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

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ABSTRACT

Chironomid larvae (Diptera: Chironomidae) 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. 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., 1995). 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., 2010). 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., 2010; 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 ≈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 (Fig. 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 et al. (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).
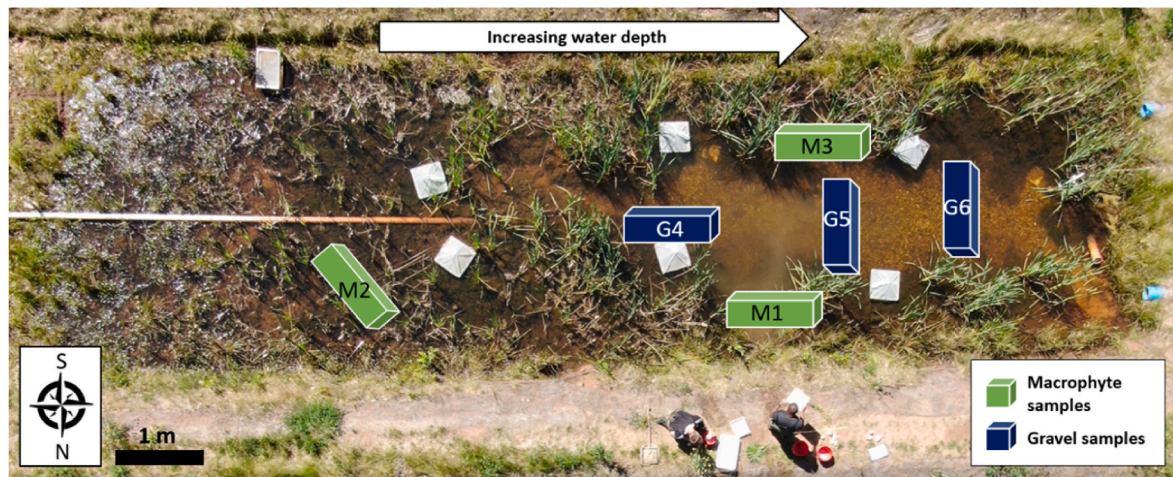
Fig. 1. Top view of one floodplain pond mesocosm (A ≈ 104 m^2) with three sampling transects (1 m) for each habitat (macrophytes as green polygons and gravel as blue polygons) using a standard kicknet sampler (25 × 25 cm; mesh size = 500 μm). The kicknet was dragged along the bottom of the mesocosm while disturbing the sediment by foot. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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 (Fig. 1). In order to collect samples representative for the benthic invertebrate community and cover FPM habitat variability, we sampled three 1-m transects for each habitat in each FPM (see Fig. 1) using a kicknet sampler (25 × 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 macro-invertebrates (see taxonomic resolution in Table 1), we used a stereo-microscope (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 & Seid-enbusch (2002).

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 min. 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 plots of residuals versus fitted values (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

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

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 ≈ 0.04; Fig. 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; Fig. 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 ≈ 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 ≈ 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 ≈ 0.02), and a similar pattern, although not statistically significant, for the other subfamilies of Orthocladiinae (~62%; $F_{1,10} = 2.92$; p ≈ 0.12) and Tanypodinae (~18%; $F_{1,10} = 0.17$; p ≈ 0.69; Fig. 2b).

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 (Fig. 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.

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).

3.3. Emerging Odonata

A total of 1717 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; Fig. 4) in Bti-treated FPMs. Aeshnidae and Libellulidae were largely dominated by two genera, *Anax* and *Sympetrum*, respectively.
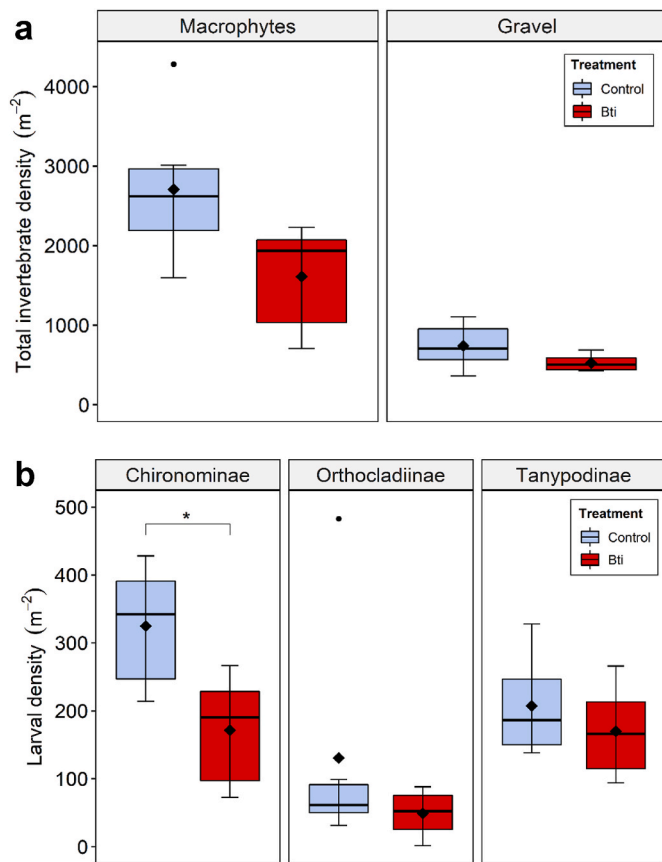
Fig. 2. (a) Total invertebrate density (number of individuals per m²) in macrophytes and gravel habitats and in control (blue; n = 6) and Bti-treated FPMs (red; n = 6), based on habitat means (n = 3) within each FPM. (b) Chironomid larval density of the three identified chironomid subfamilies in control 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 (*). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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	

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., 2022). Since non-target chironomids and target mosquitoes are physiologically similar, chironomids
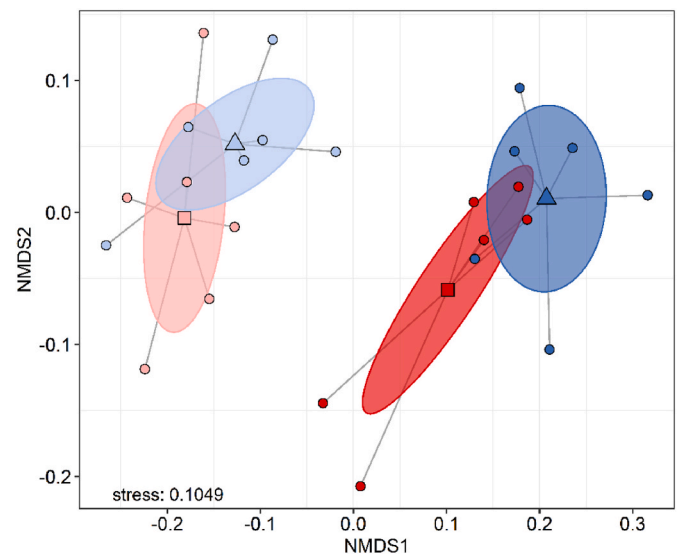

Fig. 3. Non-metric multidimensional scaling (nMDS) ordination plot illustrating differences in benthic macroinvertebrate community composition between treatment (red squares; n = 6) and control FPMs (blue triangles; n = 6), and habitats gravel (light color; n = 6) and macrophytes (dark color; n = 6), including 95% confidence ellipses. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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 m²) 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.

Taxa	Average density (m⁻²)		Contributing percent	Percent difference
	Control	Treatment		
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%

Table 4

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

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

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
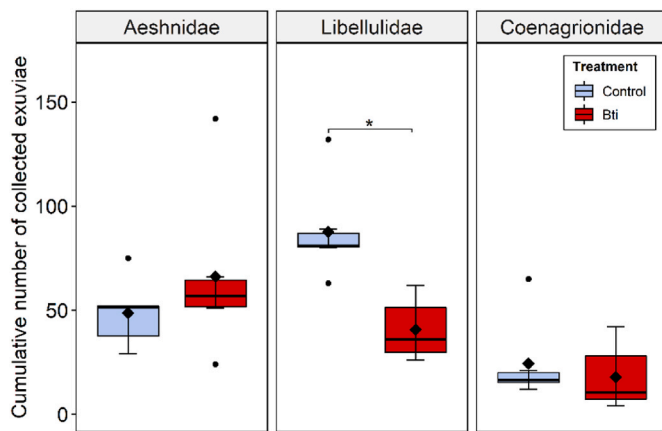
Fig. 4. Cumulative number of exuviae in control (blue; n = 6) and Bti-treated FPMs (red; n = 6) for Aeshnidae (A), Libellulidae (B), and Coenagrionidae (C), collected from June to September 2021. Lower and upper box boundaries show 25th and 75th percentiles, respectively, line inside box show medians 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 (*). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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 et al. (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. (2022) 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 10–15 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 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, Fig. 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 (Fig. 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* (van Buskirk, 1992). 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., 2022). 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.

Author contributions (Credit)

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

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.120488.

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