Application	2	270	10.100	**<0.001**
Protein carbonyl content	Treatment	1	10	0.006	0.938
	Application	2	269	6.046	**0.003**
	Treatment × Application	2	269	9.007	**0.002**

Our results from the field are comparable to the findings of Schweizer et al. (2019), who did not record any Bti-induced difference in stress-related biomarker levels in *R. temporaria* tadpoles at 15 °C. We observed a similar temperature of 13.5 °C after the second Bti application in May (Figure S1, Table S1). However, we expected the highest effect on sensitive early stage tadpoles, which we sampled in mid-April at a significantly lower temperature (7 °C), compared to Allgeier et al. (2018) and Schweizer et al. (2018) (for comparison see Figure 3). The cold-water scenario at application 1 may be due to the fact that we experienced an exceptionally cold April in 2021 compared to the previous years (Deutscher Wetterdienst, 2023), potentially buffering the effects on early stage tadpoles. Additionally, our test facility

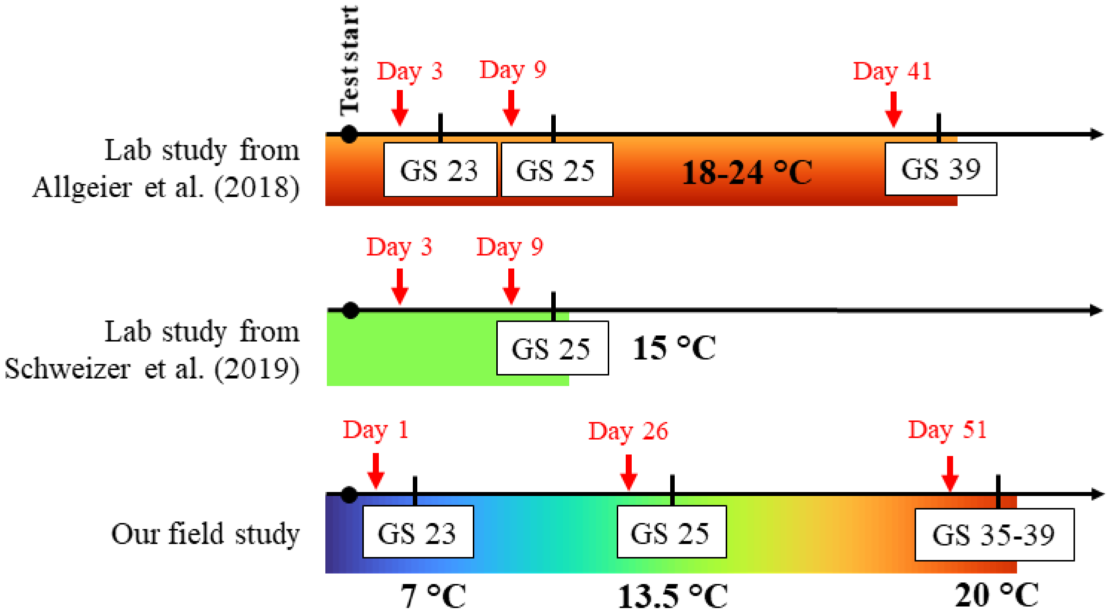

Figure 15: Comparison of water temperatures over the course of the three discussed studies. Red arrows represent Bti applications, black lines indicate tadpole samplings (48h after Bti application) with information on Gosner stages (GS) and mean temperature (printed in bold).

is located in a temperate forest with generally lower temperatures compared to water bodies in the Upper Rhine Valley, where Bti is applied. Although Schweizer et al. (2019) claimed that the effects observed by Allgeier et al. (2018) were possibly due to the high water temperatures (18-24 °C), we measured peak temperatures of up to 20 °C at the end of April (Table S1). In spring, water temperatures of up to 25 °C in ponds in our regions are common, especially in ponds located in lowlands of the Upper Rhine Valley (Adams et al., 2021), which can be considerably warmer than our FPMs. Therefore, it is possible that early and medium stage tadpoles are temporarily exposed to such temperature conditions used by Allgeier et al. (2018). Despite the lack of the effect we expected in early stage tadpoles, we also assumed a significantly higher Bti-induced effect at warmer temperatures, which was not recorded after the third application at 20 °C. We only observed a slightly increased GST activity in late stage tadpoles (Figure 2 a). However, a possible effect of warmer exposure conditions could be reduced due to higher developmental stages of tadpoles which are expected to be less sensitive. In other words, if applications of Bti in early spring coincide with peak temperatures of 20 °C, oxidative stress in early stage *R. temporaria* tadpoles is more likely to happen as reported in Allgeier et al. (2018). An increase of cellular responses to xenobiotics is linked to a higher cost of energy (Steinberg, 2012). In turn, this may impair tadpoles' behavior and development, possibly affecting amphibian populations at a later stage (Monaghan et al., 2009). However, this was not observed under the comparably cold temperatures experienced during our experiment.

Similar to GST activity levels, there was no significant Bti-induced effect on protein carbonyl contents due to oxidative stress (Figure 2 b, Table 1, Table S3, S5). In contrast, Gutierrez-Villagomez et al. (2021) exposed tadpoles of two North American frog species (*Lithobates sylvaticus* and *Anaxyrus americanus*) to two different Bti formulations in a chronic exposure laboratory experiment. Authors did not record a significant change in stress response in a dose-dependent pattern, but they observed modifications in the intestine microbiota as well as an overexpression of the genes *cyp1a* and *sod* in tails of *L. sylvaticus*. Overexpression of *cyp1a* and *sod* may indicate detoxification processes and oxidative stress, but this pattern was not observed in *A. americanus* tadpoles. The *sod* gene expresses an enzyme which is responsible for destroying radicals, such as ROS. In our experiment, we assumed ROS formation in Bti-exposed tadpoles resulting in protein damage, i.e., increased protein carbonylation. However, in our scenario, Bti did not have an effect on protein carbonyl content. Our findings suggest insignificant levels of ROS formation; thus, we assume that Bti does not affect proteins in *R. temporaria* tadpoles under the tested conditions.

4 Conclusion

This is the first study investigating oxidative stress response in tadpoles exposed to Bti under natural environmental conditions. Our results show no significant increase of biochemical biomarkers as response to stress from Bti treatment. However, we are cautious to not generalise our findings to different environmental conditions, species or different developmental stages of such a worldwide threatened vertebrate group like amphibians. During our experiment, exceptional cold temperatures in early spring prevented our pond mesocosm from being an accurate representation of wetlands in the Upper Rhine Valley, where Bti is applied and water temperatures can be considerably warmer. Additionally, with global climate change suggesting increased water temperatures in the future, it is likely that amphibians in temperate regions will face higher water temperatures during their whole aquatic development (Noyes et al., 2009). Although our results do not suggest an effect of temperature on the toxicity of Bti, natural temperature fluctuations should be considered in biocide and pesticide toxicity tests (Baier et al., 2016; Leeb et al., 2022), especially of substances intentionally applied to amphibian-rich wetlands. Indirect effects of Bti on food webs were not addressed in this study. However, recent investigations (e.g. Gutierrez-Villagomez et al., 2021; McKie et al., 2023) revealed that Bti can have implications on the trophic structure in aquatic ecosystems, potentially affecting amphibians. This highlights the importance of further research on indirect effects of Bti on aquatic food webs.

5 Funding

This study was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – 326210499/GRK2360.

6 Ethical approval

All experimental procedures in our study were evaluated and approved by the Institutional Animal Care and Use Committee at the University of Koblenz-Landau and the federal investigation office (Landesuntersuchungsamt – LUA Rhineland-Palatinate, Germany, NTP-ID: 00035820-2-7). We thank the Struktur- und Genehmigungsdirektion Süd (SGD) in Neustadt, Germany, for sampling permissions.

7 References

Adams, E., Gerstle, V., & Brühl, C. A. (2021). Dermal Fungicide Exposure at Realistic Field Rates Induces Lethal and Sublethal Effects on Juvenile European Common Frogs (*Rana temporaria*). *Environmental Toxicology and Chemistry*, *40*(5), 1289–1297. https://doi.org/10.1002/etc.4972

Allgeier, S., Frombold, B., Mingo, V., & Brühl, C. A. (2018). European common frog *Rana temporaria* (Anura: Ranidae) larvae show subcellular responses under field-relevant *Bacillus thuringiensis* var. *israelensis* (Bti) exposure levels. *Environmental Research*, *162*, 271–279. https://doi.org/10.1016/j.envres.2018.01.010

Baier, F., Gruber, E., Hein, T., Bondar-Kunze, E., Ivanković, M., Mentler, A., Brühl, C. A., Spangl, B., & Zaller, J. G. (2016). Non-target effects of a glyphosate-based herbicide on Common toad larvae (*Bufo bufo*, Amphibia) and associated algae are altered by temperature. *PeerJ*, *2016*(11). https://doi.org/10.7717/peerj.2641

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2014). Fitting linear mixed-effects models using lme4. *ArXiv Preprint ArXiv:1406.5823*.

Becker, N. (1997). Microbial Control of Mosquitoes: Management of the Upper Rhine Mosquito Population as a Model Programme. *Parasitology Today*, *13*(12), 485–487. https://doi.org/https://doi.org/10.1016/S0169-4758(97)01154-X

Becker, N. (2002). Sterilization of *Bacillus thuringiensis israelensis* products by gamma radiation. *Journal of the American Mosquito Control Association*, *18*(1), 57–62.

Becker, N., Ludwig, M., & Su, T. (2018). Lack of resistance in *Aedes vexans* field populations after 36 years of *Bacillus thuringiensis* subsp. *israelensis* applications in the Upper Rhine valley, Germany. *Journal of the American Mosquito Control Association*, *34*(2), 154–157.

Bordalo, M. D., Gravato, C., Beleza, S., Campos, D., Lopes, I., & Pestana, J. L. T. (2020). Lethal and sublethal toxicity assessment of *Bacillus thuringiensis* var. *israelensis* and *Beauveria bassiana* based bioinsecticides to the aquatic insect *Chironomus riparius*. *Science of the Total Environment*, *698*. https://doi.org/10.1016/j.scitotenv.2019.134155

Bravo, A., Gill, S. S., & Soberón, M. (2007). Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon*, *49*(4), 423–435. https://doi.org/https://doi.org/10.1016/j.toxicon.2006.11.022

Brühl, C. A., Després, L., Frör, O., Patil, C. D., Poulin, B., Tetreau, G., & Allgeier, S. (2020). Environmental and socioeconomic effects of mosquito control in Europe using the biocide *Bacillus thuringiensis* subsp. *israelensis* (Bti). In *Science of the Total Environment* (Vol. 724). Elsevier B.V. https://doi.org/10.1016/j.scitotenv.2020.137800

Charbonneau, C. S., Drobney, R. D., & Rabeni, C. F. (1994). Effects of *Bacillus thuringiensis* var. *israelensis* on non-target benthic organisms in a lentic habitat and factors affecting the efficiacy of the larvicide. In *Environmental Toxicology and Chemistry* (Vol. 13, Issue 2). https://doi.org/https://doi.org/10.1002/etc.5620130211

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., & Colombo, R. (2003). Protein carbonyl groups as biomarkers of oxidative stress. *Clinica Chimica Acta*, *329*(1–2), 23–38. https://doi.org/10.1016/S0009-8981(03)00003-2

Deutscher Wetterdienst. (2023, January 8). *Klimaüberwachung Deutschland, Monats- und Jahreszeitenbericht*. https://www.dwd.de/DE/Home/home_node.html

Empey, M. A., Lefebvre-Raine, M., Gutierrez-Villagomez, J. M., Langlois, V. S., & Trudeau, V. L. (2021). A Review of the Effects of the Biopesticides *Bacillus thuringiensis* Serotypes *israelensis* (Bti) and *kurstaki* (Btk) in Amphibians. In *Archives of Environmental Contamination and Toxicology* (Vol. 80, Issue 4, pp. 789–800). Springer. https://doi.org/10.1007/s00244-021-00842-2

Freitas, J. S., & Almeida, E. A. (2016). Antioxidant defense system of tadpoles (*Eupemphix nattereri*) exposed to changes in temperature and pH. *Zoological Science*, *33*(2), 186–194. https://doi.org/10.2108/zs150075

Freitas, J. S., Felício, A. A., Teresa, F. B., & Alves de Almeida, E. (2017). Combined effects of temperature and clomazone (Gamit®) on oxidative stress responses and B-esterase activity of *Physalaemus nattereri* (Leiuperidae) and *Rhinella schneideri* (Bufonidae) tadpoles. *Chemosphere*, *185*, 548–562. https://doi.org/10.1016/j.chemosphere.2017.07.061

Gerstle, V., Manfrin, A., Kolbenschlag, S., Gerken, M., Islam, A. S. M. M. U., Entling, M. H., Bundschuh, M., & Brühl, C. A. (2023). Benthic macroinvertebrate community shifts based on Bti-induced chironomid reduction also decrease Odonata emergence. *Environmental Pollution*, *316*. https://doi.org/10.1016/j.envpol.2022.120488

Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*, *16*(3), 183–190.

Günther, R. (1996). *Die Amphibien und Reptilien Deutschlands*. Springer.

Gutierrez-Villagomez, J. M., Patey, G., To, T. A., Lefebvre-Raine, M., Lara-Jacobo, L. R., Comte, J., Klein, B., & Langlois, V. S. (2021). Frogs Respond to Commercial Formulations of the Biopesticide *Bacillus thuringiensis* var. *israelensis*, Especially Their Intestine Microbiota. *Environmental Science and Technology*, *55*(18), 12504–12516. https://doi.org/10.1021/acs.est.1c02322

Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974). Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry*, *249*(22), 7130–7139. https://doi.org/https://doi.org/10.1016/S0021-9258(19)42083-8

Kästel, A., Allgeier, S., & Brühl, C. A. (2017). Decreasing *Bacillus thuringiensis israelensis* sensitivity of Chironomus riparius larvae with age indicates potential environmental risk for mosquito control. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-14019-2

Kolbenschlag, S., Gerstle, V., Eberhardt, J., Bollinger, E., Schulz, R., Brühl, C. A., & Bundschuh, M. (2023). A temporal perspective on aquatic subsidy: Bti affects emergence of Chironomidae. *Ecotoxicology and Environmental Safety*, *250*, 114503. https://doi.org/10.1016/j.ecoenv.2023.114503

Lajmanovich, R. C., Junges, C. M., Cabagna-Zenklusen, M. C., Attademo, A. M., Peltzer, P. M., Maglianese, M., Márquez, V. E., & Beccaria, A. J. (2015). Toxicity of *Bacillus thuringiensis* var. *israelensis* in aqueous suspension on the South American common frog *Leptodactylus latrans* (Anura: Leptodactylidae) tadpoles. *Environmental Research*, *136*, 205–212. https://doi.org/10.1016/j.envres.2014.10.022

Leeb, C., Schuler, L., Brühl, C. A., & Theissinger, K. (2022). Low temperatures lead to higher toxicity of the fungicide folpet to larval stages of *Rana temporaria* and *Bufotes viridis*. *PLoS ONE*, *17*(8 August). https://doi.org/10.1371/journal.pone.0258631

Lenth, R. v. (2016). Least-squares means: the R package lsmeans. *Journal of Statistical Software*, *69*, 1–33.

Madeira, D., Narciso, L., Cabral, H. N., Vinagre, C., & Diniz, M. S. (2013). Influence of temperature in thermal and oxidative stress responses in estuarine fish. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, *166*(2), 237–243. https://doi.org/10.1016/j.cbpa.2013.06.008

Margalith, Y., & Ben-Dov, E. (2000). Biological Control by *Bacillus thuringiensis* subsp. *israelensis*. In J. E. Rechcigl & N. A. Rechcigl (Eds.), *Insect pest management: techniques for environmental protection* (pp. 243–281). CRC Press.

McKie, B. G., Taylor, A., Nilsson, T., Frainer, A., & Goedkoop, W. (2023). Ecological effects of mosquito control with Bti: evidence for shifts in the trophic structure of soil- and ground-based food webs. *Aquatic Sciences*, *85*(2). https://doi.org/10.1007/s00027-023-00944-0

Mingo, V., Lötters, S., & Wagner, N. (2017). The use of buccal swabs as a minimal-invasive method for detecting effects of pesticide exposure on enzymatic activity in common wall lizards. *Environmental Pollution*, *220*, 53–62.

Monaghan, P., Metcalfe, N. B., & Torres, R. (2009). Oxidative stress as a mediator of life history trade-offs: Mechanisms, measurements and interpretation. In *Ecology Letters* (Vol. 12, Issue 1, pp. 75–92). https://doi.org/10.1111/j.1461-0248.2008.01258.x

Munstermann, M. J., Heim, N. A., McCauley, D. J., Payne, J. L., Upham, N. S., Wang, S. C., & Knope, M. L. (2022). A global ecological signal of extinction risk in terrestrial vertebrates. *Conservation Biology*, *36*(3). https://doi.org/10.1111/cobi.13852

Noyes, P. D., McElwee, M. K., Miller, H. D., Clark, B. W., van Tiem, L. A., Walcott, K. C., Erwin, K. N., & Levin, E. D. (2009). The toxicology of climate change: Environmental contaminants in a warming world. In *Environment International* (Vol. 35, Issue 6, pp. 971–986). Elsevier Ltd. https://doi.org/10.1016/j.envint.2009.02.006

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Heisterkamp, S., van Willigen, B., & Maintainer, R. (2017). Package 'nlme.' *Linear and Nonlinear Mixed Effects Models, Version*, *3*(1).

Poulin, B., Lefebvre, G., Hilaire, S., & Després, L. (2022). Long-term persistence and recycling of Bacillus thuringiensis israelensis spores in wetlands sprayed for mosquito control. *Ecotoxicology and Environmental Safety*, *243*. https://doi.org/10.1016/j.ecoenv.2022.114004

R Core Team. (2013). *R: A language and environment for statistical computing*.

Schweizer, M., Miksch, L., Köhler, H. R., & Triebskorn, R. (2019). Does Bti (*Bacillus thuringiensis* var. *israelensis*) affect *Rana temporaria* tadpoles? *Ecotoxicology and Environmental Safety*, *181*, 121–129. https://doi.org/10.1016/j.ecoenv.2019.05.080

Sparling, D. W., Fellers, G. M., & McConnell, L. L. (2001). Pesticides and amphibian population declines in California, USA. *Environmental Toxicology and Chemistry: An International Journal*, *20*(7), 1591–1595.

Stehle, S., Manfrin, A., Feckler, A., Graf, T., Joschko, T. J., Jupke, J., Noss, C., Rösch, V., Schirmel, J., Schmidt, T., Zubrod, J. P., & Schulz, R. (2022). Structural and functional development of twelve newly established floodplain pond mesocosms. *Ecology and Evolution*, *12*, 8674. https://doi.org/10.1002/ece3.8674

Steinberg, C. E. W. (2012). Multiple stressors as environmental realism: synergism or antagonism. In *Stress Ecology* (pp. 295–309). Springer.

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S. L., Fischman, D. L., & Waller, R. W. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science*, *306*(5702), 1783–1786.

Tetreau, G., Alessi, M., Veyrenc, S., Périgon, S., David, J. P., Reynaud, S., & Després, L. (2012). Fate of Bacillus thuringiensis subsp. Israelensis in the field: Evidence for spore recycling and differential persistence of toxins in leaf litter. *Applied and Environmental Microbiology*, *78*(23), 8362–8367. https://doi.org/10.1128/AEM.02088-12

Venturino, A., & D'Angelo, A. M. P. de. (2005). Biochemical targets of xenobiotics: Biomarkers in amphibian ecotoxicology. *Applied Herpetology*, *2*(3), 335–353.

Wickham, H., Chang, W., & Wickham, M. H. (2016). Package 'ggplot2.' *Create Elegant Data Visualisations Using the Grammar of Graphics. Version*, *2*(1), 1–189.

Zuur, A. F., Ieno, E. N., Walker, N. J., Saveliev, A. A., & Smith, G. M. (2009). *Mixed effects models and extensions in ecology with R* (Vol. 574). Springer.

Supporting information for A-3

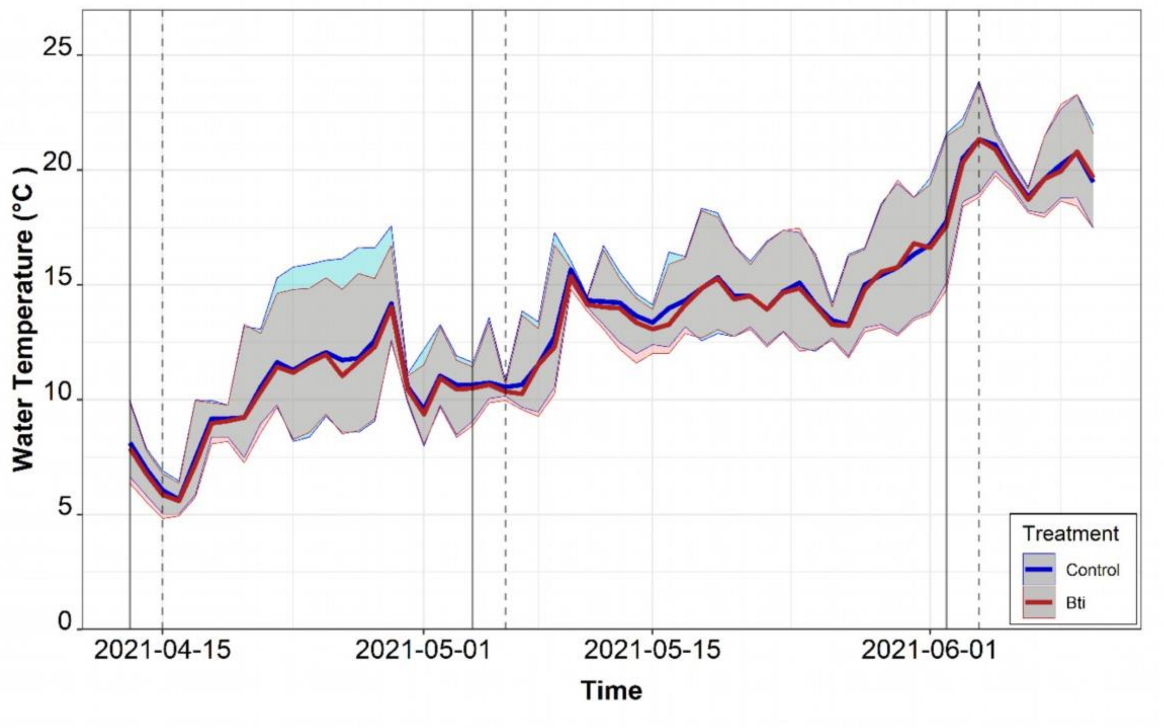

Figure S 3: Water temperature in control (blue; n=6) and Bti-treated FPMs (red; n=6) over the course of the experiment. Shown are the median temperatures with lower and upper quartiles and the date of the Bti application (solid line) and tadpole samplings (dashed line).

Table S 4: Environmental parameters measured inside the cages in each FPM. Sampling dates are printed in bold.

Date	FPM	Time	Water level (cm)	pH	Conductivity (µS/cm)	O_2 (mg/L)	O_2 (%)	Temperature (°C)
2021 Apr 13	1	12:33	53	7.62	99.9	10.48	86.8	6.8
	2	12:31	52	7.74	124.4	11.19	92.9	6.9
	3	12:29	51	7.75	125.5	11.29	95.0	7.5
	4	12:27	52	7.58	126.2	11.16	94.0	7.6
	5	12:24	51	7.72	98.3	11.34	95.9	7.8
	6	12:22	52	7.68	124.2	11.22	94.8	7.7
	7	12:20	52	7.55	115.5	10.90	90.6	7.1
	8	12:44	52	7.48	104.6	11.41	95.5	7.3
	9	12:43	51	7.74	97.8	11.11	93.5	7.5
	10	12:42	52	7.72	139.2	11.10	91.1	6.6
	11	12:41	52	7.72	122.5	10.32	84.9	6.7
	12	12:39	48	7.68	152.1	10.78	87.9	6.5
2021 Apr 15	1	10:27	53	7.68	106.2	11.45	87.9	3.9
	2	10:25	52	7.78	136.6	11.91	91.8	4.0
	3	10:23	51	7.78	136.3	12.05	92.2	3.7
	4	10:21	52	7.68	130.0	11.70	89.6	3.6
	5	10:19	50	7.86	105.7	11.99	92.0	4.0

	6	10:17	50	7.76	132.6	11.62	87.6	3.2
	7	10:15	52	7.71	123.2	12.02	92.1	3.9
	8	10:13	52	7.82	109.8	11.61	88.4	3.8
	9	10:11	50	7.82	105.6	11.72	88.7	3.1
	10	10:09	52	7.95	145.7	12.21	93.7	3.9
	11	10:07	52	7.89	138.3	11.38	87.8	4.1
	12	10:04	48	8.08	157.2	11.86	90.5	3.7
2021 Apr 22	1	15:32	30	7.66	132.5	10.99	114.0	16.2
	2	15:29	30	8.65	150.6	15.13	156.7	16.1
	3	15:27	30	8.14	156.1	13.32	140.6	17.6
	4	15:25	30	8.13	140.4	12.36	131.0	17.3
	5	15:22	31	8.46	115.9	13.37	142.5	17.5
	6	15:20	30	8.12	152.5	13.23	139.6	17.1
	7	15:18	31	8.1	154.2	13.48	143.5	17.5
	8	15:16	30	7.91	122.3	13.03	133.0	15.4
	9	15:13	31	8.45	112.1	14.14	148.5	16.8
	10	15:11	30	7.86	184.5	13.01	132.4	15.3
	11	15:09	31	7.93	179.7	12.07	128.0	17.2
	12	15:06	31	7.31	173.2	11.50	115.6	15.1
2021 Apr 29	1	16:10	29	8.23	124.8	11.35	126.6	18.7
	2	16:05	29	9.61	151.0	16.79	184.2	18.8
	3	16:03	29	8.76	168.0	13.79	152.7	18.9
	4	16:01	29	8.87	153.6	13.04	148.2	19.8
	5	15:59	30	9.12	112.3	13.70	154.7	19.4
	6	15:56	28	8.81	164.1	11.94	135.0	19.4
	7	15:54	30	8.88	140.4	13.27	148.6	18.8
	8	15:51	29	9.08	122.4	13.38	147.0	18.4
	9	15:49	30	9.61	136.3	16.49	183.6	19.0
	10	15:46	30	8.97	156.9	11.64	126.0	17.7
	11	15:44	30	7.8	197.7	10.91	120.8	18.8
	12	15:41	30	7.31	185.4	10.04	108.8	17.6
2021 May 10	1	11:07	52	7.58	97.3	9.72	87.0	9.5
	2	11:05	50	7.94	129.9	10.73	93.8	8.9
	3	11:03	52	7.83	131.2	10.73	95.1	9.1
	4	10:59	51	7.7	118.6	10.76	95.6	9.2
	5	10:57	51	7.64	102.3	10.32	92.5	9.5
	6	10:54	49	7.62	122.9	10.65	94.2	9.0
	7	10:52	52	7.56	122.6	9.97	87.7	8.8
	8	10:49	51	7.66	107.7	10.59	92.6	8.5
	9	10:47	50	7.79	107.3	10.68	92.2	8.0
	10	10:45	52	7.78	133.8	10.34	88.3	7.7
	11	10:43	53	7.74	136.1	9.55	81.7	7.8
	12	10:41	52	7.72	141.7	9.69	83.0	7.8
2021 May 17	1	16:32	30	7.37	123.6	9.98	107.2	17.3
	2	16:30	30	7.22	126.3	14.08	151.0	17.0
	3	16:27	30	7.78	150.1	11.99	129.7	17.5
	4	16:25	31	7.85	120.1	12.52	136.6	17.9
	5	16:23	30	7.85	98.2	13.97	151.0	17.5
	6	16:21	31	7.50	127.3	12.12	130.7	17.3
	7	16:19	29	7.40	143.7	11.57	124.9	17.4
	8	16:16	31	7.40	109.7	10.64	115.2	17.0
	9	16:14	29	7.37	132.7	12.44	132.6	17.1
	10	16:11	30	7.31	211.0	7.83	83.2	16.6

	11	16:08	32	7.43	230.0	10.08	108.2	17.1
	12	16:06	30	7.41	187.1	10.30	109.2	16.5
2021 May 25	1	13:25	33	7.54	93.7	10.60	103.3	12.0
	2	13:23	32	7.58	151.7	9.05	85.7	12.1
	3	13:21	32	7.97	151.4	10.71	101.9	12.0
	4	13:19	31	8.49	105.3	11.91	113.9	12.4
	5	13:17	32	8.44	86.7	12.92	123.4	12.3
	6	13:15	32	7.75	132.7	10.76	102.5	12.3
	7	13:12	32	7.6	116.1	11.68	111.5	12.3
	8	13:10	32	7.75	90.7	10.45	99.4	12.2
	9	13:08	32	8.21	127.4	12.03	114.0	11.9
	10	13:06	34	7.65	148.1	10.29	96.7	11.9
	11	13:04	35	7.50	189.8	8.95	84.7	12.0
	12	13:02	33	6.93	170.2	9.98	93.3	12.2
2021 June 04	1	10:59	51	7.28	102.9	5.91	66.7	20.4
	2	10:57	50	7.48	147.9	6.83	76.2	19.8
	3	10:55	52	7.44	142.6	7.33	81.8	19.7
	4	10:53	51	7.37	122.3	6.78	75.4	20.2
	5	10:51	51	7.55	101.0	8.44	94.3	20.0
	6	10:49	50	7.49	115.6	9.64	106.2	19.3
	7	10:47	51	7.56	124.9	8.77	96.0	19.0
	8	10:45	51	7.52	103.3	9.42	103.4	19.1
	9	10:43	50	7.42	107.2	8.16	89.1	18.8
	10	10:41	52	7.62	135.7	8.41	91.1	18.4
	11	10:39	53	7.64	134.8	8.27	89.7	18.4
	12	10:37	52	7.49	137.9	7.02	76.3	18.6

Table S 5: GST activity (mean ± SD; nmol/mg protein min) in *Rana temporaria* tadpoles sampled 48h after each Bti application.

FPM	**Treatment**	**Application 1**		**Application 2**		**Application 3**	
		GST activity	**n**	**GST activity**	**n**	**GST activity**	**n**
1	Control	4.03 ± 1.89	8	6.33 ± 3.40	8	16.06 ± 9.05	8
2	Bti	3.97 ± 2.16	7	9.50 ± 8.76	8	23.13 ± 4.77	8
3	Control	7.94 ± 3.63	7	6.64 ± 4.78	8	11.11 ± 8.20	8
4	Bti	0.57 ± 0.93	8	2.67 ± 2.65	8	14.53 ± 9.35	8
5	Control	7.00 ± 6.35	8	11.20 ± 3.47	8	18.71 ± 3.36	8
6	Bti	3.87 ± 3.27	8	14.04 ± 1.96	8	24.54 ± 6.91	8
7	Control	3.76 ± 6.13	8	13.55 ± 4.66	8	13.82 ± 8.11	8
8	Bti	1.41 ± 1.75	8	17.31 ± 2.89	8	35.36 ± 10.34	8
9	Control	8.38 ± 2.80	8	11.05 ± 3.72	8	20.02 ± 9.45	8
10	Bti	4.28 ± 5.56	8	9.52 ± 3.67	8	14.97 ± 5.71	8
11	Control	8.93 ± 4.25	8	9.23 ± 3.08	8	7.61 ± 5.22	8
12	Bti	4.99 ± 4.99	8	14.64 ± 3.72	8	8.60 ± 4.95	8

Table S 6: Protein carbonyls (mean ± SD; nmol/mg protein) in *Rana temporaria* tadpoles sampled 48h after each Bti application.

FPM	**Treatment**	**Application 1**		**Application 2**		**Application 3**	
		Protein carbonyl	**n**	**Protein carbonyl**	**n**	**Protein carbonyl**	**n**
1	Control	24.13 ± 6.63	7	36.50 ± 7.90	8	53.16 ± 19.69	8
2	Bti	28.77 ± 11.21	8	22.43 ± 2.61	8	35.95 ± 10.61	8
3	Control	16.71 ± 3.11	8	20.93 ± 3.07	8	28.17 ± 5.30	8
4	Bti	16.02 ± 4.81	7	22.27 ± 3.71	8	79.44 ± 51.22	7
5	Control	97.29 ± 20.05	8	72.85 ± 38.02	8	34.62 ± 4.90	8
6	Bti	80.33 ± 21.45	8	39.70 ± 6.97	8	35.79 ± 12.61	8
7	Control	55.44 ± 20.54	8	33.89 ± 6.36	8	23.45 ± 4.61	8
8	Bti	64.61 ± 17.19	8	29.56 ± 3.87	8	27.34 ± 10.60	8
9	Control	67.71 ± 27.18	8	30.20 ± 6.85	8	36.93 ± 8.58	8
10	Bti	52.25 ± 21.42	8	35.90 ± 16.19	8	118.91 ± 38.65	8
11	Control	40.46 ± 12.23	8	38.18 ± 13.04	8	41.66 ± 11.22	8
12	Bti	40.93 ± 14.35	8	28.89 ± 4.41	8	38.63 ± 7.63	8

Table S 7: Comparison of groups using least squares-means on log-transformed GST activity levels with standard errors (SE), degrees of freedom (df) and T ratio. Significant differences are printed in bold.

Comparison	Estimate	SE	df	T ratio	P value
Bti 1 - Control 1	-0.347	0.112	10	-3.093	**0.019**
Bti 1 - Bti 2	-0.540	0.076	270	-7.064	**<0.001**
Bti 1 - Control 2	-0.617	0.103	10	-5.994	**<0.001**
Bti 1 - Bti 3	-0.897	0.077	270	-11.584	**<0.001**
Bti 1 - Control 3	-0.738	0.105	10	-7.014	**<0.001**
Control 1 - Bti 2	-0.193	0.103	10	-1.875	0.104
Control 1 - Control 2	-0.270	0.076	270	-3.524	**<0.001**
Control 1 - Bti 3	-0.549	0.105	10	-5.224	**<0.001**
Control 1 - Control 3	-0.390	0.077	270	-5.043	**<0.001**
Bti 2 - Control 2	-0.077	0.093	10	-0.827	0.427
Bti 2 - Bti 3	-0.357	0.063	270	-5.659	**<0.001**
Bti 2 - Control 3	-0.197	0.095	10	-2.077	0.081
Control 2 - Bti 3	-0.280	0.095	10	-2.945	**0.022**
Control 2 - Control 3	-0.121	0.063	270	-1.919	0.076
Bti 3 - Control 3	0.159	0.098	10	1.631	0.144

Table S 8: Comparison of groups using least squares-means on log-transformed GST activity levels with standard errors (SE), degrees of freedom (df) and T ratio. Significant differences are printed in bold.

Comparison	Estimate	SE	df	T ratio	P value
Bti 1 - Control 1	0.043	0.086	10	0.502	0.672
Bti 1 - Bti 2	0.163	0.044	269	3.738	**0.002**
Bti 1 - Control 2	0.068	0.083	10	0.827	0.642
Bti 1 - Bti 3	-0.012	0.044	269	-0.281	0.779
Bti 1 - Control 3	0.104	0.083	10	1.250	0.550
Control 1 - Bti 2	0.119	0.083	10	1.445	0.537
Control 1 - Control 2	0.025	0.043	269	0.572	0.655
Control 1 - Bti 3	-0.056	0.083	10	-0.667	0.650
Control 1 - Control 3	0.061	0.044	269	1.394	0.537
Bti 2 - Control 2	-0.094	0.078	10	-1.203	0.550
Bti 2 - Bti 3	-0.175	0.036	269	-4.913	**<0.001**
Bti 2 - Control 3	-0.058	0.079	10	-0.735	0.650
Control 2 - Bti 3	-0.081	0.079	10	-1.014	0.558
Control 2 - Control 3	0.036	0.035	269	1.017	0.558
Bti 3 - Control 3	0.117	0.080	10	1.451	0.537

Appendix A-4: Author contributions

Contributions to A-1

Benthic macroinvertebrate community shifts based on Bti-induced chironomid reduction also decrease Odonata emergence

Verena Gerstle: Conceptualization, Methodology, Formal Analysis, Investigation, Writing – original draft, Writing – review & editing.
Alessandro Manfrin: Conceptualization, Methodology, Formal Analysis, Writing – review and editing, Supervision.
Sara Kolbenschlag: Methodology, Writing – review and editing.
Maximilian Gerken: Investigation, Formal Analysis, Writing – review and editing.
A.S.M.M.U. Islam: Investigation, Writing – review and editing.
Martin H. Entling: Investigation, Writing – review and editing, Supervision.
Mirco Bundschuh: Methodology, Writing – review and editing.
Carsten A. Brühl: Conceptualization, Methodology, Investigation, Writing – review and editing, Supervision.

Contributions to A-2

Trophic effects of Bti-based mosquito control on two top predators in floodplain pond mesocosms

Verena Gerstle: Conceptualization, Methodology, Formal Analysis, Investigation, Writing – original draft, Writing – review & editing.
Eric Bollinger: Conceptualization, Methodology, Formal Analysis, Investigation, Writing – review & editing.
Alessandro Manfrin: Conceptualization, Formal Analysis, Writing – review and editing, Supervision.
Sebastian Pietz: Formal Analysis, Investigation, Writing – review & editing.
Sara Kolbenschlag: Methodology, Writing – review and editing.
Alexander Feckler: Investigation, Writing – review & editing.
Martin H. Entling: Conceptualization, Writing – review & editing.
Carsten A. Brühl: Conceptualization, Writing – review and editing, Supervision.

Contributions to A-3

Stress response of European common frog (*Rana temporaria*) tadpoles to Bti exposure in an outdoor pond mesocosm

Verena Gerstle: Conceptualization, Methodology, Formal Analysis, Investigation, Writing – original draft, Writing – review & editing.
Priyanka Solanki: Formal Analysis, Investigation, Writing – review & editing.
Alessandro Manfrin: Formal Analysis, Writing – review & editing.
Sara Kolbenschlag: Methodology, Writing – review & editing.
Carsten A. Brühl: Conceptualization, Writing – review & editing, Supervision.

Appendix A-5: Declaration

I hereby declare that I independently conducted the work presented in this thesis entitled "ECOLOGICAL EFFECTS OF BTI-BASED MOSQUITO CONTROL ON AQUATIC-TERRESTRIAL LINKAGES". All used assistances are mentioned and involved contributors are either co-authors of or are acknowledged in the respective publication.

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

Place, date Signature

Appendix A-6: Curriculum Vitae

PERSONAL INFORMATION

Name	Verena Elsa Gerstle
Nationality	German

EDUCATION

10/2019 – 03/2023	PhD student at the Rheinland-Pfälzische Universität Kaiserslautern-Landau (RPTU), part of the DFG research training group 2360 *SystemLink*. Title of thesis: “Ecological effects of Bti-based mosquito control on aquatic-terrestrial linkages” Reviewers: Dr. habil. Carsten A. Brühl, Prof. Dr. Martin H. Entling
10/2016 – 08/2019	Master degree in Ecotoxicology (M. Sc.) at the University of Koblenz-Landau. Title of thesis: “Sublethal effects of folpet and glyphosate on *Rana temporaria* and *Bufo bufo* at environmentally relevant field rates”. Reviewers: Dr. habil. Carsten Brühl, Dr. Kathrin Theissinger
10/2012 – 08/2016	Bachelor degree in Environmental Sciences (B. Sc.) at the University of Koblenz-Landau. Title of thesis: “Effects of nano-TiO_2 on the photolysis of pesticides under differing UV intensities on the test organism *Daphnia magna*”. Reviewers: Jun- Prof. Dr. Mirco Bundschuh, Prof. Dr. Ralf Schulz
03/2012	German High School degree Abitur at the Otto Hahn-Gymnasium, Landau.

PROFESSIONAL EXPERIENCE

10/2019 – 03/2023	Scientific research associate, University of Koblenz-Landau, Germany.
02/2019 – 12/2019	Laboratory assistant at nEcoTox Consult GbR, Annweiler am Trifels, Germany.
09/2018 – 12/2018	Student’s assistant at working group Environmental Chemistry, University of Koblenz-Landau, Germany.
10/2017 – 03/2018	Tutor for the Master’s lecture “Advanced Environmental Chemistry” as part of the scholarship *Docendo discimus* by Studierendenkolleg, University of Koblenz-Landau, Germany.

06/2017 – 08/2017	Internship at Innovative Environmental Services Ltd. (IES), Witterswil, Switzerland.
07/2016 – 09/2016	Internship at the University of Sydney, Faculty of Agriculture and Environment, Sydney, Australia.
08/2014 – 09/2014	Internship at the Julius Kühn-Institute (Federal Research Center for Cultivated Plants), Siebeldingen, Germany.
09/2014	Internship at the Forest Research Institute Rheinland-Pfalz (Forest Ecology and Forestry), Trippstadt, Germany.

CONTRIBUTIONS TO SCIENTIFIC CONFERENCES

11/2022	SFE^2 / GfÖ / EES Joint Meeting, International Conference on Ecological Sciences, Metz, France (Onsite poster presentation). “Bti-based mosquito control agent alters natural benthic invertebrate community composition and Odonata emergence success in replicated pond systems”
08/2022	36th Congress of the Society of Limnology, Berlin, Germany (Onsite oral presentation). “Bti-based mosquito control agent alters natural benthic invertebrate community composition and Odonata emergence success in replicated pond systems”
07/2022	Young Researchers Symposium, Kaiserslautern, Germany. (Onsite oral presentation) “Bti-based mosquito control alters benthic invertebrate communities and reduces dragonfly emergence“
05/2022	32nd Annual Meeting of the Society of Environmental Toxicology and Chemistry, Copenhagen, Denmark (Onsite poster presentation). “Bti-based mosquito control agent alters natural benthic invertebrate community composition and Odonata emergence success in replicated pond systems”
05/2021	31st Annual Meeting of the Society of Environmental Toxicology and Chemistry Europe (Online poster presentation). “Specific mosquito control agent? Reduction of non-target organisms Chironomidae and Odonata observed in semi-field mesocosms treated with *Bacillus thuringiensis* var. *israelensis*” **PRICE FOR ‘BEST POSTER PRESENTATION – YOUNG SCIENTIST AWARD**

02/2019	8th Young Environmental Scientists (YES) Meeting, Ghent, Belgium (Onsite Poster Presentation). "Lethal and sublethal effects of folpet and glyphosate formulations on *Rana temporaria* at field relevant exposure levels."

PEER-REVIEWED PUBLICATIONS – PART OF THIS THESIS

Gerstle V, Manfrin A, Kolbenschlag S, Gerken M, Islam ASMMU, Entling MH, Bundschuh M, Brühl CA (2023). Benthic macroinvertebrate community shifts based on Bti-induced chironomid reduction also decrease Odonata emergence. *Environmental Pollution*, 316, 120488.

Gerstle V, Solanki P, Manfrin A, Kobenschlag S, Brühl CA (2023). Stress response of European common frog (*Rana temporaria*) tadpoles to Bti exposure in an outdoor pond mesocosm. *Bulletin of Environmental Contamination and Toxicology, 110*(4), 70.

Gerstle V, Bollinger E, Manfrin A, Pietz S, Kolbenschlag S, Feckler A, Entling MH, Brühl CA (submitted). Trophic effects of Bti-based mosquito control on two top predators in floodplain pond mesocosms.

OTHER PEER-REVIEWED PUBLICATIONS

Adams E, **Gerstle V**, Schmitt T, & Brühl CA. (2021). Co-formulants and adjuvants affect the acute aquatic and terrestrial toxicity of a cycloxydim herbicide formulation to European common frogs (*Rana temporaria*). *Science of The Total Environment*, *789*, 147865.

Adams E, **Gerstle V**, & Brühl CA. (2021). Dermal fungicide exposure at realistic field rates induces lethal and sublethal effects on juvenile European common frogs (*Rana temporaria*). *Environmental Toxicology and Chemistry*, *40*(5), 1289-1297.

Feckler A, Pietz S, Gonçalves S, **Gerstle V**, Risse-Buhl U, & Bundschuh M (2024). Detritivore physiology and growth benefit from algal presence during microbial leaf colonization. *Limnology and Oceanography*, 9999, 12530.

Ganglo C, Mendoza-Lera C, Manfrin A, Bolpagni R, **Gerstle V**, Kolbenschlag S, Bollinger E, Schulz R, & Lorke A (2023). Does biocide treatment for mosquito control alter carbon dynamics in floodplain ponds?. *Science of The Total Environment*, 161978.

Kolbenschlag S, **Gerstle V**, Eberhardt J, Bollinger E, Schulz R, Brühl CA, & Bundschuh M (2023). A temporal perspective on aquatic subsidy: Bti affects emergence of Chironomidae. *Ecotoxicology and Environmental Safety*, 250, 114503.

Kolbenschlag S, Bollinger E, **Gerstle V**, Brühl CA, Entling MH, Schulz R, & Bundschuh M (2023). Impact across ecosystem boundaries – Does Bti application change quality and composition of the diet of riparian spiders?. *Science of The Total Environment*, 162351.

Lüderwald S, Meyer F, **Gerstle V**, Friedrichs L, Rolfing K, Schreiner VC, Bakanov N, Schulz R, & Bundschuh M. (2020). Reduction of Pesticide Toxicity Under Field-Relevant

Conditions? The Interaction of Titanium Dioxide Nanoparticles, Ultraviolet, and Natural Organic Matter. *Environmental Toxicology and Chemistry*, *39*(11), 2237-2246.

Manfrin A, Schirmel J, Mendoza-Lera C, Ahmed A, Bohde R, Brunn M, Brühl CA, Buchmann C, Bundschuh M, Burgis F, Diehl D, Entling MH, Ganglo C, Geissler S, **Gerstle V**, Girardi JP, Graf T, Huszarik M, Jamin J, Joschko TJ, Jungkunst HF, Knäbel A, Kolbenschlag S, Lorke A, Muñoz K, Ogbeide C, Osakpolor SE, Pietz S, Riess K, Roodt AP, Rovelli L, Röder N, Rösch V, Schaumann GE, Schäfer RB, Schmitt T, Schmitz D, Schützenmeister K, Schwenk K, Stehle S, & Schulz R (2023). SystemLink: Moving beyond Aquatic–Terrestrial Interactions to Incorporate Food Web Studies. Bulletin Limnology and Oceanography, 32(2), 10557.

Stoll VS, Röder N, **Gerstle V**, Manfrin A, & Schwenk K (under review). Effects of Bti on the diversity and community composition of three Chironomidae subfamilies across different micro-habitats.