Mosquito control based on *Bacillus thuringiensis israelensis* (Bti) - Ecological effects on wetland food chains and public acceptance of control alternatives

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Stechmückenbekämpfung mit *Bacillus thuringiensis israelensis* (Bti) – ökologische Effekte auf die Nahrungskette in Feuchtgebieten und Akzeptanz von Alternativen in der Bevölkerung

by

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LIST OF PUBLICATIONS

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

I. 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, 13565. DOI: 10.1038/s41598-017-14019-2 **[APPENDIX I]**

II. **Allgeier, S.,** Kästel, A., Brühl, C.A. (2019). 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. DOI: 10.1016/j.ecoenv.2018.11.050 **[APPENDIX II]**

III. Theissinger, K., Kästel, A., Elbrecht, V., Makkonen, J., Michiels, S., Schmidt, S., **Allgeier, S**., Brühl, C.A. (2018). Using DNA metabarcoding for assessing chironomid diversity and community change in mosquito controlled temporary wetlands. *Metabarcoding and Metagenomics* 2: e21060. DOI: 10.3897/mbmg.2.21060 **[APPENDIX III]**

IV. Theissinger, K., Röder, N., **Allgeier, S**., Beermann, A., 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. DOI: 10.1111/mec.15214 **[APPENDIX IV]**

V. **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. DOI: 10.1016/j.envres.2018.01.010 **[APPENDIX V]**

VI. **Allgeier, S.,** Friedrich, A., Brühl, C.A. (2019). Mosquito control based on Bacillus thuringiensis israelensis (Bti) interrupts artificial wetland food chains. *Science of the Total Environment* 686: 1173-1184. DOI: 10.1016/j.scitotenv.2019.05.358 **[APPENDIX VI]**

Publications I and IV are available open access and II, V and VI are reprinted with permission of the publisher.

TABLE OF CONTENT

ABBREVIATION LIST

AChE	Acetylcholinesterase
BG	Biogents
Bti	*Bacillus thuringiensis israelensis*
CI	Confidence interval
CVM	Contingent valuation method
EC_{50}	Effect concentration 50%
ERA	Environmental risk assessment
FR	Field rate
GR	Glutathion reductase
GST	Glutathion-S-transferase
HLR	Human Landing Rate
ITU	International Toxic Units
GMCA	German mosquito control association (German: KABS e.V.)
LC_{50}	Lethal Concentration 50%
NTO	Non-target organism
OTU	Operational taxonomic unit
PEC	Predicted environmental concentration
PNEC	Predicted no effect concentration
SE	Standard error
SETAC	Society of Environmental Toxicology and Chemistry
SVL	snout to vent length
TTM	time to metamorphosis
OECD	Organization for Economic Co-operation and Development

THESIS SUMMARY

English abstract

The bio-insecticide *Bacillus thuringiensis israelensis* (Bti) has worldwide become the most commonly used agent in mosquito control programs that pursue two main objectives: the control of vector-borne diseases and the reduction of nuisance, mainly coming from mosquitoes that emerge in large quantities from seasonal wetlands. The Upper Rhine Valley, a biodiversity hotspot in Germany, has been treated with Bti for decades to reduce mosquito-borne nuisance and increase human well-being. Although Bti is presumed to be an environmentally safe agent, adverse effects on wetland ecosystems are still a matter of debate especially when it comes to long-term and indirect effects on non-target organisms. In light of the above, this thesis aims at investigating direct and indirect effects of Bti-based mosquito control on non-target organisms within wetland food chains. Effects were examined in studies with increasing eco(toxico)logical complexity, ranging from laboratory over microcosm to field approaches with a focus on the non-biting Chironomidae and amphibian larvae (*Rana temporaria, Lissotriton sp.*). In addition, public acceptance of environmentally less invasive alternative mosquito control methods was evaluated within surveys among the local population.

Chironomids were the most severely affected non-target aquatic invertebrates. Bti substantially reduced larval and adult chironomid abundances and modified their species composition. Repeated exposures to commonly used Bti formulations induced sublethal alterations of enzymatic biomarkers activity in frog tadpoles. Bti-induced reductions of chironomid prey availability indirectly decreased body size of newts at metamorphosis and increased predation on newt larvae in microcosm experiments. Indirect effects of severe reductions in midge biomass might equally be passed through aquatic but also terrestrial food chains influencing predators of higher trophic levels. The majority of affected people in the Upper Rhine Valley expressed a high willingness to contribute financially to environmentally less harmful mosquito control. Alternative approaches could still include Bti applications excepting treatment of ecologically valuable areas. Potentially rising mosquito levels could be counteracted with local acting mosquito traps in domestic and urban areas because mosquito presence was experienced as most annoying in the home environment.

As Bti-based mosquito control can adversely affect wetland ecosystems, its large-scale applications, including nature conservation areas, should be considered more carefully to avoid harmful consequences for the environment at the Upper Rhine Valley. This thesis emphasizes the importance to reconsider the current practice of mosquito control and encourage research on alternative mosquito control concepts that are endorsed by the local population. In the context of the ongoing amphibian and insect declines further human-induced effects on wetlands should be avoided to preserve biodiversity in functioning ecosystems.

German abstract (Zusammenfassung)

Das biologische Insektizid *Bacillus thuringiensis israelensis* (Bti) ist das weltweit am häufigsten eingesetzte Mittel zur Stechmückenbekämpfung. Die Bekämpfung verfolgt dabei zwei Hauptziele: die Kontrolle vektorübertragener Krankheiten und die Reduktion der Belästigung durch Stechmücken, die massenhaft aus temporären Feuchtgebieten schlüpfen. Eines der behandelten Gebiete ist das Oberrheintal, ein Biodiversitäts-Hotspot in Deutschland. Bti wird dort seit Jahrzehnten ausgebracht, um die Belästigung durch Stechmücken zu reduzieren und somit das menschliche Wohlbefinden zu steigern. Obwohl Bti bisher als umweltfreundlicher Wirkstoff angesehen wird, werden negative Auswirkungen auf die Ökosysteme von Feuchtgebieten und insbesondere die langfristigen und indirekten Auswirkungen auf Nichtzielorganismen, fortlaufend wissenschaftlich diskutiert. Angesichts dessen hat diese Arbeit zum Ziel, direkte und indirekte Auswirkungen der Stechmückenbekämpfung mit Bti auf Nichtzielorganismen innerhalb der Nahrungsketten von Feuchtgebieten zu untersuchen. Betrachtet wurden dabei die nicht stechenden Zuckmücken (Chironomidae) und Amphibienlarven (*Rana temporaria, Lissotriton sp.*) in Studien mit zunehmender öko(toxiko)logischer Komplexität, von Laborexperimenten über Mesokosmos- bis hin zu Freilandstudien. Darüber hinaus wurde die Akzeptanz umweltschonenderer alternativer Methoden zur Stechmückenbekämpfung auf der Grundlage von Befragungen der betroffenen Bevölkerung bewertet. Zuckmücken waren die am stärksten betroffenen aquatischen Nichtziel-Invertebraten. Die Behandlung mit Bti führte zu einer beträchtlichen Reduktion der Anzahl von Larven als auch adulten Zuckmücken und änderte deren Artzusammensetzung. Eine wiederholte Exposition gegenüber häufig verwendeten Bti-Formulierungen führte bei Kaulquappen zu subletalen Effekten, gezeigt durch Veränderungen von enzymatischen Biomarkern. Durch die verringerte Verfügbarkeit von Zuckmücken als Beute wurden Molchlarven in Mesokosmen indirekt beeinflusst, indem sie erhöhter Prädation ausgesetzt und bei der Metamorphose etwas kleiner waren. Die Effekte einer verringerten Mückenbiomasse könnten sich gleichermaßen auf aquatische sowie terrestrische Nahrungsketten auswirken und indirekt auch Räuber höherer trophischer Ebenen beeinflussen. Die Mehrheit der betroffenen Bevölkerung wäre unter solchen Umständen bereit, einen finanziellen Beitrag zu einer umweltschonenderen Stechmückenbekämpfung zu leisten. Alternative Bekämpfungskonzepte könnten weiterhin eine Anwendung von Bti beinhalten, jedoch unter Ausnahme der Behandlung in ökologisch wertvollen Gebieten. Da Stechmücken in der häuslichen Umgebung als besonders lästig empfunden wurden, könnte einem erhöhten Vorkommen von Stechmücken in häuslichen und städtischen Gebieten mit lokal-wirkenden Stechmückenfallen vorgebeugt werden. Aufgrund der möglichen negativen Auswirkungen auf die Ökosysteme in Feuchtgebieten sollte die großflächige Ausbringung von Bti vor allem in Naturschutzgebieten sorgfältiger geprüft werden, um schädliche Folgen für die Umwelt im Oberrhein zu vermeiden. Diese Arbeit verdeutlicht, dass die derzeitige

Praxis der Stechmückenbekämpfung überdacht und die Forschung an alternativen, von der lokalen Bevölkerung unterstützen Konzepten in Zukunft intensiviert werden sollte. Um die Biodiversität in Feuchtgebieten zu erhalten sollten weitere vom Menschen verursachte Auswirkungen weitgehend vermieden werden, vor allem vor dem Hintergrund des anhaltenden Populationsrückgangs bei Insekten und Amphibien.

1 INTRODUCTION

1.1 Mosquitoes and the human population

Mosquitoes affect the health and well-being of human populations for two main reasons: the transmission of mosquito-borne diseases and the nuisance associated with mosquito bites (Becker et al., 2010). On a global scale, the greatest concern about mosquitoes is their vector competence for transmitting diseases such as malaria, dengue, Zika, Chikungunya or West Nile virus. The threat of these diseases has increased over the last three decades and accounted for 17% of the estimated global burden of all infectious illnesses (Vega Rúa and Okech, 2019). The worldwide spread of tropical vector mosquitoes was facilitated by accelerated urbanization, global warming and the intensification of trade and travel which led to a migration into temperate zones in the United States and Europe (Enserink, 2008; Medlock et al., 2012; Vega Rúa and Okech, 2019). From more than 3500 recorded mosquito species worldwide, about 100 traverse the European continent (Becker et al., 2010). After the first record of the invasive Asian tiger mosquito (*Aedes albopictus*) in Albania in 1979 (Adhami and Reiter, 1998), its rapid spread and establishment in Europe has given major rise to public concern (Becker et al., 2017; Medlock et al., 2012). Autochthonous transmission of dengue and Chikungunya by *Ae. albopictus* were recently recorded in Southern Europe (Calba et al., 2017; Succo et al., 2016). Nevertheless, Germany is free of autochthonous transmission since the elimination of malaria in the 50's which was mainly achieved due to socio-economic improvements (Falkenhorst et al., 2018; Zhao et al., 2016).

Aside from the global impact of disease-carrying species on public health, temperate regions face seasonal outbreaks of mosquitoes that cause nuisance in recreational and residential areas. Mosquitoes are widely considered incompatible with human life quality as they prevent people from enjoying outdoor activities and can also negatively affect the economy by discouraging recreation, tourism and outdoor labor (Halasa et al., 2014; Hirsch and Becker, 2009). Nuisance due to high mosquito activity and their bites is considered even more restrictive for people than being exposed to vector-borne diseases (Dickinson and Paskewitz, 2012). Because of these implications for the human population, extensive mosquito control activities were implemented (Becker et al., 2010; Bithas et al., 2018; Fonseca et al., 2013; Medlock et al., 2012).

1.2 Mosquito control

In the late nineteenth century, first mosquito control attempts focused on environmental management designed to suppress mosquito populations with the help of mosquito predators such as fish (Bellini et al., 1994; Lamborn, 1890). The invention of highly effective synthetic insecticides such as DDT led to an increased usage of chemicals belonging to the major groups of chlorinated hydrocarbons, organophosphates, carbamates and pyrethroids (Becker et al., 2010; van den Berg et al., 2012). The widespread application of chemical insecticides had long-term side effects such as the development of insect resistances over many successive generations (Coetzee and Koekemoer, 2013; Hemingway and Ranson, 2000; van den Berg et al., 2015) and adverse effects on the environment and human health (Boyce et al., 2007; Hemingway and Ranson, 2000; Rogan and Chen, 2005; Sharma, 2001). Consequently, the usage of more specifically acting and less harmful bio-insecticides increased substantially over the last decades.

Mosquitoes breed in a variety of aquatic habitats with stagnant water which is why control strategies have been adapted to their breeding sites. For instance, vectors such as *Ae. albopictus* and *A.s aegypti* are typical container-inhabiting mosquitoes associated with human habitats where they utilize artificial human-made breeding sites such as tires, catch basins, bins but also knotholes (Becker et al., 2017; Medlock et al., 2012; Vega Rúa and Okech, 2019). Conversely, floodwater species such as *Ae. vexans* and *Ae. sticticus* hatch simultaneously in massive numbers after flooding events along rivers which poses immense nuisance for people living next to inundation areas (Becker, 2006; Schäfer and Lundström, 2014). There are different wetland types that produce a considerable number of floodwater mosquitoes ranging from coastal wetlands influenced by ocean tides to constructed and freshwater wetlands that are regularly flooded (Becker et al., 2010; Rey et al., 2012). Nuisance control requires extensive treatments of mosquito larvae in such wetlands while vector control is mostly performed locally around urban breeding sites (van den Berg et al., 2012).

Wetlands represent important habitats providing essential social, economic and ecological services for the human population. However, there is a controversy about the need for mosquito control in wetlands and the environmental compatibility of these anthropogenic interferences (Rey et al., 2012). Seasonal wetlands and flood areas of small creeks are also suitable breeding sites for many valuable aquatic organisms since they provide an aquatic environment with standing water free of predatory fish (Batzer and Wissinger, 1996;

Blaustein and Margalit, 1996; Rubbo et al., 2011). In Europe, mosquito control is often implemented in wetlands situated in national nature conservation sites and areas of international importance (Ramsar Convention, Natura 2000) that are designed to preserve biodiversity (Lagadic et al., 2014; Land and Miljand, 2014; Schrauth and Wink, 2018; Swedish Chemicals Agency, 2015).

Simultaneously protecting human health and the environment has always been the major challenge in large-scale mosquito control. Novel and eco-friendly strategies that are not based on the use of insecticides are increasingly investigated (Benelli, 2015; Benelli et al., 2016; Benelli and Mehlhorn, 2016). These methods include the usage of repellents (Park et al., 2005; Semmler et al., 2009; Sharma, 2001), natural predators (Acquah-Lamptey and Brandl, 2018; Brodman and Dorton, 2006; Elono et al., 2010; Soumare and Cilek, 2011), natural ecological traps influencing oviposition (Gardner et al., 2018), mechanical traps for adults (Englbrecht et al., 2015; Jackson et al., 2012; Poulin et al., 2017), nanoparticles (Govindarajan et al., 2016), and active citizen participation (Johnson et al., 2018). In addition, the modification and eradication of vector mosquitoes is achieved by gene-editing (CRISPR-Cas9) (Gantz et al., 2015; Kyrou et al., 2018) or the release of mosquito males infected with the bacteria *Wolbachia pipientis* (Walker et al., 2011) or sterilized with gamma-radiation (Ricardo Machi et al., 2019). The ecological impact of eradicating mosquitoes is still difficult to predict because mosquitoes also serve important functions in numerous ecosystems (Fang, 2010; Pugh, 2016; Webber et al., 2015). Mosquitoes are a substantial part of the biomass in a wide range of wetlands and prey for many aquatic and terrestrial predators (Becker et al., 2010; Shaalan and Canyon, 2009). In addition, adult mosquitoes play a so far underestimated role in pollination (Lahondère et al., 2019; Peach and Gries, 2016).

1.3 The biocide *Bacillus thuringiensis* var. *israelensis* (Bti)

Subspecies of the bacterium *Bacillus thuringiensis* (Bt) are the most successful insect pathogens used to protect crops and forests from insect pests (Hilbeck et al., 2017; Tabashnik et al., 2013; UBA and BfN, 2018) but also animals and humans from midges (Després et al., 2011; Lawler, 2017). The subspecies *Bacillus thuringiensis* var. *israelensis* (Bti) acts as bacterio-insecticide against the larvae of Culicidae (mosquitoes), black flies (Simuliidae) and several other midges (Boisvert and Boisvert, 2000). On a global scale, Bti is largely applied for human health issues in subtropical and tropical urban breeding sites (van den Berg et al., 2012) with application amounts of 70-300 tons of formulated product per year (van den Berg et al., 2012). Comparatively, the output quantity of organophosphates for mosquito control amounts to 163 tons per year (van den Berg et al., 2012). In Europe, insecticidal products that are not used in an agricultural context are regulated according to the biocide regulation EU 528/2012. Their impact on humans, animals and the environment is required to be assessed within this framework. There are currently ten products containing the active ingredient Bti strain AM65-52 (serotype H-14) that are approved to be applied in 22 European countries aerially by helicopter and on the ground by spray or hand (ECHA, 2019). Due to its high specificity against midges, Bti it is the only agent allowed to be applied directly into the water phase of European wetlands (Benelli et al., 2016; Després et al., 2011). Bti-treated water bodies range from human-constructed habitats (irrigation ditches, containers, water tanks) to natural wetlands including marshes, temporary flooded wetlands and ponds (Becker et al., 2010; Lawler, 2017).

1.3.1 Toxic mode of action

The toxicity and specificity in the mode of action is related to a toxin mixture of four toxins produced during bacterial sporulation: three Cry toxins (Cry4Aa, Cry4Ba and Cry11Aa) and one Cyt toxin (Cyt1Aa) (Ben-Dov, 2014; Vachon et al., 2012). After ingestion, Bti crystals are solubilized in the alkaline larval midgut where they get activated *in vivo* into toxins by midgut proteases (Fig. 1). Following this activation, toxins bind to specific receptor sites in the outer membrane of the gut cells, oligomerize and form pores in cell membranes (Fig. 1).

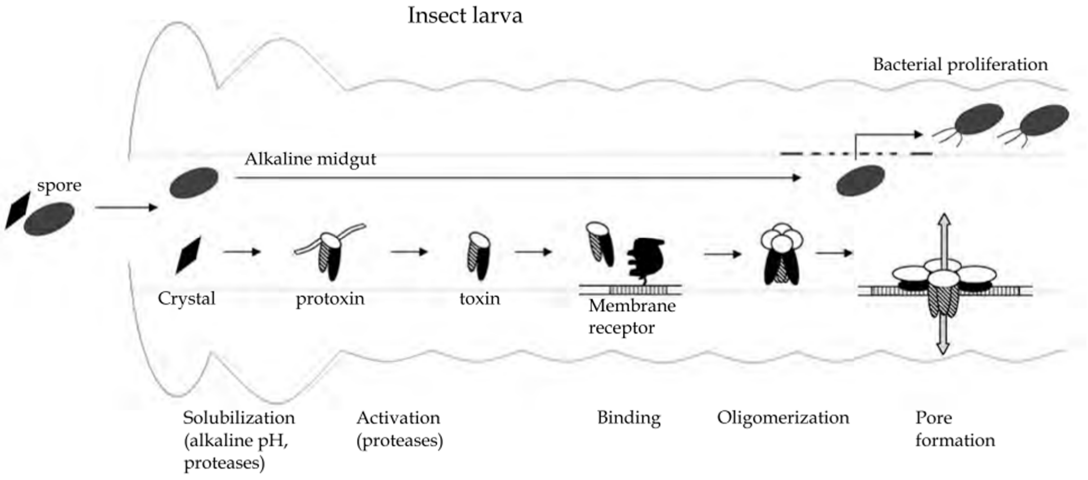


Figure 1: Model of the mode of action of Bti Cry toxins in the insect midgut (figure from Després et al., (2011))

Depending on the ingested amount, a fast larval death (minutes to hours) is induced by the formation of pores that ultimately leads to gut disruption (Ben-Dov, 2014; Bravo et al., 2011; Vachon et al., 2012). The preceding activation of the toxins depends on several factors such as the alkaline condition and the number of receptors in the midgut membrane resulting in varying susceptibilities of targeted species (Bravo et al., 2007). Cry and Cyt toxins have major structural and functional differences, e.g. the Cyt toxin increases toxicity of Cry toxins and most importantly, impede the development of insect resistance (Pardo-López et al., 2013; Soberón et al., 2013; Wirth et al., 2005). Whereas the development of resistance to individual toxins is possible (Stalinski et al., 2014; Wirth et al., 2012), resistance has not been observed to the toxin mixture in the field (Becker et al., 2018; WHO, 2005).

In mosquito control, the larvicide is applied or sprayed as formulations containing the active ingredient Bti (strain AM 65-52) as a mixture of spores and toxin-including crystals (Fig. 1). The persistence of individual crystals varies and depends on the environment whereas immobilized crystals can conserve insecticidal activity up to three weeks (Boisvert and Boisvert, 1999; Ohana et al., 1987; Sheeran and Fisher, 1992; Tetreau et al., 2012). The spores, as the long-lived components of the bacterium, can still be detected months to years after continuous applications (Duchet et al., 2014; Guidi et al., 2011; Respinis et al., 2006). Thus, Bti that is applied in Germany must be sterilized by gamma radiation before application to remove viable spores or cells and prevent de novo sporulation and recycling of the spores (Becker et al., 2018; Melo-Santos et al., 2009; Tilquin et al., 2008).

1.3.2 Effects on non-target fauna

Due to the specific toxic mode of action and the absence of receptor sites in other organisms, Bti is considered to be harmless for vertebrates and aquatic non-target organisms (NTO) such as molluscs, crustacean and most insects (Boisvert and Boisvert, 2000; Lacey and Merritt, 2003; Lagadic et al., 2014; Lawler, 2017; WHO, 1999). However, non-biting midges of the family Chironomidae also show a high susceptibility to Bti because they are phylogenetically closely related to mosquitoes (Hughes et al., 2005; Stevens et al., 2005). Bti is even actively applied against chironomids to reduce nuisance associated with their ability to form huge swarms upon emergence (Ali et al., 2008; Vaughan et al., 2008). Since most chironomids are assumed to react less sensitive than mosquitoes (Becker and Lüthy, 2017; Boisvert and Boisvert, 2000; Yiallouros et al., 1999) it is deduced that field populations are not impaired at regular mosquito control rates (Lundström et al., 2010a; WHO, 1999). Chironomid communities in the field can be highly diverse in terms of species compositions and age structures (Armitage et al., 1995), which influences their sensitivity. Younger larvae are more susceptible to Bti (Kästel et al., 2017) and different sensitivities occur among species (Yiallouros et al., 1999) and subfamilies (Liber et al., 1998). The impact of Bti treatment on chironomids in the field is heavily debated in the scientific community. Some studies have shown reduced chironomid abundances in wetlands after regular Bti-based mosquito control activities (Hershey et al., 1998; Jakob and Poulin, 2016) while others did not find negative effects of Bti on chironomids in the field (Duchet et al., 2015; Lagadic et al., 2016; Lundström et al., 2010b; Wolfram et al., 2018).

Chironomids usually constitute a major proportion of invertebrate biomass and secondary production in lotic and lentic systems (Allgeier et al., 2019; Leeper and Taylor, 1998; Lundström et al., 2010b; Williams, 2006). They also contribute considerably to species diversity (Lundström et al., 2010a; Theissinger et al., 2018; Wolfram et al., 2018) and ecological diversity (Ferrington, 2008). Their high protein content and digestibility make them a quality food resource for both aquatic (amphibians, fish, insects) and terrestrial (birds, bats, spiders, insects) predators (Armitage et al., 1995; Arnold et al., 2000; Fard et al., 2014; Gergs et al., 2014; Jakob and Poulin, 2016; Pfitzner et al., 2015; Poulin et al., 2010; Quirino et al., 2017; Wesner, 2010). Chironomids represent important links between the aquatic and the terrestrial food web (Hoekman et al., 2011). Consequently, Bti-induced changes in chironomid abundances may have severe consequences on wetland food webs (Jakob and Poulin, 2016; Poulin et al., 2010; Schulz et al., 2015).

Shortly after discovery of its toxic effects on nematocerans insect larvae (Diptera: Nematocera) in Israel (Goldberg and Margalit, 1977), research focused on the efficacy of Bti against its target species and the direct effects on non-target fauna (Poulin, 2012) (Fig. 2). However, (semi-)field investigations addressing indirect effects via the food web have received less attention in the first 25 years of globally increasing Bti usage (Fig. 2). The number of studies that deal with the effects on trophic interactions within ecosystems has not substantially increased in recent years despite this apparent knowledge gap (Jakob and Poulin, 2016; Pauley et al., 2015; Poulin, 2012).

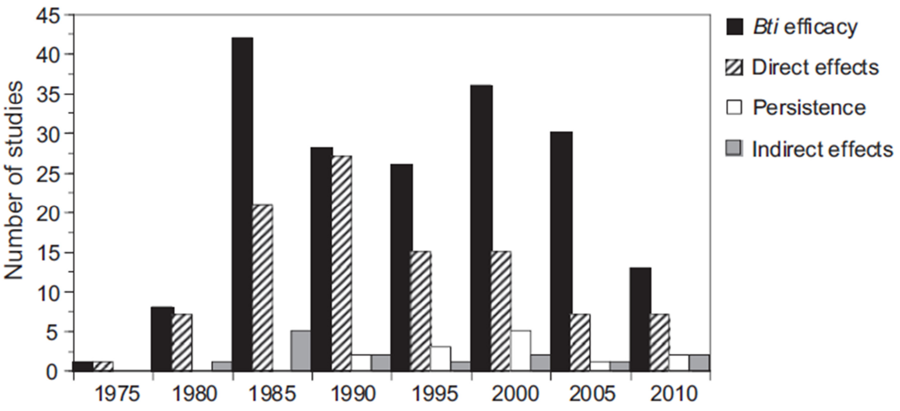


Figure 2: Studies dealing with Bti use in the environment including efficacy against the target organism, persistence, direct and indirect effect on the non-target fauna from 311 studies published between 1974 and 2010 (Figure taken from Poulin (2012)).

1.4 Human-induced loss of biodiversity

The ongoing global loss of biodiversity is one of the most critical environmental problems which threatens crucial ecosystem processes and services that are partly essential services for human societies and their well-being (Cardinale et al., 2012; Díaz et al., 2006; Mace et al., 2012). The continued growth of human population which is accompanied by habitat construction, the release of toxic pollutants, transport of invasive species and climate disruption further intensified population and species losses that led to an accelerated human-induced sixth mass extinction crisis (Butchart et al., 2010; Ceballos et al., 2015; Ceballos and Ehrlich, 2010). Most attention was previously given to worldwide population declines of common and rare species of vertebrates (Ceballos et al., 2017, 2015), but entomofauna is also heavily affected (Hallmann et al., 2017; IPBES, 2016; Powney et al., 2019).

In 2019, there were 98.512 species evaluated on the International Union for Conservation of Nature Red List, of which 28% are threatened with extinction (IUCN, 2019). Among all vertebrates, amphibians are currently the most globally threatened group (40% classified as endangered) with declining populations at alarming rates (IUCN, 2019; Stuart et al., 2004; Wake and Vredenburg, 2008). Most negative effects on amphibians are byproducts of human activities. Amphibians have been severely impacted by habitat destruction, climate change, disease spreading and the growing rate of human-induced environmental contamination, most notably the increasing usage of pesticides in agricultural landscapes (Brühl et al., 2013; Gallant et al., 2007; Pounds et al., 2006; Sparling et al., 2001). Declining amphibian populations were also observed in protected, natural areas where amphibians are exposed to other human-induced modifications of natural wetlands such as the introduction of nonnative species (Collins and Storfer, 2003; Davidson and Knapp, 2007; Knapp et al., 2001).

Many insect species are undergoing declines in their diversity and abundance. This pattern is well studied for certain flower visiting insect taxa such as bees (Goulson et al., 2015, 2007; Potts et al., 2010; Vray et al., 2019) and moths (Dennis et al., 2017; van Swaay et al., 2013). There is continuously growing evidence that the declines in individual taxa reflect the status of the entire entomofauna (Hallmann et al., 2017; Lister and Garcia, 2018; Shortall et al., 2009). Urbanization, agricultural intensification and pollution through pesticides are among the main drivers of insect decline (Dennis et al., 2017; Hahn et al., 2015; IPBES, 2016; Jones and Leather, 2013; Ollerton, 2017). Apart from agroecosystems, substantially declining flying insect biomass over a span of 27 years was also recorded in German nature reserves (Hallmann et al., 2017). Insects fulfill several ecosystem services such as dung burial, pest control, nutrient cycling, pollination or wildlife nutrition (Kremen and Chaplin-Kramer, 2007; Losey and Vaughan, 2006; Saunders, 2018; Yang and Gratton, 2014). Insect decline is therefore also threatening human welfare as well as nature conservation e.g. by cascading effects in food webs (Benton et al., 2002; Schrauth and Wink, 2018; Stanton et al., 2017; Vickery et al., 2001).

2 STUDY AREA

The study area was the Upper Rhine Valley (Germany) where wetlands have been extensively treated with Bti for 40 years with the objective of reducing nuisance for the local population (Fig. 3) (Becker et al., 2018). The Upper Rhine Valley is one of 30 biodiversity hotspots in Germany (Ackermann et al., 2012). Moreover, it is registered as a wetland of international importance (Ramsar Convention) and includes several protected areas ranging from national to international conservation areas (bird sanctuaries, nature reserves, Natura 2000 sites) with temporary pools comprising high biodiversity (Biggs et al., 2017; Lukács et al., 2013). Bti is largely applied in wetlands that are part of this nature conservation network. The vast majority (87%) of all Bti-treated wetlands in the federal state Rhineland-Palatinate are located in nature protection.

Figure 3: Bti-treated wetlands in the Upper Rhine Valley ranging from forests (A), over floodplains (B) to meadows (C) and the Bti application conducted by helicopter (D).

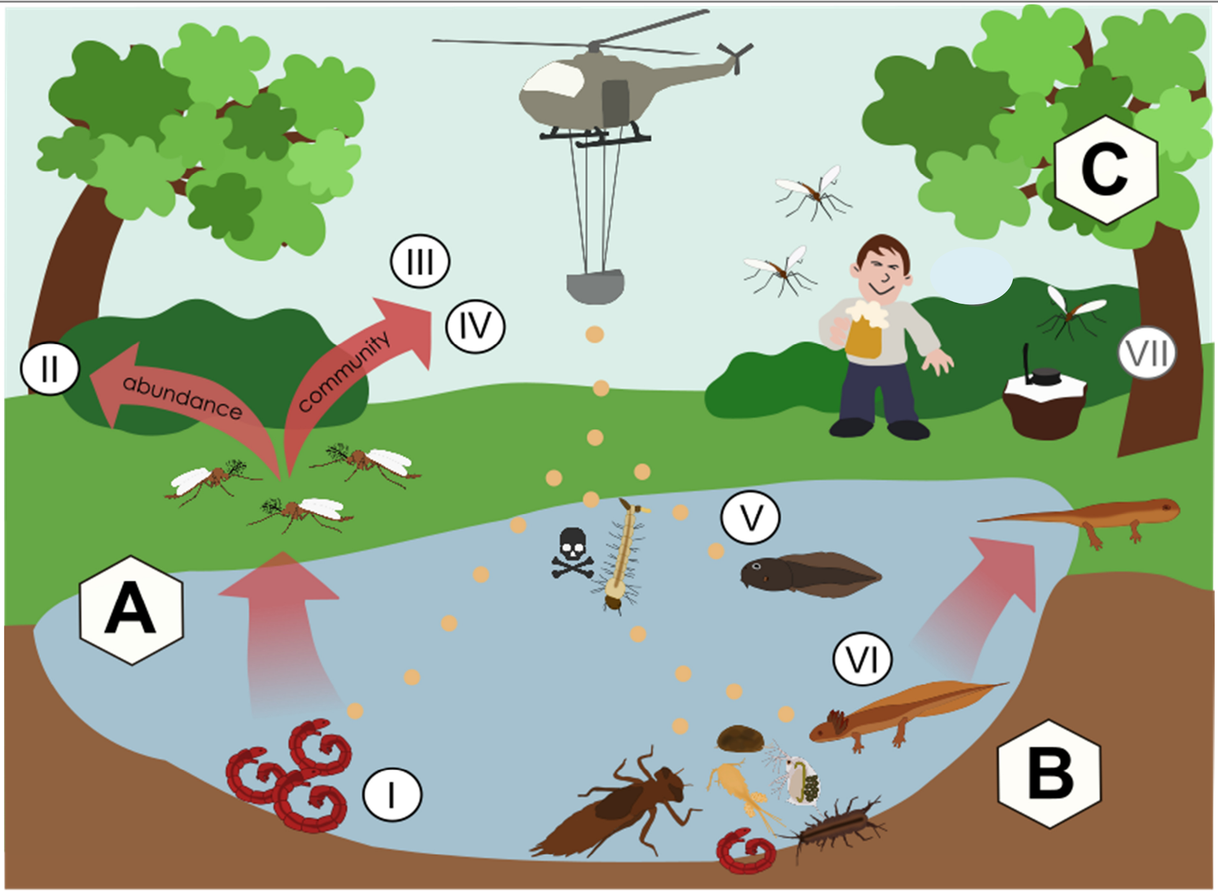
The GMCA regularly applies two commercially available Bti formulations - a water dispersible granule formulation named VectoBac®WG and an aqueous suspension called VectoBac®12AS - in three delivery forms depending on the application type (Table 1). Two thirds of the application are treated aerially by helicopters using Bti ice granules (IcyPearls®) (Fig. 3D) while knapsack sprayers and sand granules are deployed for ground application of the remaining area (Becker et al., 2018). At sites with deeper water (> 10 cm), high mosquito populations densities and older mosquitoes (3rd, 4th instar stages), the low field rate (FR) is routinely increased to a high FR to achieve sufficient control (BAuA, 2018; Becker, 2003). All Bti applications conducted in this thesis were performed by or adapted to the mosquito control strategy of the GMCA.

Table 1: Application rates and toxicity (ITU = International Toxic Unit) of three Bti delivery forms (Ice, Liquid, Sand) in their low (1x) and high (2x) field rate (FR) frequently applied in the German mosquito control strategy [Table adapted from Appendix V].

Delivery form	**IcyPearls® (Ice)**	**Sand granules (Sand)**	**Solution (Liquid)**
Formulation	VectoBac®WG 37.4% a.i., 3000 ITU/mg	VectoBac®WG 37.4% a.i., 3000 ITU/mg	VectoBac®12AS 11.6% a.i., 1200 ITU/mg
Low FR (1x)	15 kg (1.44 × 10^9 ITU) /ha	25 kg (1.2 × 10^9 ITU) /ha	2 L (1.92 × 10^9 ITU) /ha
High FR (2x)	30 kg (2.88 × 10^9 ITU) /ha	50 kg (2.4 × 109 ITU) /ha	4 L (3.84 × 10^9 ITU) /ha

3 OBJECTIVES AND THESIS STRUCTURE

This thesis focused on the identification and characterization of the eco(toxico)logical implications associated with Bti-based mosquito control on NTO with special emphasis on chironomids and amphibians. In light of the potential adverse effects of the current large-scale mosquito control strategy, this thesis further assessed local populations' attitude towards mosquitoes and the acceptance of alternative control methods based on mosquito traps. Thus, this thesis is subdivided into three main research objectives (A, B, C) to answer three overall research questions (RQ) displayed in Figure 4. Research objective A is based on four underlying scientific publications [Appendix I-IV], B includes two scientific publications [Appendix V, VI] and C is based on unpublished data [Appendix VII].



A

UNDERESTIMATED EFFECTS ON NON-TARGET CHIRONOMIDAE

RQ-A: Does regular Bti-based mosquito control adversely affect abundance and community composition of wetland chironomids?

[Scientific publications I, I, III, IV – Appendices I - IV]

B

BTI AS A POTENTIAL THREAT TO AMPHIBIANS

RQ-B: Does Bti-based mosquito control have direct effects on amphibians at realistic treatment schemes or indirect effects through Bti-induced alterations in wetland food chains?

[Scientific publications V, VI – Appendices V, VI]

C

PUBLIC ACCEPTANCE OF MOSQUITO CONTROL ALTERNATIVES

RQ-C: How important is the reduction of nuisance associated with mosquitoes in light of nature conservation and would modifications of the current large-scale Bti-based mosquito control to local control strategies be accepted by the population in the Upper Rhine Valley?

[Unpublished data – Appendices VII]

Figure 4: Schematic overview of the thesis outline, including the three main research objectives (A, B, C), the six underlying scientific publications (Appendix I-VI) and unpublished data (Appendix VII).

4 METHODS OVERVIEW

This thesis applied a tiered experimental testing approach ranging from laboratory (4.1) over microcosm studies (4.2) to semi-field and field studies (4.3) to assess the ecotoxicological implications of Bti applications on wetland invertebrates and amphibians (RQ-A , RQ-B). In addition, the attitude of the affected local population towards mosquito control and the implementation of less invasive control alternatives using traps (4.4) was determined with the use of socioeconomic surveys (4.5) (RQ-C).

4.1 Laboratory study

4.1.1 Chironomidae

The first laboratory experiment aimed at assessing the acute toxicity of Bti on the entire aquatic life cycle of *Chironomus riparius,* the ecotoxicological standard test species for chironomids. The experiment followed a consecutive test design with one acute toxicity test being conducted at each day of the larval development of *C. riparius* (28 days) according to OECD guideline 235 using the Bti formulation VectoBac®WG [**Appendix I**]. The endpoints were immobility (EC50) at each of the 28 test days and in the four larval stages.

4.1.2 Amphibians

Another laboratory experiment examined acute effects of Bti in three delivery forms (Ice, Liquid, Sand) on the common frog *Rana temporaria*, a surrogate for herbivorous amphibians that is widely distributed throughout Europe. Its spawning habitats range from stagnant shallow to temporary ponds where it co-occurs with mosquito larvae and thus exposed to Bti (Schlüpmann and Günther, 2004). Tadpoles were consecutively exposed to environmentally relevant application frequencies (up to three times) with three application rates (1× FR, 2 × FR, 10 × FR) at three different stages during their larval development (Fig. 5, **Appendix V**). Mortality, physiological fitness (evaluated by growth) and time to metamorphosis (TTM) of froglets were assessed at the end of complete metamorphosis. Sublethal effects were examined 48h after each exposure using three well studied enzymatic biomarkers of effect in anuran larvae: glutathione-S-transferase (GST), glutathione reductase (GR) and acetylcholine esterase (AChE) (Venturino and D’Angelo, 2005).

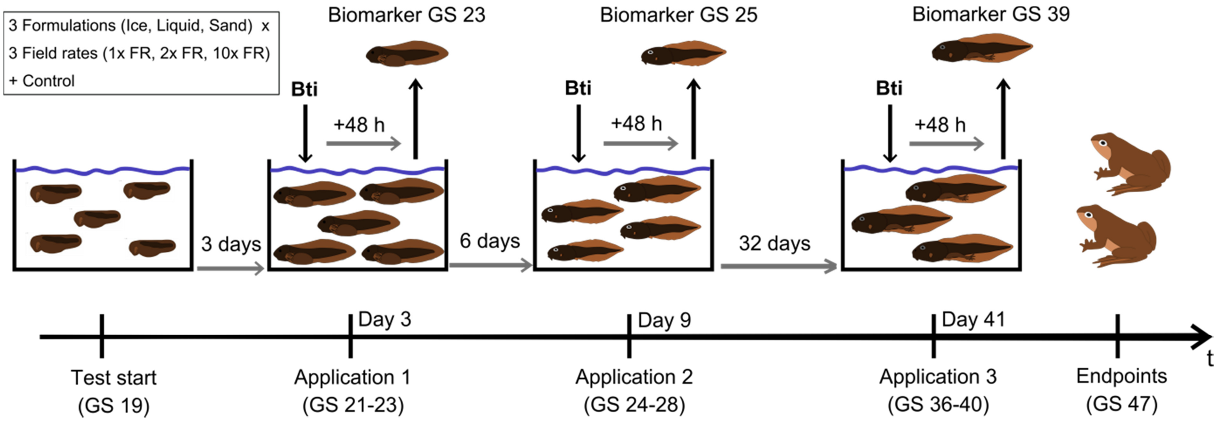


Figure 5: Schematic overview of the experimental design of the multiple exposure experiment using *R. temporaria* tadpoles. Three formulations (Ice, Liquid, Sand) at three field rates (FR) (1×, 2×, 10×) were applied three times (Application 1-3) at three consecutive time points (Day 3, 9, 41) after the test start at Gosner stage (GS) 19 until recording of endpoints (mortality, physiological parameters) at GS 47 (n=10). Tadpoles for biomarker analysis were sampled 48 h after each application in GS 23, 25 and 39 (n=10) [Figure taken from Appendix V].

4.2 Microcosm studies

To assess effects on chironomids and newt larvae in an ecologically more complex design, two experiments were conducted in artificial freshwater microcosms (90 L) at the Landau Stream Mesocosm Facility at the University of Koblenz-Landau (Germany). Both microcosm experiments lasted nine weeks and followed the same study system but were implemented in two subsequent years (Experiment 1: 2015, Experiment 2: 2016). A schematic overview of the contribution of the study endpoints to the underlying scientific papers [**Appendix II, VI**] is displayed in Figure 6. For both experiments, the microcosms were filled with tab and pond water and supplied with quartz sand, a black alder/oak leaf mixture typically found in swamp forests and two aquatic plants (*Elodea canadensis, Myrophyllum spicatum)*. Several aquatic invertebrates were obtained from local wetlands (*Asellus aquaticus*, snails (Physidae, Planorbidae, Lymnidae), zooplankton (Cladocera, Copepoda, Ostracoda), chironomids, mosquitoes) and in-house cultures (*Culex pipiens*, *C. riparius*, *Daphnia magna*) to establish standardized invertebrate communities and suitable prey organisms for higher predators. The microcosms were covered with emergence traps to prevent colonization by external organisms and catch emerging insects (Fig. 6). Aquatic invertebrate community and emergence were sampled once a week.

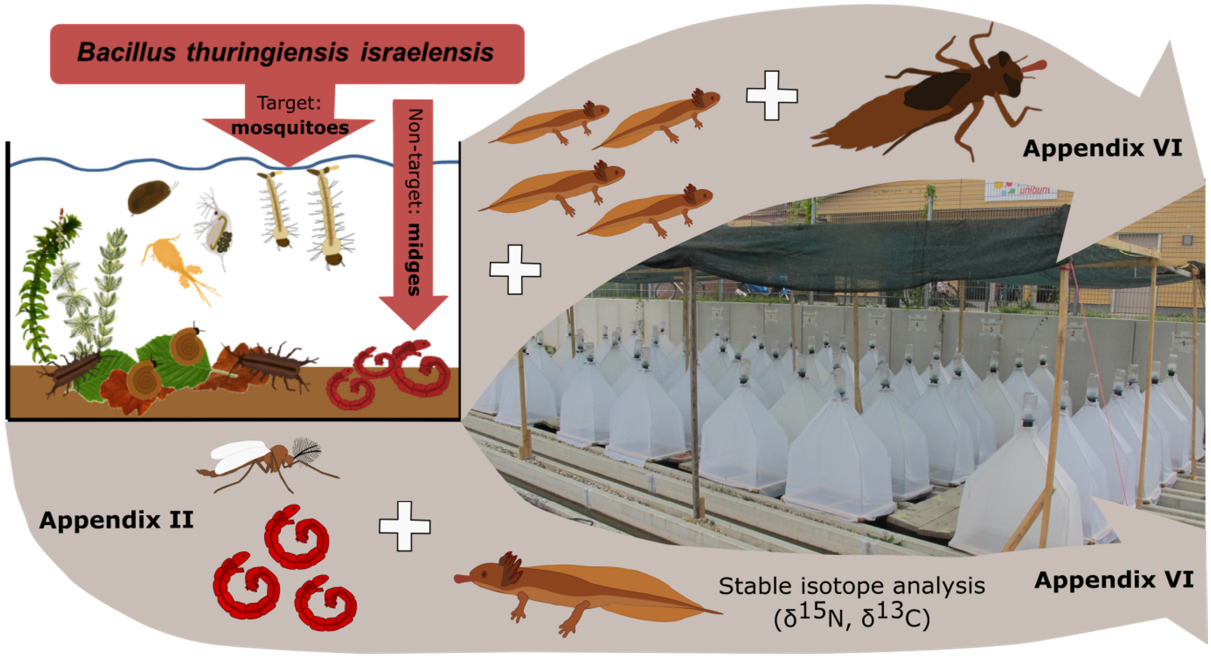


Figure 6: Schematic overview of microcosm experiments and their contribution to the scientific papers [Figure adapted from Appendix VI].

Experiment 1 was comprised of 48 microcosms in a randomized 3 × 2 factorial design manipulating two factors in the aquatic communities to assess the impacts on chironomids [**Appendix II**] and the food chain [**Appendix VI**]: three levels of predators [*no predators (predation control (PC))/ newt larvae (N)/ newts and dragonfly larvae (ND)*] and two levels of Bti treatment [*control/ Bti-treated (1× high FR VectoBac®WG)*]. Regarding food chain effects, this experiment focused on the dietary intake and survival of a natural newt larvae assemblage (*Lissotriton helveticus*, *Lissotrition vulgaris)* under potential intraguild predation by the generalist dragonfly predator *Aeshna cyanea*. The dragonfly nymph is efficient in consuming a wide range of prey in the wild including zooplankton, amphipods and amphibian larvae (Van Buskirk and Schmidt, 2000). Both newt species are common in Central Europe and among the smallest newts in seasonal wetlands which is why they share a similar feeding behavior and have a high food niche overlap (Griffiths, 1986). They breed in early spring and their aquatic larval period coincides spatially and temporally with the application of Bti for mosquito control (Günther, 1996). The use of stable isotope ratios (δ^{13}C and δ^{15}N) enabled insights into dietary composition of isotopic niche widths of the predators.

Experiment 2 focused on the development of individual *L. helveticus* larvae and comprised 16 microcosms including eight control and Bti (2 × high FR IcyPearls®) treatments respectively [**Appendix VI**]. Recorded endpoints were TTM, length, snout to vent length (SVL) and mass of newts after successful metamorphosis.

4.3 (Semi)field studies

The semi-field and field studies facilitated the interpretation of the previous results in the context of field relevance. Hence, the effects on overall abundances of adult chironomids [**Appendix II**] and their community level were assessed using state-of-the-art DNA-metabarcoding [**Appendix III, IV**]. Metabarcoding is a genetic method of biodiversity assessment. The revealed metabarcode data sets contain presence/absence data of operational taxonomic units (OTUs) that display a pragmatic proxy for different chironomid taxa.

Semi-field study - The semi-field study was conducted in a river floodplain (hereinafter referred to as floodplain) that was never treated with Bti before since it is officially listed as off-limits zone for Bti-based mosquito control (Fig. 7, **Appendix II**). In total, 24 enclosures (300 L) were driven into the wetland sediment of which 12 were treated with Bti IcyPearls® in the low FR. For the comparison of chironomid abundances in and outside of the enclosures, six floating traps were installed next to the enclosures (Fig. 8).

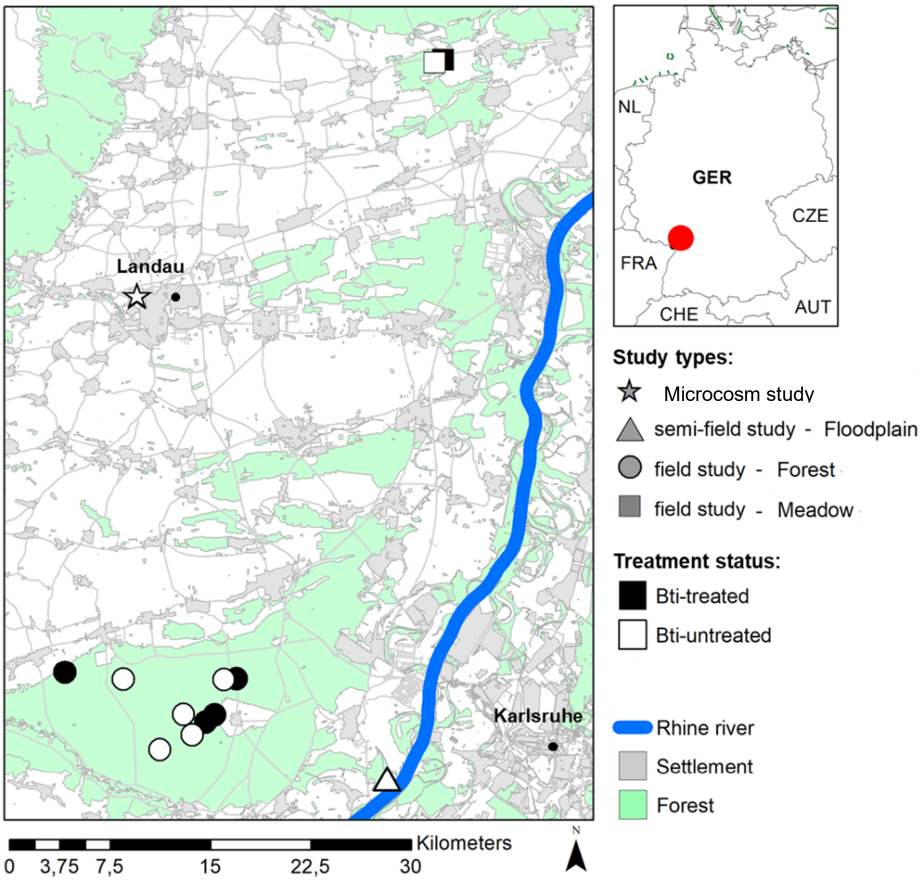


Figure 7: Study sites in the southwest of Germany. Semi-field study with enclosures located in an untreated temporary flooded pond next to the Rhine (Δ). Field studies were conducted in a forest temporary wetland (○) with Bti-treated (black, n = 4) and control ponds (white, n = 5) and a meadow temporary wetland (□) that was divided in one treated (black) and one untreated (white) part by split-design [Figure taken from Appendix II].

Figure 8: Enclosures with emergence traps next to floating emergence traps in the semi-field study [Figure adapted from Supplementary material of Appendix II].

Field studies - Additionally, two field studies were conducted at two sites that are subject of regular mosquito control activities and differed in structure and vegetation which is generally reflected in varying chironomid compositions (Brink et al., 1991). Aquatic emergence was sampled with floating emergence traps as shown in Figure 3C. Sampling emergence enables the detection of effects on 1st or 2nd instar chironomids which are difficult to count in natural samples due to their small sizes.

One study was conducted in forest temporary wetlands (referred to as forest) that were part of a forest called Bienwald next to the French border. Forest ponds around settlements are routinely treated with Bti against univoltine snowmelt mosquitoes. Bti treatment is prohibited in core zones for nature protection (off-limits zones) that were chosen as controls (Fig. 7). Mosquito control was performed by the local GMCA operators on foot by applying the low Bti FR with backpack sprayers.

The other study site was a meadow temporary wetland (referred to as meadow) regularly flooded in spring and dry during summer and is located close to Neustadt/ Weinstraße (Fig. 7). In spring 2013, the flooded grassland was divided into two parts that are naturally separated by some trees and a path. While one part was still treated with Bti, the other part was left untreated for the first time for over 20 years and further served as control. During the study time, the high FR was applied by helicopter by local GMCA operators on the still treated site. The chironomid community of the first year after the implementation of the split field design was assessed in **Appendix III**. **Appendix IV** focused on potential recovery effects of the chironomid species composition after three consecutive years of this split field design.

4.4 Mosquito traps

A prerequisite for the success of alternative concepts for mosquito control in the Upper Rhine Valley is the capability to reduce nuisance associated with the mass occurrences of *Ae. vexans*. A promising alternative approach that was recently applied in the Camargue is based on the use of mosquito traps (Poulin et al., 2017). So far, traps were mainly used for the surveillance of a large range of mosquito species in Central Europe (Lühken et al., 2014; Meeraus et al., 2008). This thesis investigated the effectivity of the Biogents (BG)-Mosquitaire CO_2 mosquito trap (Kröckel et al., 2006) concerning the reduction of mosquito-borne nuisance in the town Berg/ Pfalz [**Appendix VII**]. In addition, the species spectrum of the caught mosquitoes was assessed. Three different concepts for trap-based mosquito control were applied, namely, installing traps at five private properties distributed on the outskirts and center of the town (single houses), at a row of six linear neighboring houses (wall) and around an area of seven houses (cluster). Nuisance reduction was assessed within 15 minutes human landing rate (HLR) periods (Englbrecht et al., 2015; Kröckel et al., 2006).

4.5 Socio-economic surveys

The attitude of the local population in the Upper Rhine Valley towards mosquito control was assessed by implementing two socio-economic surveys. A preliminary survey (August 2016) comprised 300 face-to-face interviews in front of 14 local supermarkets in GMCA member municipalities between Berg (Pfalz) and Altrip. This survey assessed the opinion of the affected population to the nuisance accompanied by mosquitoes and their control activities. The main survey (September 2017) focused on the populations' attitude towards mosquito control, taking into account possible risks to nature and environment. Hence, the willingness to pay for an alternative concept that may conform to nature conservation was assessed among 635 randomly selected households in 12 locations between Speyer and Neuburg. The survey was based on the so-called Contingent Value Method (CVM), which is a standardized method for determining the social value of environmental goods (Whitehead, 2013). Interviewees were proposed an alternative mosquito control concept based on the omission of Bti treatment in ecologically important areas to better protect ecosystems along the Upper Rhine. The proposed alternative control also includes the implementation of mosquito traps to counteract potentially higher nuisance levels in residential areas due to less Bti application.

5 UNDERESTIMATED EFFECTS ON NON-TARGET CHIRONOMIDAE (A)

The results contributing to research objective A were documented in four manuscripts focusing on chironomids. Three were published in three peer-reviewed journals while one was submitted [**Appendix I – IV**].

5.1 Effects in laboratory experiments and artificial freshwater microcosms

Laboratory study - During the 28-day long larval development, *C. riparius* displayed a decreasing sensitivity towards Bti with the free-swimming 1st larval stage being 100-fold more sensitive than the fourth larval stage (Fig. 9). This thesis determined the ecotoxicological relevant EC_{50} value for first instar *C. riparius* at 6.9 ITU/L which is 209 times below the low FR applied in the Upper Rhine Valley (1440 ITU/L). Based on the laboratory data first instar chironomids are likely to show effects in the field at realistic mosquito control rates.

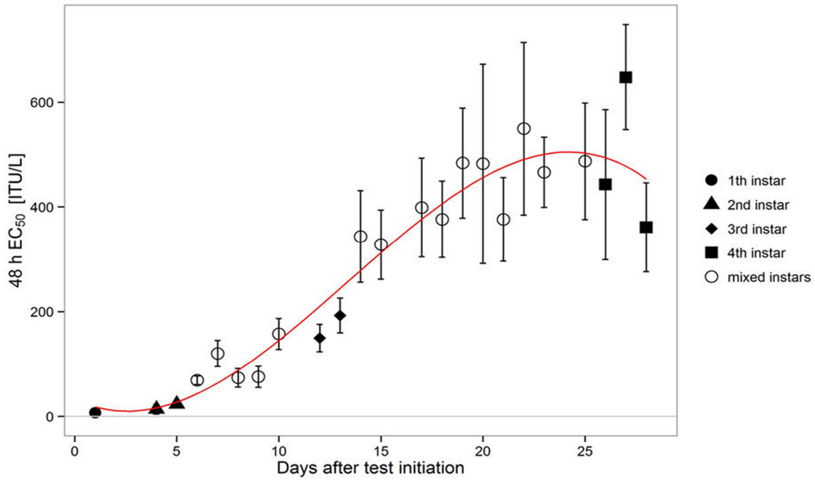


Figure 9: EC_{50} values with 95% confidence intervals (CI) on each test day during the 28 day test period. Days with more than 90% of the individuals attributed to a specific larval stage were assigned to first until fourth instars (filled symbols). The EC_{50} values where this criterion was not met are marked as mixed instars (open symbol). [Figure taken from Appendix I].

The authorization of biocides requires an ecological risk assessment (ERA) where data on the effects on surrogate organisms are compiled and compared to the predicted environmental concentrations (PEC). The newly generated EC_{50} value equals the no effect concentration (PNEC) for chironomids as the most sensitive organism. A potential risk is characterized by an exposure/effect ratio (PEC/PNEC) that exceeds the trigger value of 1 which subsequently might require risk reduction measures. This ratio highly exceeds 1 when applying the PEC reported in the assessment report (PEC = 74 ITU/L) (European Commission, 2011) indicating an underestimated risk of first instar *C. riparius*. The ratio was even higher using a more realistic exposure estimate (PEC = 1440 IT/L). Using the available literature EC_{50} values for varying chironomid species and larval stages as PNEC, all species can be considered at risk at the low FR exposure estimate (Fig. 10B) [**Appendix I**]. .

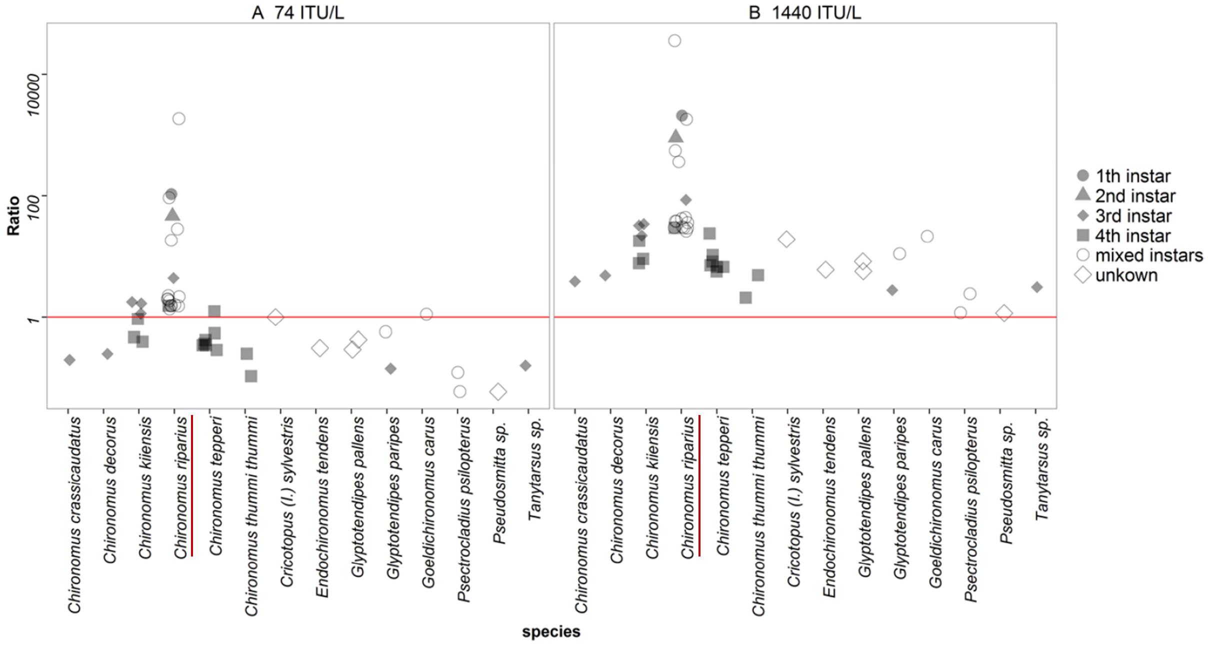


Figure 10: Calculated PEC/PNEC ratios based on a PEC of A) 74 ITU/L obtained from the recent assessment report of Bti (European Commission, 2011) and B) 1440 ITU/L as the actual field rate. The red line marks the trigger value of PEC/PNEC = 1 [Figure taken from Appendix I].

Microcosm study - Several factors such as substrate, macrophyte density, larval instar distributions and densities are known to diminish the larvicidal efficacy on benthic chironomids when compared to acute exposure conditions in the laboratory (Cao et al., 2012; Stevens et al., 2004). Hence, this thesis provided the first investigation of chironomids in artificial freshwater pond microcosms with Bti treatment as the only modified stressor. Larval chironomids were the most adversely affected amongst all non-target aquatic invertebrates in the altered communities of the Bti-treated microcosms [**Appendix II**]. Within six weeks after application, chironomid abundance was cumulatively reduced by around half at Bti concentrations that were three times lower than the EC_{50} of the least sensitive fourth instar stage of *C. riparius* in the laboratory (Table 2, Fig. 9). The major part of the identifiable chironomid adults belonged to the subfamilies Orthocladiinae and Chironominae that have been shown to be adversely affected by Bti treatments (Boisvert and Boisvert, 2000; Liber et al., 1998). Predatory Tanypodinae were not to affected by the Bti treatment which is accordance with a semi-field study that applied ten-fold the operational rates (Liber et al., 1998).

Table 2: Comparison of aquatic invertebrates and emergence between Bti treatment and control in the microcosms, semi-field and field studies. Forest (-C5): Exclusion of one control pond that was highly productive in generating mosquitoes and chironomids. Statistically significant reductions due to the Bti treatment indicated in bold [Table taken from Appendix II].

		sampling weeks	traps	overall organisms abundances	Culicidae		Chironomidae	
					cumulative abundance	reduction [%]	cumulative abundance	reduction [%]
microcosm (aquatic invertebrates)								
	control	7		8797	27		1985	
	Bti-treated	7		7363	4	**85.2**	928	**53.3**
microcosm (emergence)								
	control	7	8	2282	21		2154	
	Bti-treated	7	8	1213	2	**90.5**	1106	**48.7**
semi-field (emergence)								
	control	15	12	1473	17		923	
	Bti-treated	15	12	1088	13	23.5	542	41.3
field (emergence)								
forest	control	6	15	5458	2978		1522	
	Bti-treated	6	12	942	38	**98.7**	354	76.7
forest (-C5)	control	6	12	1424	1037		228	
	Bti-treated	6	12	942	38	**96.3**	354	- 55.3
meadow	control	14	12	5489	165		3527	
	Bti-treated	14	12	3436	13	**92.1**	1138	**67.7**

5.2 Effects on abundance and community structure in (semi)field studies

Based on the high sensitivity of chironomids that was revealed in the first experiments of this chapter, the subsequent (semi-)field experiments investigated the impact of Bti applications on chironomid species communities under natural wetland conditions. This thesis established DNA metabarcoding for chironomid diversity assessment as an alternative to challenging morphological determination of chironomids which can only be performed by taxonomic experts (Boix and Batzer, 2016). Applying metabarcoding increased species identification rates by 70% compared to traditional taxonomic determination of larval samples [**Appendix III**].

Overall, the three study sites floodplain, meadow and forest differed in their chironomid community composition (Fig. 11). They only shared 10% of OTUs which provided a good basis for assessing Bti-induced non-target effects across three different mosquito-control relevant wetland types [**Appendix IV**]. Communities were characterized by a few highly dominant species that are adapted generalists of temporary wetlands such as *Polypedilum uncinatum* and several Lymnophyes species, but also numerous rare species. Bti treatment affected chironomid abundances (Table 2) [**Appendix II**] and species composition (Fig. 11)

[**Appendix IV**]. However, hydraulic and other abiotic conditions (i.e., connection to permanent water bodies, springs or ground water) mainly influenced chironomid communities.

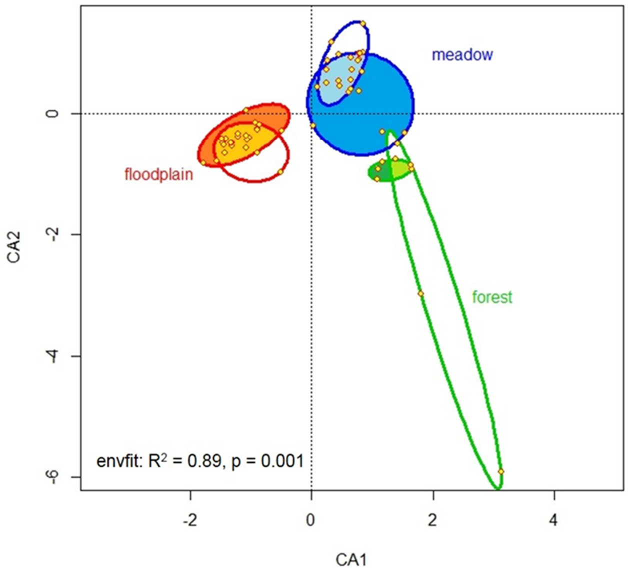


Figure 11: Correspondence analysis with dots representing samples and ellipsoids the significant best fit of OUT composition on the environmental samples. Filled ellipsoids: Bti-treated samples; transparent ellipsoids: control samples [Figure taken from Appendix IV].

Semi-field study – Chironomidae dominated insect emergence (57% of total emergence) in the floodplain site while Culicidae only made up 1% of all organisms. The installation of the enclosures influenced the chironomid community concerning abundances (2.5 times less than in floating traps) and species composition. Enclosures contained a shifted community rather than depleted chironomid species diversity. Equally to the microcosm, overall chironomid emergence rates in the enclosures were reduced by 41% in the Bti-treated enclosures (Table 2). The effect on chironomid abundances was observed eight weeks after the initial Bti application, when the majority (90%) of chironomids emerged (Fig. 12B). Control success of mosquitoes was only low (24%) which may indicate that the observed effect on chironomids may also be ranked as low.

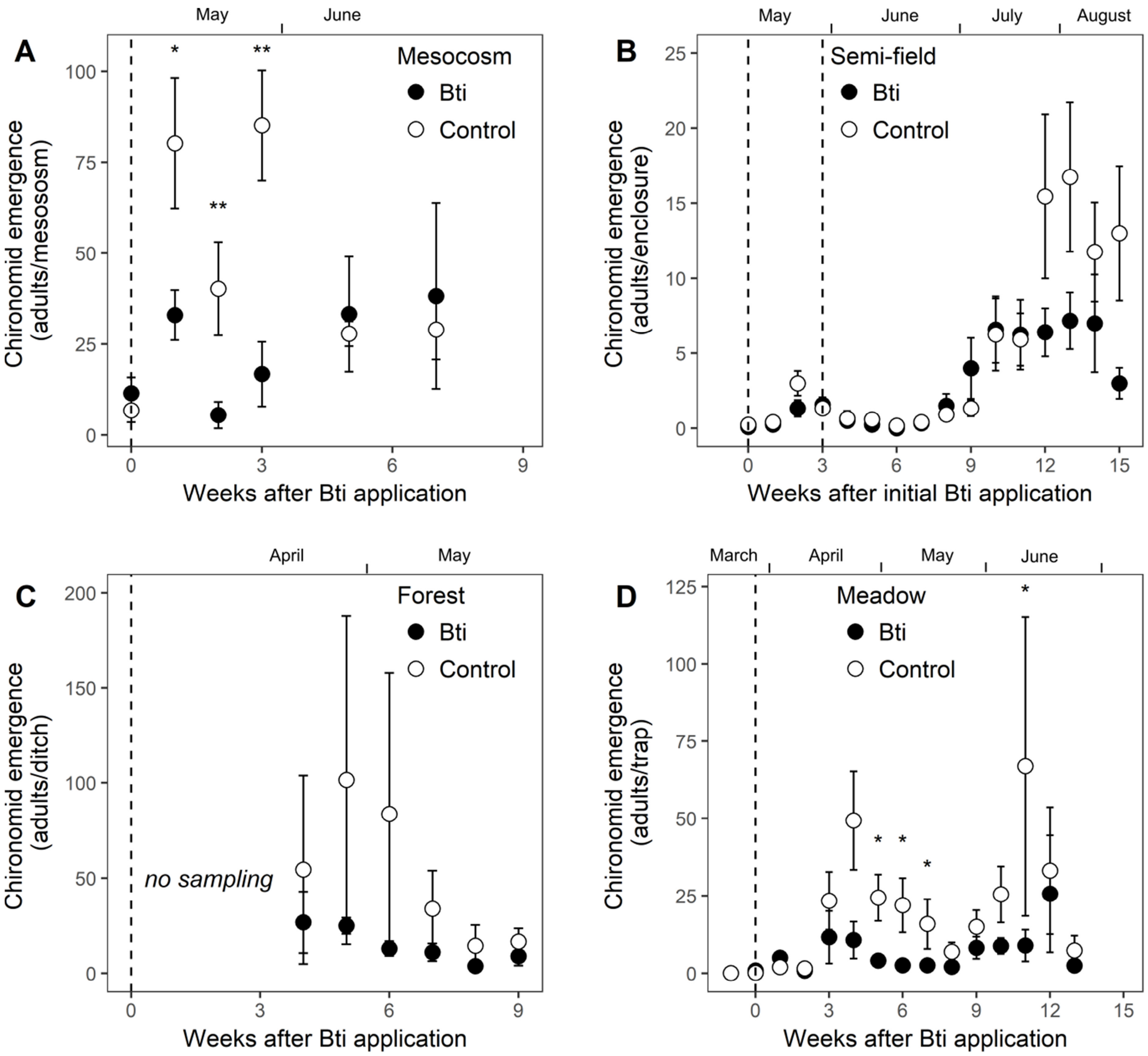


Figure 12: Comparison of mean chironomid emergence (±SE) between the control (white) and Bti treatments (black) in the A: microcosms (n = 8), B: semi-field study (n = 12), C: forest (n = 4 resp. 5) and D: meadows (n = 12) after initial Bti application (dashed lines). Significantly different from control, *: $p < 0.05$; **: $p < 0.01$ [Figure taken from Appendix II].

Field studies – Insect emergence in the forest ponds was dominated by Culicidae (47%) that were efficiently reduced by 99% within six weeks after the Bti treatment. Chironomids amounted to 29% of the emergence and were observed in the lowest diversity among the three study sites (Fig. 13). Due to the annual droughts of the ponds, chironomid community was mainly comprised of species that are typical for temporary ponds, whereas ubiquitous species that are typical for persistent water bodies were missing. Bti reduced chironomid abundances by 77% but showed a high variance in control ponds (Fig. 12C, Table 2) [**Appendix II**]. The OTU dissimilarity analysis for the forest as well as the floodplain site indicated an OTU turnover with quite similar species numbers in Bti treatment and controls (Fig. 13) [**Appendix IV**].

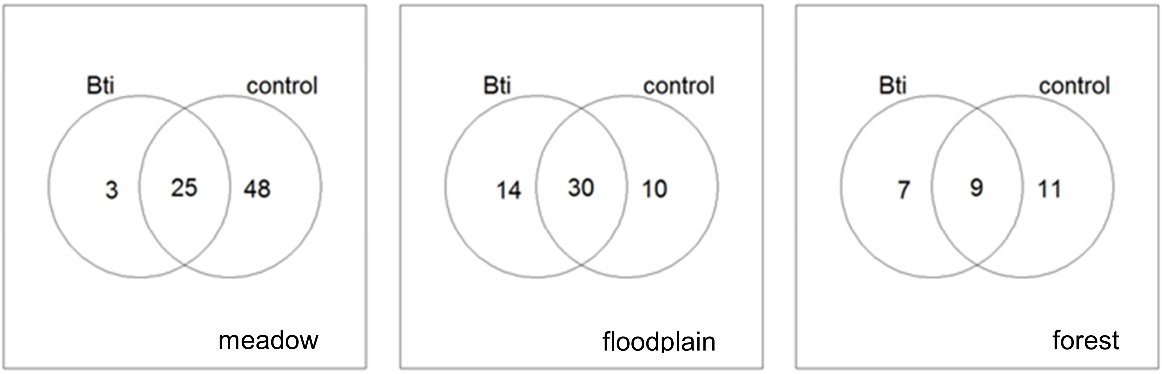


Figure 13: Venn diagram showing the numbers of detected and shared OTUs per study site and treatment (Bti vs control) [Figure adapted from Appendix IV].

Chironomids made up more than half (52%) of all collected insects in the emergence traps of the meadow site which is comparable to the floodplain site. Chironomid abundances fluctuated over time showing one spring emergence peak at the end of April and a summer emergence peak in June (Fig. 12D). Both times, the Bti-treated wetland produced less chironomids, resulting in a cumulative two-thirds (68%) reduction during three months of sampling (Table 2) The meadow site had the highest chironomid species richness and experienced the strongest Bti influence on abundances and community composition (Fig. 11, Table 2) [**Appendix II, VI**]. The still Bti-treated part of the meadow showed a 63% lower chironomid diversity compared to the part that was left untreated for four consecutive years (Fig. 13). In contrast to the other sites, the meadow was treated with the high FR in order to reach sufficient mosquito control efficiency.

Moreover, the meadow set-up allowed the examination of potential recolonization of the previously Bti-treated control part when comparing the first and fourth year after the Bti treatment was stopped. Mosquito control reduced chironomid production consistently by two-thirds at the still Bti-treated site in both study years [**Appendix III, IV**]. While only minor effects on chironomid community composition occurred in the first year of intermittence, the effect was more pronounced after four years. In addition, 27 species were newly detected in the meadow after three years from which almost 80% were solely found on the control site (Fig, 13). This implied a recolonization by new chironomid species after continued (fourth year) Bti intermittence and a positive effect on the biodiversity of NTO in a temporary wetland ecosystem.

It was suggested that predatory subfamily Tanypodinae are less susceptible to Bti than filter-feeder or grazer that are exposed to Bti as food in the water or after deposition on the sediment (Appendix II, Liber et al., 1998). This hypothesis could not be confirmed in the field studies [**Appendix IV**]. Feeding strategy may not be the main driver for Bti effects in natural wetlands (Kondo et al., 1995).

5.3 Discussion RQ-A: Effects on chironomid abundance and community composition

Laboratory effects on chironomids were also observed in ecologically more complex study systems under field conditions. Chironomid emergence rates were comparatively reduced by about half throughout study systems of increasing complexity. Remarkably, the most distinct effect did not occur under controlled conditions in the microcosm but in the meadow field study where regular Bti treatment affected chironomids quantitatively (abundance) and qualitatively (species composition).

The extent of the observed effects on chironomids in all study systems of this thesis is in agreement with studies of mosquito control areas in the salty marshes of the Camargue (48% less adult chironomids) (Jakob and Poulin, 2016) and freshwater wetlands in Minnesota (60 to 80% decrease two and three years after initiation of Bti application) (Hershey et al., 1998). Another study even found 94% decreased aquatic chironomids emergence rates in a wetland in the Upper Rhine Valley which is comparable to the study sites in this thesis (Fillinger, 1998).

While an increasing number of studies on Bti have been conducted in brackish coastal wetlands or saltmarshes (Caquet et al., 2011; Duchet et al., 2015; Jakob and Poulin, 2016; Lagadic et al., 2014, 2016; Pont et al., 1999), long-term field data on the impact of Bti on chironomids in freshwater ecosystems is rather scarce (Fillinger, 1998; Hershey et al., 1998; Lundström et al., 2010b). The latest study is a six-year monitoring of floodplains along river Dalälven in Sweden that did not record changes in chironomid production and species richness (Lundström et al., 2010a, 2010b). These results from Sweden are, however, hardly transferable to the floodplains of the Upper Rhine Valley. While Swedish wetlands experienced a maximum of five applications within six years (Lundström et al., 2010a, 2010b), floodplains along the river Rhine are treated on average five times per season (Becker et al., 2018).

It has been proposed that the occurrence of flooding or drought and other habitat parameters are more important than Bti in explaining chironomid abundances particularly in long-term field investigations (Lagadic et al., 2016; Lundström et al., 2010b). However, most discrepancies between field studies can be explained by differences in study design, particularly timing of the Bti treatment and length of examination period. Most chironomid species have a developmental time of two to four months that is much longer than for mosquitoes which emerge in less than three weeks during summer (Becker et al., 2010; Pillot, 2014a, 2014b). Short-term studies that run for few days to a maximum of eight weeks are therefore not suitable to investigate Bti-related effects on chironomid abundances (Charbonneau et al., 1994; Duchet et al., 2015; Liber et al., 1998; Pont et al., 1999; Wolfram et al., 2018). The results of this thesis suggest that field approaches should in any event also sample chironomid emergence as it enables the detection of effects on small-sized, free-swimming 1st but also 2nd instar larvae that are difficult to count in natural samples (Armitage et al., 1995). Effects on sensitive larval stages of chironomid species should be examined within at least three months after an initial Bti application. Moreover, unwanted effects on NTO should always be evaluated in relation to the achieved mosquito control rate. It is important to stress that many semi-field and field studies that did not find adverse effects on chironomids under operational application rates, have not investigated treatment success on mosquitoes (Caquet et al., 2011; Duchet et al., 2015; Lagadic et al., 2016, 2014; Liber et al., 1998; Pont et al., 1999). The absence of any effects on chironomids should only be seen as reliable prediction if mosquitoes are statistically significant reduced due to the Bti treatment at the same time. Otherwise it could also be possible that the applied concentration was ineffective due to environmental conditions.

In comparison to permanent water bodies such as lakes, seasonal wetlands subjected to mosquito control are occasionally classified as untypical chironomid habitats and less important in terms of chironomid productivity (Becker and Margalit, 1993; Lundström et al., 2010b). This thesis showed that chironomids actually often represent the most dominant taxa in the invertebrate community in seasonal wetlands [**Appendix II, III**]. Moreover, chironomids depict an reliable insect food resource as they are independent of flooding events and show continued availabilities because of a wide distribution of emergence peaks within the year (Armitage et al., 1995). The family Chironomidae is an ecologically highly diverse group with a broad range of feeding types and life cycles (Ferrington, 2008), different sensitivities for anthropogenic stressors (Carew et al., 2007; Nicacio and Juen, 2015; Serra et al., 2017) and a high adaptability for changing environmental conditions (Raunio et al., 2011).

The considerable reduction in overall abundances and the alteration of chironomid communities could have far-reaching implications on the ecological systems of temporary wetlands (Gopal and Junk, 2000). Bti-induced losses of rare species in particular could lead to increasingly homogeneous chironomid communities with a decreased adaptive potential.

Moreover, chironomids play a key role in the trophic structure in these freshwater ecosystems due to their high protein content, digestibility and their ability to form large swarms over their breeding waters (Armitage et al., 1995; De La Noüe and Choubert, 1985). In early spring, when terrestrial insect emergence is still reduced, chironomids are an important food source, e.g. for bats that primarily hunt above wetlands (Arnold et al., 2000). The ecological risk of Bti-based mosquito control is underestimated when only considering the reduced mosquito biomass available for predators (Arnold et al., 2000; Blum et al., 1997). Wetland mosquitoes represent a food resource that only peaks after flooding events and therefore less reliable than ubiquitous chironomids. The almost complete reduction of mosquitoes (Becker et al., 2010) and a substantial decrease in chironomid abundances adds up to a significant loss of major food resources for aquatic as well as terrestrial predators. Indirect effects of Bti due to a reduced availability of midges were already demonstrated for dragonflies and birds in the Camargue (Jakob and Poulin, 2016; Poulin et al., 2010).

Decreasing populations of flying insects have already been linked to the decline in aerial insectivorous birds in North America (Nebel et al., 2010; Stanton et al., 2016) or Britain (Benton et al., 2002; Vickery et al., 2001). Due to the highly specialized diets of insectivorous birds such as swallows, even subtle changes in insect prey availability can induce behavioral changes and physiological costs (Stanton et al., 2017, 2016). While those studies focused on intensified agricultural landscapes, decreasing populations of aerial insectivorous birds have also been detected over wetlands located in nature conservation areas in Germany that have been subject to regular Bti treatments for 30 years (Schrauth and Wink, 2018). Although the authors suspected surrounding farmlands to be responsible for a lack of food resources, the long history of Bti treatment could also have contributed to the observed trends more than previously assumed.

The European approval for the biocidal usage of Bti considered the crustacean *D. magna* as most susceptible NTO within the aquatic invertebrates based on an 21-day chronic tests (European Commission, 2011). Based on the severe effects on chironomids, the incorporation of appropriate additional endpoints is recommended for the upcoming re-evaluation of the active substance Bti AM65-52 in the year 2023. Applying the ERA procedure to several chironomid species at different larval stages shows that the existing assessment of Bti is not protective for this group. Thus, the risk of Bti-based mosquito control was previously underestimated and needs to be re-adapted to protect NTO and chironomids in particular.

6 BTI AS A POTENTIAL THREAT TO AMPHIBIANS (B)

The results on amphibians contributing to research objective B were published in two peer-reviewed journals [**Appendix V, VI**].

6.1 Direct effects after sublethal exposures

Neither of the applied Bti formulations (VectoBac®12AS and ®WG), in any of the different delivery forms (Liquid, Ice, Sand) induced acute mortality in *R. temporaria* tadpoles after their application. Tadpoles developed faster than in the Bti treatment (8 days prior to control). They may have escaped unfavorable conditions in their larval environment as soon after they reach a certain threshold body mass (Cauble and Wagner, 2005; Morey and Reznick, 2000). Tadpoles showed no differences in length, mass and body condition.

Consecutive Bti applications in a short period of time increased the risk for the induction of detoxification (GST) and antioxidant responses (GR) [**Appendix V**]. GST showed increased activity levels in tadpoles by 37% after the first, 150% after the second and 550% after the third application (Fig. 14). Mean GR actvities increased after the Bti applications, showing the highest increase of 140% after the second application at GS 25 (Application 1: 5%, Application 3: 24%) [**Appendix V**]. The application of higher field rates significantly increased GR activities which was different to GST results [**Appendix V**].

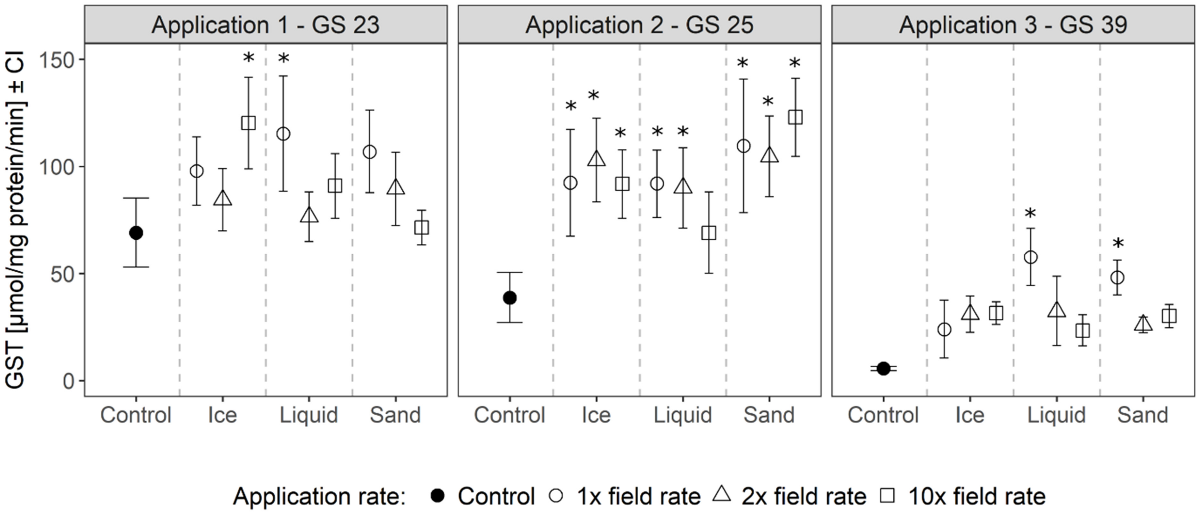


Figure 14: Mean GST activity rates (± 95% CI) of *R. temporaria* larvae in control and Bti treatments (Ice, Liquid, Sand) at different application rates (1x, 2x, 10x field rate) for three Gosner stages (GS) 23, 25, 39 (n=10, except n=5 in 2x and 10x FR at Gosner 39). Asterisks indicate statistically significant differences to the respective control p<0.05 [Figure taken from Appendix V].

The activity pattern of AChE was similiar to GST and GR responses (Fig. 15). After the first two applications, AChE levels of all treatments increased by an average of 38% and 137%

while the third Bti application showed no increases compared to controls (Fig. 15) [**Appendix V**]. The experiments showed that field-relevant Bti applications did not induce mortality in frog tadpoles but rather metabolic detoxification processes, antioxidant defenses as well as altered neuronal activity. Bti-exposed tadpoles experienced sublethal effects demonstrated by deviations in the level of enzyme activities regardless of applied formulation, application rate or delivery form. These alterations indicate that tadpoles were subjected to increased stress conditions [**Appendix V**].

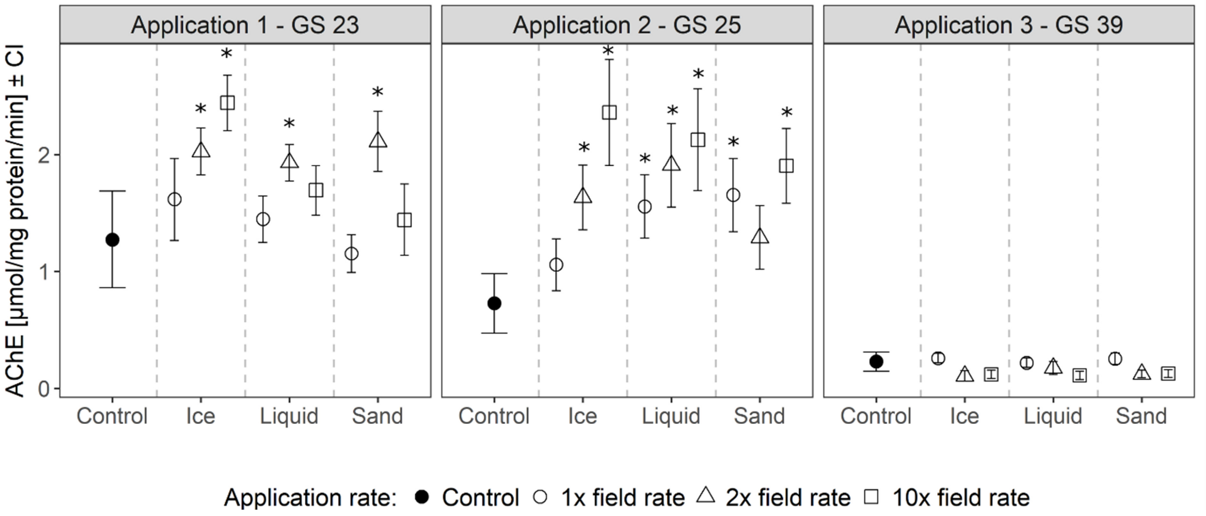


Figure 15: Mean AChE activity rates (± 95% CI) of *R. temporaria* larvae in control and Bti treatments (Ice, Liquid, Sand) at different application rates (1×, 2×, 10× field rate) for three Gosner stages (GS) 23, 25, 39 (n=10, except n=5 in 2× and 10× FR at Gosner 39). Asterisks indicate statistically significant differences to the respective control p<0.05 [Figure taken from Appendix V].

6.2 Indirect effects of Bti-modified food chains

Invertebrate community structure in the microcosms differed significantly as a result of proceeding time, the Bti treatment and the presence of predators [**Appendix VI**]. Bti treatment resulted in altered invertebrate communities. Mosquitoes as the target organism were negatively affected but non-target chironomid densities also decreased between 53 and 87%. Chironomid larvae and small-bodied zooplankton (Cladocera, Ostracoda, Copepoda) were highly abundant in all treatments. Bti treatment reduced the occurrence of the zooplankton groups Cladocera and Copepoda whereas the presence of newts affected the abundances of Cladocera and Ostracoda.

The use of bulk stable isotope analysis (δ^{13}C and δ^{15}N) enabled insights into the food web structure of the artificial pond microcosms. Newt diet was dominated by a combination of mostly chironomids (>56%) and zooplankton (>25%, Fig. 16). Newt larvae preyed preferentially on chironomids instead of feeding opportunistically as a result of higher availability of other prey such as zooplankton [**Appendix VI**].

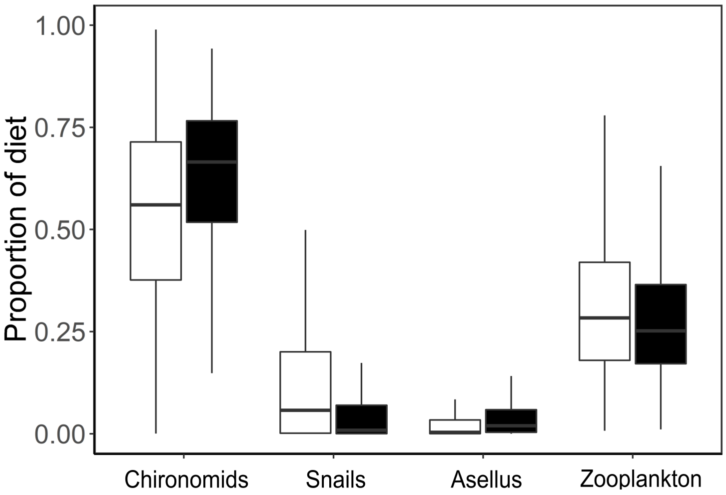


Figure 16: Proportional contributions (lines = median, box = 50% CI, error bars = 95% CI) of each prey item to the diet of *Lissotriton* newts calculated with the abundance data (abundance prior) in control (white) and Bti (black) microcosms [Figure taken from Appendix VI].

Newt metamorphs were slightly smaller (7% lower body weight) in Bti-treated microcosms despite no change in their dietary spectrum was detected. However, newts might have eaten less chironomids in the Bti treatment which is indicated by the difference in chironomid emergence rates. Chironomid emergence rates were highly reduced in both predator treatments suggesting that chironomid larvae were not only the preferred prey of newts but also of dragonfly nymphs [**Appendix VI**].

In addition, the experiments showed that 27% less newts survived the experiment when they were exposed to the dragonfly predator *A. cyanea* and Bti (Fig.17). In contrast, *A. cyanea* had only a minor effect on newt survival in control microcosms even though dragonflies are one of the most efficient predators in seasonal wetlands (Van Buskirk and Schmidt, 2000; Wilbur and Fauth, 1990).

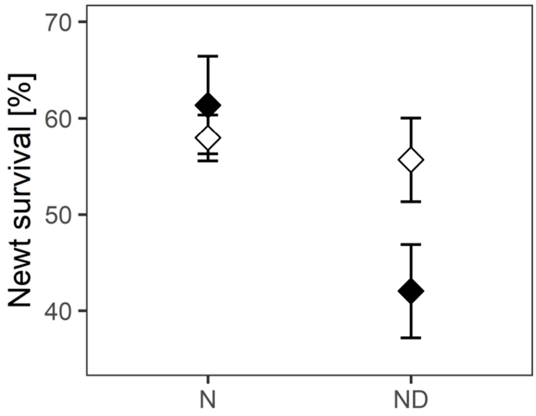


Figure 17: Mean proportion (±SE) of surviving newts (*L. helveticus, L. vulgaris*) in the absence (N) and presence (ND) of one *A. cyanea* nymph under control (white) and Bti treatment (black) (initially introduced newt larvae: n = 11) [Figure taken from Appendix VI].

Unfortunately, the time late-instar dragonfly nymphs spent in the experiment was not long enough to assimilate enough tissue to incorporate isotopic signals prevailing in the microcosms. Therefore, an assessment of dragonflies' dietary composition by mixing models was not possible. Nevertheless, isotopic niche widths of *A. cyanea* revealed large differences, namely a five times wider niche width when dragonflies fed in Bti-treated microcosms with just a small niche overlap (20%) to the dragonflies of the controls (Fig.18). At the same time, control dragonfly nymphs were larger (higher body condition) at the assessment after 38 days [**Appendix VI**].

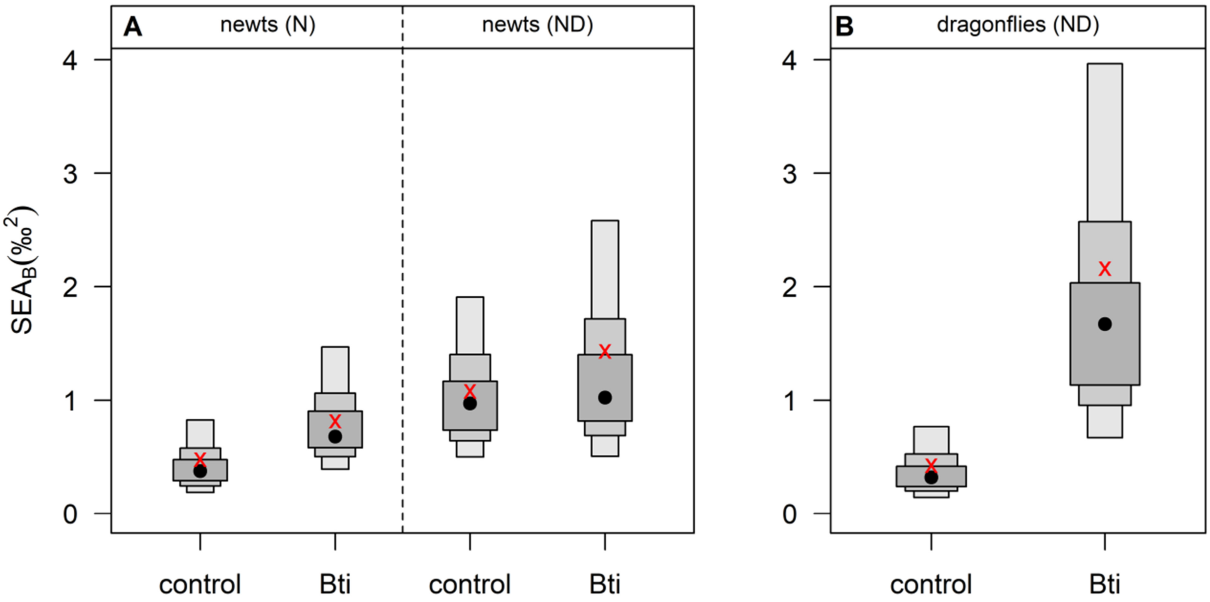


Figure 18: Density plots of standard ellipse area (SEA) displaying the isotopic niche width for (A) newts and (B) *A. cyanea* in predator (N/ ND) and Bti treatments (control/Bti). Boxed areas represent SEA_B value (black dot) with Bayesian 50, 70 and 95% credible intervals (grey bars). The red cross indicates the sample size corrected SEA_C value [Figure taken from Appendix VI].

6.3 Discussion RQ-B: Direct and indirect effects on amphibians

Exposure to Bti treatment as the only stressor did not lead to acute toxic effects on amphibian larvae (tadpoles, newts) which is consistent with the majority of studies on vertebrates in general (Boisvert and Boisvert, 2000; Lacey and Merritt, 2003) and amphibians in particular (Becker and Margalit, 1993; Boisvert and Boisvert, 2000; Schweizer et al., 2019). Lethal effects on amphibians are unlikely, due to the absence of suitable receptor sites in the neutral intestine of amphibians (Broderick et al., 2006; McDiarmid and Altig, 1999). Thus, mortality recorded after exposing frogs to another liquid Bti formulation (Introban®) is assumed to be driven by formulation additives (Lajmanovich et al., 2015). In fact, many studies concerning the risks of pesticide applications for human health and wildlife have already highlighted the importance of additives and surfactants as drivers of toxicity (Cox and Surgan, 2006; Puglis and Boone, 2011; Wagner et al., 2013).

Bti formulations applied in Germany induced sublethal effects such as increased detoxification processes, antioxidant defenses and altered neuronal activity that may subsequently lead to oxidative stress (Monaghan et al., 2009). Most recently, a study commissioned by the GMCA found no alterations of another stress protein (Hsp70) in *R. temporaria* tadpoles (Schweizer et al., 2019). These contrasting results leave the question about sublethal effects still open to new research. Nevertheless, any investment in subcellular responses to xenobiotics is an energy demanding process for animals (Steinberg, 2012). There are trade-offs of the increased energy consumption which may impair their fitness or lead to direct mortality in the worst case (Lushchak, 2011; Monaghan et al., 2009). Furthermore, sublethal effects in early larval development can result in latent damage that may be exhibited not before juvenile or adult stages (Pechenik, 2006).

This thesis further highlights the crucial role of chironomids for food chains of confined aquatic wetlands as their decreased availability had severe consequences for newt development and survival probability. Reduced newt growth rates are most likely a result of the Bti-induced chironomid prey limitation. However, smaller sizes and higher vulnerability to predation could also be influenced by the higher energetic investment in detoxification mechanisms or an altered swimming behavior (Junges et al., 2017; Wright and Wright, 1996). Both was shown to occur in tadpoles after the exposure to Bti (Allgeier et al., 2018; Junges et al., 2017). While activity reduction is a common behavioral response to avoid short-term predation (Relyea, 2001), a reduced activity induced by contaminants has no clear benefits for tadpoles but rather long-term implications such as less foraging time, smaller sizes and longer time to metamorphosis (Relyea, 2007). Contaminant-induced sublethal effects often increase susceptibility to predation in amphibians, e.g. inhibiting the development of an antipredator behavior (Hayden et al., 2015; Polo-Cavia et al., 2016; Reeves et al., 2011). Furthermore, a higher vulnerability to predation would be favored by smaller body sizes (Formanowicz, 1986; Smith, 1987).

High chironomid prey abundances in the controls facilitated the coexistence of newts and dragonflies by suppressing intraguild predation. A correlation between the presence of a more preferable alternative prey and decreased predation rates was recently shown for amphibian larvae and a mosquitofish predator in another outdoor microcosm study (Preston et al. 2017). Increased amphibian mortality caused by additional dragonfly stress and Bti was already revealed in Gray treefrog tadpoles that experienced mortalities of more than 80% (Pauley et al. 2015). The mechanism behind this synergistic effect was not explicitly studied but did not involve intraguild predation as herbivorous treefrogs are no competitors to dragonflies. Amphibian larvae are exposed to several stressors in natural wetlands e.g. more predators, low food resources, higher competition or various chemical contaminants that often have interactive effects (Boone et al., 2007; Rohr et al., 2004). Additional stressors were not incorporated in the microcosm experiments but can increase the observed effect on newt larvae.

This thesis is the first to show indirect effects of Bti-based mosquito control on aquatic food webs. So far, indirect effects were found in terrestrial systems where birds and odonates were adversely affected in abundances and breeding success (Jakob and Poulin, 2016; Poulin et al., 2010; Poulin and Lefebvre, 2016). Compared to mobile insectivorous birds, aquatic predators cannot escape from Bti-treated ponds and visit additional feeding grounds. Except a study that found increasing abundances of medium-sized diving beetles after controlling floodwater mosquitoes with Bti (Vinnersten et al., 2009), no other studies investigated indirect effects of altered wetland food resources aside from mosquitoes on aquatic predators.

Bti-based mosquito control may threaten entire amphibian populations as individuals can be affected directly or indirectly by entailing altered fitness levels at juvenile stages or less metamorphs that leave Bti-treated ponds. Amphibian populations are already declining as a result of habitat loss, amongst other things (Gallant et al., 2007; Hartel et al., 2011). In this light, it is important to protect amphibian freshwater habitats outside of agricultural areas against additional stressors. Adult as well as larval amphibians largely contribute to habitat interconnectivity due to their biphasic life cycle and to several ecosystem services including the biological control of mosquitoes, pests and related diseases (Hocking and Babbitt, 2014). Hence, a reduction of amphibian metamorphs would have implications on entire ecosystems e.g. by reducing habitat interconnectivity and the transfer of biomass and energy across ecosystem boundaries (Gibbons et al., 2006).

7 PUBLIC ACCEPTANCE OF MOSQUITO CONTROL ALTERNATIVES (C)

7.1 Efficiency of mosquito traps

Mosquito traps caught 1575 individuals during the entire sampling period. Most of these individuals belonged to the genus *Culex* (93.9%), followed by *Aedes* (3%), *Anopheles* (2.5%), *Culiseta* (0.4%) and *Coquillettidia* (0.1%). Within the genus *Culex* the majority of individuals were members of the *Cx. pipiens* complex including the northern and southern house mosquito. *Culex pipiens* lays eggs in standing waters ranging from puddles to a variety of water-filled containers around human settlements (Becker et al., 2010). Traps caught individuals of *Ae. japonicus,* an invasive vector mosquito from Southeast Asia that is already widely distributed in Central Europe since 2000 (Jansen et al., 2018). Catchment rates of the floodwater mosquito, *Ae. vexans*, were very low (<3%) due to missing large-scale flooding events during the hot summer. Therefore, this study could not finally judge the effectiveness of BG-Mosquitaire CO_2 traps against huge quantities of notorious *Ae. vexans* in the Upper Rhine Valley. However, traps succeeded in reducing mosquito nuisance. 89% less mosquito biting attempts were recorded around single houses equipped with traps within five weeks after their activation [**Appendix VII**].

Although mosquito traps are largely used for monitoring purposes (Lühken et al., 2014), there have been successful attempts to apply traps in outdoor control strategies to reduce biting rates of tropical disease and nuisance mosquitoes (Jackson et al., 2012; Poulin et al., 2017). For instance, traps reduced *Ae. albopictus* by 64 to 100% in Europe (Akhoundi et al., 2018; Englbrecht et al., 2015), *Ae. aegypti* by 68% in Brazil (Kröckel et al., 2006) or *Anopheles gambiae* by 76% and *Cx. quinquefasciatus* by 84% in Tanzania (Kitau et al., 2010). In addition, a CO_2 baited trap barrier system similar to the ones used in this thesis reduced marine nuisance species *Ochlerotatus caspius* and *Oc. detritus* in a French village by 74 and 98% respectively (Poulin et al., 2017). Both species are mainly responsible for nuisance in the Camargue and routinely controlled with Bti spraying. *Aedes vexans* was not very abundant in the French saltmarshes, however, traps also reduced their biting incidences by 94% (Poulin et al., 2017).

The use of trapping systems has the advantage that the equipment is environmentally friendly because no toxins are used, taps are only deployed locally an there is negligible impact on the non-target fauna (Akhoundi et al., 2018; Poulin et al., 2017). In addition, mosquito larvae as well as adults are preserved as a food source until they are caught which decreases the potential for negative effects on food webs. Disadvantages are the costs for CO_2 that is needed to increase the mosquito attraction capacity of the BG-Mosquitaire traps (Degener et al., 2019) and the local effective radius that makes trapping systems unsuitable to reduce nuisance in seasonal wetlands or forests.

7.2 Public attitude towards mosquito control

The local population in the Upper Rhine Valley agreed that mosquito control enhances life quality in their communities (Fig. 19). Nevertheless, the majority of people would even endorse an expansion of mosquito control activities as they still feel nuisance (Fig. 19). In contrast, just 12% would agree to expand mosquito control after being informed about the actual share payed by their municipality. Without being explicitly informed about potential environmental consequences a higher mosquito pressure is not accepted by 70% of the people. The majority of the respondents (70%) are worried about the spreading of mosquito-transmitted diseases in the Upper Rhine Valley.

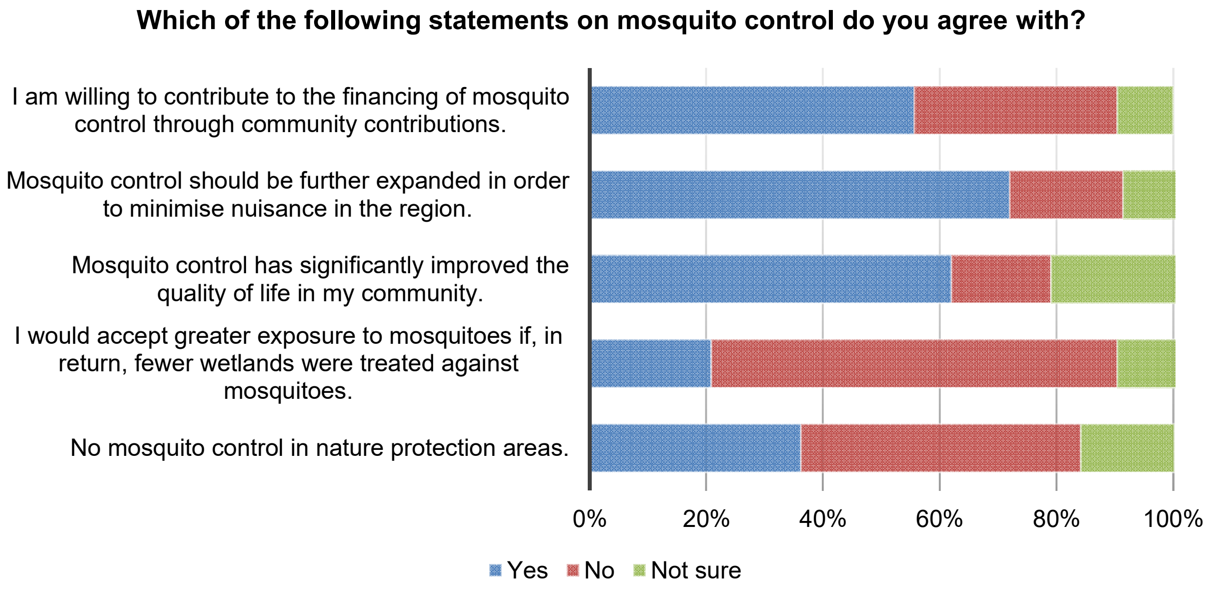


Figure 19: Attitude towards mosquito control revealed by the preliminary survey (N=294)

Despite regular mosquito control actions in wetlands, half of the respondents in the main survey still feel strongly or very strongly annoyed and use additional measures such as skin protection (sprays, clothing), fly screens or avoiding being out at dusk. However, nobody mentioned the use of mosquito traps that seems to be not well-known in the Upper Rhine Valley. Outdoor mosquito control is presumably believed to be outside of the personal sphere of influence and subsequently left to the official control program by the GMCA. Moreover, both surveys showed that strongest nuisance was felt on interviewees' properties (garden, terrace, and balcony) (Fig. 20). Nuisance in domestic areas was even felt to be comparable to the nuisance occurring in actual mosquito breeding sites such as floodplains along the Rhine and in forests (Fig. 20). Hence, an adaptation of the previous control practice could only be achieved and accepted by local population if it is ensured that the nuisance in domestic areas will not increase further.

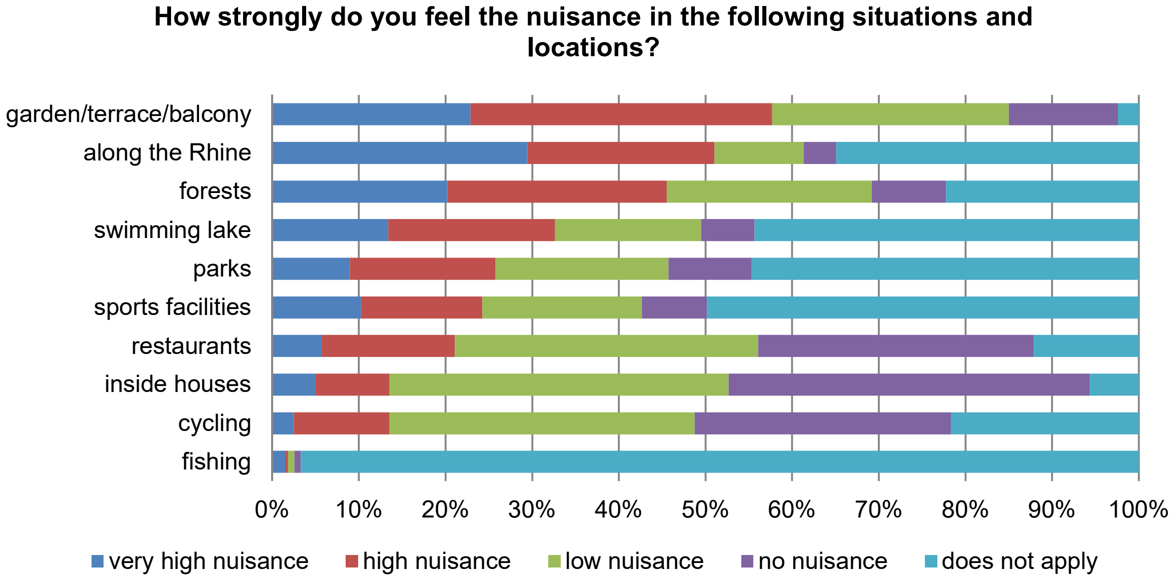


Figure 20: Assessment of nuisance though mosquitoes in the preliminary survey (N=294)

The population is poorly informed about potential ecological effects after applying Bti in natural wetlands. Most people did not link mosquito control to environmental harm. On the one hand the majority highly appreciates nature and believes that it should be left untouched by humans in protection areas (Fig. 21). On the other hand around half of the interviewees knew that mosquito control is executed in nature protection areas but less than one quarter of the interviewees found this to be worrying.

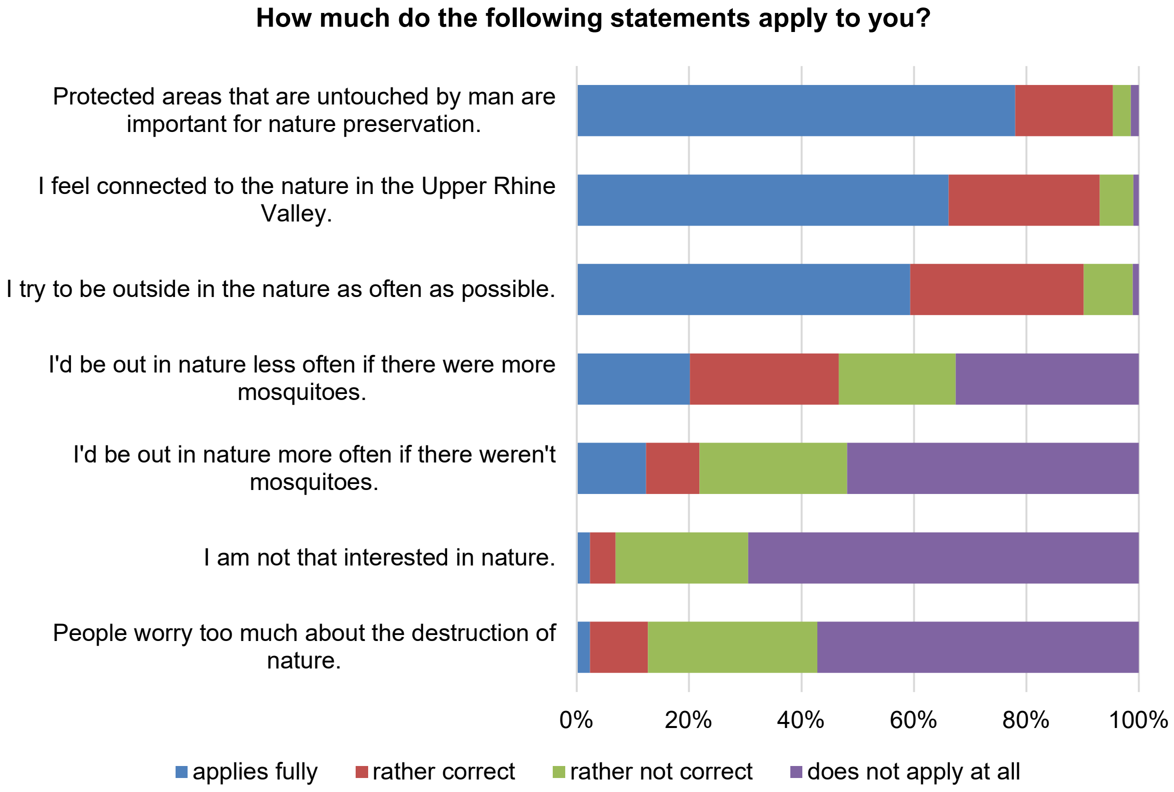


Figure 21: Attitude to nature and outside activities revealed by the main survey (N=635)

When being confronted with possible side effects on wetland food chains and thus nature at the Upper Rhine Valley, a large part of the interviewees was skeptical about the current control practice. Thus, two-thirds (67%) of the respondents were in favor of reconsidering and adapting the current practice of mosquito control based on Bti spraying as long as the level of protection against mosquitoes at home and in urban areas remains the same. In contrast, 19% see no need for adaptations and want adhere to the current control practice.

The survey showed that the population attributes great importance to pristine nature that is unimpaired by biocide applications for mosquito control purposes. Many respondents would even accept that there are more mosquitoes outside the villages to achieve a higher level of nature protection (Fig. 22). Nevertheless, an entire omission of all mosquito control practices was just claimed by very few people because mosquito control was largely seen to improve the quality of life.

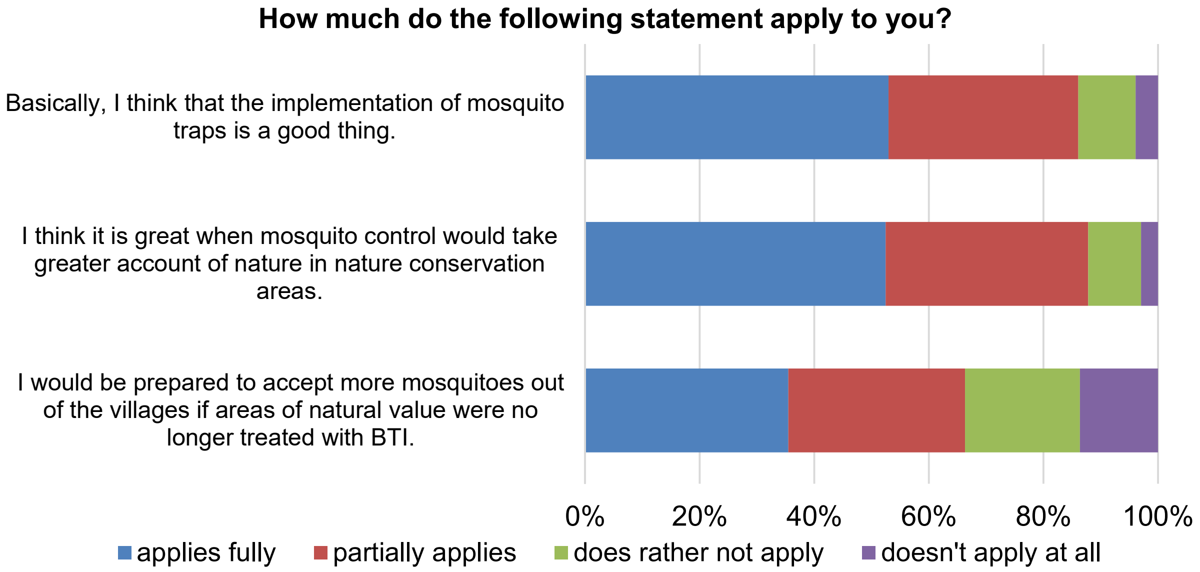


Figure 22: Opinion on the alternative concept on a combination of traps and reduced Bti spraying (N=635)

The interviewees were very willing to bear additional costs of a mosquito control that is in accordance with nature conservation regulations. This is expressed in their willingness to spend on average 75 € per household and year. In comparison, a survey in the early 2000s revealed a mean willingness-to-pay of 3.5 € per person for Bti-based GMCA mosquito control (Hirsch and Becker, 2009). Thus, mosquito control is perceived as a great benefit because the actual costs for mosquito control in the Upper Rhine Valley are much lower (1.3 € per person per year) (Becker and Lüthy, 2017). Nevertheless, environmentally less invasive mosquito control is even more important for the respondents than mosquito control in general.

The proposed alternative including the use of traps is quite well received by the majority of interviewees (Fig. 22). Nevertheless, many respondents expressed their lack of trust in the effectivity of this new technology. The general disapproval of the proposed alternative and increasing mosquito levels outside villages were the most important reason not to accept the proposed alternative**.**

7.3 Discussion RQ-C: Public acceptance of control alternatives

Environmental side-effects of Bti applications were previously considered to be negligible (Boisvert and Boisvert, 2000; Lawler, 2017) and have therefore not been considered when assessing the added value of mosquito control for human well-being in the Upper Rhine Valley (Hirsch and Becker, 2009). However, this thesis showed that the interviewed population is willing to financially support environmental friendly alternatives if available. This is in line with residents at Texas gulf coast that were willing to pay double when control measures are ecologically sensitive (John et al., 1992). A similar preference was expressed by inhabitants of the Marais de Baux wetland in southern France. Two-thirds of the respondents attributed positive values to natural mosquito control programs including water management and biological control by fish compared to the application of Bti as it is performed in the neighboring Camargue (Westerberg et al., 2010). Thus, public concerns about environmental consequences of mosquito control need to be addressed in alternative concepts.

Although nature protection is deemed a future priority, the local population seemed undecided when being asked about the proposed alternative including mosquito traps. For one thing, they were receptive a new method inducing less environmental harm in nature protection areas. However, a complete cessation of the Bti spraying was not desired by most interviewees as upcoming mosquito plagues should be averted by this trusted method. The solution of omitting Bti treatment in ecologically valuable areas combined with increased control actions in places where mosquitoes were most annoying (private properties and villages), was met with great approval. Interviewees were still struggling to judge and trust the effectiveness of the presented mosquito traps because they are not well-known for private usage. Although this thesis could not finally assess the effectivity of traps against floodwater mosquitoes, Poulin et al. (2017) effectively applied a mosquito trap network functioning as protecting belt against other nuisance mosquitoes that are usually treated with Bti. Deploying traps in urban areas could also be cost-efficient compared to large-scale wetland monitoring and Bti application that amounts to 3.4 million € each year in the Upper Rhine region (Becker and Lüthy, 2017).

When reducing mosquito control in ecologically valuable wetlands, parts of the municipal budget could be used to intensify urban mosquito control which continues to loom large due to several reasons. Firstly, even in years with few flooding events and associating mass occurrence of *Ae. vexans*, a large number of domestic Culex mosquitoes can lead to nuisance within residential areas [see traps study, **Appendix VII**]. Secondly, the ongoing spread of container-breeding vector mosquitoes such as *Ae. albopictus* (Becker et al., 2017; Thomas et al., 2018) led to a growing fear in the population that vector-borne diseases are transmitted in the Upper Rhine Valley. The vector competence of domestic mosquito species has recently received more attention (Heitmann et al., 2017; Jansen et al., 2019). Container-breeding species such as *Ae. albopictus* are rarely targeted by large-scale Bti spraying over nature conservation areas and require a much more targeted control strategy by focusing on small water bodies (vases, knotholes or rain barrels). There seems to be growing importance to raise awareness among the population about control measures and their targets. Residents can substantially contribute to reduce nuisance and risk of peridomestic mosquitoes by reducing breeding site which was shown to be very effective when actively integrating and educating the public (Healy et al., 2014). A conceivable concept in the framework of an integrated mosquito control management with less environmental interventions might include traps that are used synergistically with other control methods targeting the immature population e.g. wetland management, reduced use of Bti and domestic source reduction (Akhoundi et al., 2018; Healy et al., 2014). Such concepts should be evaluated by means of public participation before they are incorporated into decision and policy-making processes.

8 CONCLUSION AND OUTLOOK

In light of global climate change, proceeding globalization and the ongoing spread of tropical mosquito species in Europe future mosquito control will become more important to safeguard human health. The local and small-scale use of Bti against container-breeding mosquito species is among the most human and environmental friendly methods available in mosquito control. However, applying Bti against nuisance mosquitoes in wetlands to increase human convenience might induce severe adverse environmental consequences. The present thesis provides extensive evidence that field-relevant Bti levels used in mosquito control negatively affect non-target chironomid abundance and diversity [**Appendix I-IV**]. Since chironomids play a crucial role in wetland food webs, Bti-induced reductions in their populations could lead to unwanted indirect effects on aquatic and terrestrial food webs (Jakob and Poulin, 2016; Poulin et al., 2010; Poulin and Lefebvre, 2016). In addition, amphibian larvae experienced subcellular sublethal effects after consecutive exposures to Bti and were highly vulnerable to predation in artificial study systems [**Appendix V, VI**]. Both combined effects could severely impact amphibian populations in the long-run and therefore contribute to ongoing amphibian declines. Thus, large-scale applications of Bti for nuisance mosquito control in seasonal flooded wetlands might affect biodiversity and the environmental functioning of wetlands.

To preserve biodiversity around Bti-treated wetlands, it is necessary to consider direct as well as indirect effects originating from reduced food sources. To achieve such protection, the current ERA for Bti (European Commission, 2011) needs to be adapted and improved to consider the sensitivity of chironomid communities. Long-term research in different wetland ecosystems is necessary to better understand bottom-up effects in aquatic and terrestrial food webs and further assess ecological risks of Bti-based mosquito control. While long-term monitoring programs of ecological impacts were launched simultaneously to the implementation of mosquito control in France or Sweden (Lagadic et al., 2016, 2014; Lundström et al., 2010b; Poulin, 2012), no comparable field monitoring data was performed at the Upper Rhine Valley. Increased knowledge of trophic indirect effects in particular would also help decision and policy-making processes. A monitoring would not only provide necessary inside for the Upper Rhine Valley but for other Central European regions where a Bti-based mosquito control is frequently demanded by local inhabitants, e.g. along the Danube in Austria or around German lakes such as the Ammersee or Chiemsee.

The ecological consequences are of particular concern because large parts of Bti-treated regions all across Europe are situated in nature conservation areas that have been created to focus on the preservation of nature over human convenience. Adopting existing mosquito control measures within nature reserves that are located further apart from human residential areas would be an important first step towards environmentally safe mosquito control programs. In fact, reduced Bti spraying would be accepted by the population as long as mosquito control will be ensured in domestic and urban areas. The high environmental awareness in the surveyed population should provide an incentive for further research on alternative concepts that are adapted to the demands of the local population. Moreover, it would be important to include awareness campaigns to inform people about differences in control strategies (container-breeding mosquitoes vs. floodwater mosquitoes) and the possibility to participate in urban mosquito control.

Developing environmentally less invasive mosquito control alternatives is further important when wetlands are created to restore ecological and social functions such as floodwater protection. These restorations are often only perceived as beneficial by the population when measures to control rising numbers of mosquitoes are taken into account directly at the planning state (Westerberg et al., 2010). Nevertheless, wetlands are valuable parts of our landscape as they largely contribute to ecosystem services and freshwater biodiversity further maintaining human future (Biggs et al., 2017; Mitsch et al., 2015; Rands et al., 2010). In light of the ongoing declines in biodiversity of insects and vertebrates in particular (Ceballos et al., 2017, 2015; IPBES, 2016), wetlands should be managed in a sustainable manner minimizing human-induced threats. Thus, the design of future mosquito control programs and their implementation is a complex challenge that needs to find a balance between social demand and nature conservation.

9 REFERENCES

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APPENDICES

Appendix I: Scientific publication I

Decreasing *Bacillus thuringiensis israelensis* sensitivity of *Chironomus riparius* larvae with age indicates potential environmental risk for mosquito control

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Authors’ contribution:

Study concept: CAB, AK; Experimental work: AK, SA; Data analysis: AK; Manuscript preparation: AK, SA, CAB

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Abstract

Mosquito control based on the use of *Bacillus thuringiensis israelensis* (Bti) is regarded as an environmental friendly method. However, Bti also affects chironomid midges recognized as central resource in wetland food webs. To evaluate the risk for different larval instars of *Chironomus riparius* we performed a test series of daily acute toxicity laboratory tests following OECD guideline 235 over the entire aquatic life cycle of 28 days. Our study is the first approach that performs an OECD approved test design with Bti and *C. riparius* as a standard organism in ecotoxicological testing, First-instar larvae of *C. riparius* show an increased sensitivity towards Bti which is two orders of magnitude higher than for fourth instar larvae. Most EC50 values described in the literature are based on acute toxicity tests using third and fourth instar larvae. The risk for chironomids is underestimated when applying the criteria of the biocide regulation EU 528/2012 to our data and therefore the existing assessment approval is not protective. Possible impacts of Bti induced changes in chironomid abundances and community composition may additionally affect organisms at higher trophic levels, especially in spring when chironomid midges represent a key food source for reproducing vertebrates.

Introduction

Bacillus thuringiensis var. *israelensis* (Bti) formulations are commonly used agents for mosquito and black fly control worldwide[1,2]. More than 200 tons of Bti were applied annually in global mosquito control programs in the 1990s[3]. Bti is considered as the most environmental friendly alternative to chemical pesticides due to a high specifity to mosquito larvae and minimal effects to non-target organisms in closely related dipterans[4]. Within the group of Diptera the non-biting midges (Chironomidae) are the most Bti sensitive family[2]. In temperate regions chironomids are regarded as non-target organisms in mosquito control while in tropical countries they are also recognized as pests (and therefore target organism) in rice culture[5]. In this case Bti is used as control agent for chironomids with maximum density reductions between 65% and 88% in experimental ponds [6,7].

In most European control programs Bti products are usually applied over large areas by helicopter using a sling-bucket system while small wetlands are treated by hand[8]. In the Upper Rhine Valley (Germany) two different Bti formulations are used for mosquito control along 350 km of river: Vectobac® WG and Vectobac® 12 AS applied up to 12 times/season[9]. The nominal field rate depends on the occurring larval instars of the mosquito larvae, their density and flood water levels and is fixed at 1440 or doubled at 2880 ITU (International Toxic Units)/L[3,8,10]. Bti kills mosquito larvae by crystal and cytolitic-proteins that are built-up during sporulation of the bacteria[11]. Mosquito larvae consume these proteins which are activated in the alkaline milieu of the midgut subsequently. After activation they form pores in the epithelium leading to disruption of the midgut cells and finally to death of the larvae within a few hours[11,12]. The same mode of action takes place in the midgut of chironomids[13].

Chironomids are the most abundant group among aquatic macroinvertebrates in aquatic habtitats[14–16]. The life cycle of chironomids comprises four larval instars, a pupal life stage and the flying midge as imago[14,15]. Their ubiquity, species richness, high ecological diversity and high numbers of individuals in all kind of lentic and lotic habitats makes them an central food resource in wetland food webs[14]. Adult chironomids form huge swarms and can dominate insect emergence with over 90% of all individuals[17]. Additional to their availability and high biomass ranging between 1.0 and 100 g dry weight per year and square meter[14] chironomid larvae have a high protein content and digestibility[18]. All in all, chironomids are not only a frequent but also a valuable food resource for various insects and crustaceans as well as amphibians, birds, fish and mammals at higher trophic levels[14].

Chironomid larvae are routinely tested as standard organism representing aquatic insects in environmental risk assessment for pesticide regulation[19,20]. Acute toxicity is measured with first instar larvae as value for the effective concentration where 50% of the individuals are immobile[19]. Since mortality is difficult to assess in first-instar larvae immobility is used as alternative to mortality. Therefore EC50 values and LC50 values (lethal concentration where 50% of the individuals are dead) are equivalent. Acute laboratory tests are conducted without sediment to represent a worst case scenario. The first larval instar of chironomids is free-swimming and hence not affected by the absence of sediment[20]. Furthermore first instar chironomids is free-swimming and hence not affected by the absence of sediment[20]. Furthermore, first instar chironomids showed higher sensitivity to certain stressors such as heavy metals and chemicals [21,22]. The sensitivity regarding the EC50 values between first and fourth larval stage of *C. riparius* could differ by e.g. a factor up to 950 for Cadmium[23]. Although Bti products are applied directly to water bodies no EC50 values for first instar chironomids could be found in the literature and documents for Bti registrations[24].

In Europe, Bti (Serotype H-14, strain AM65-52) is regulated as biocide under the guideline 528/2012. The guideline pursues the protection of non-target organisms, environment, biodiversity and ecosystem[25]. The crucial instrument for granting authorization is the PEC (Predicted environmental concentration)/PNEC (Predicted no effect concentration) ratio which should not exceed 1 to assure the protection goals. Since 2011, Bti is approved under the former biocide directive 98/8/EC. For the assessment report[26] no ecotoxicological values for Chironomidae were available and included in the risk assessment.

Several toxicity studies with different designs produced EC50 values to pestiferous chironomid larvae using effective concentrations for different products and study designs. However, data are only available for third and fourth larval instars [22,23,27]. In contrast to these efficiency studies, we tested larvae of *C. riparius* as a non-target organism. We followed the standardized study design according to OECD (Organization for Economic Co-operation and Development) Guideline 235 to obtain comparable 48 h EC50 values for the different larval stages in the aquatic life cycle [19]. The tests were conducted daily over the entire aquatic life cycle of 28 days in order to test how sensitivity changes during larval development. Mean EC50 values for every larval stage were calculated and compared to reviewed literature values and field application rates for mosquito control. Furthermore, we compared and evaluated the different EC50 values of previous studies that addressed Bti sensitivity of chironomids. PEC/PNEC ratios for the different species and larval instars were calculated to simulate a risk assessment under the guideline EU 528/2012[25].

Results

During its larval development *C. riparius* showed a broad spectrum of sensitivity to Bti with EC50 values ranging from 6.9 ITU/L up to 607.8 ITU/L (Fig. 1). The first and most sensitive larval instar was 100-fold more sensitive than the fourth larval instar. Within this range the decrease in sensitivity of developing larval stages could be described with a sigmoid curve fit ($f(x) = 37.2 - 20.1x + 4.1x^2 - 0.1x^3$, adjusted $R^2 = 0.92$) suggesting an overall increase in sensitivity for older larvae (except for the last days, when pupation occurred, and individuals stop feeding and are therefore also not exposed to Bti).

In four of 28 tests the control immobilisation exceeded 15% or did not achieve 100% immobilization in the highest concentration which is why these studies were excluded from the analysis and do not appear in Fig. 1 (see Supplemental Information, Table S1). Days with less than 90% individuals of the same larval instar stage in the corresponding test are declared as 'mixed instars' (further details can be found in Table S1, Supplemental Information). Out of 28 tests 8 were identified as mixed instars even though all larvae were from one age cohort (within 24 h).

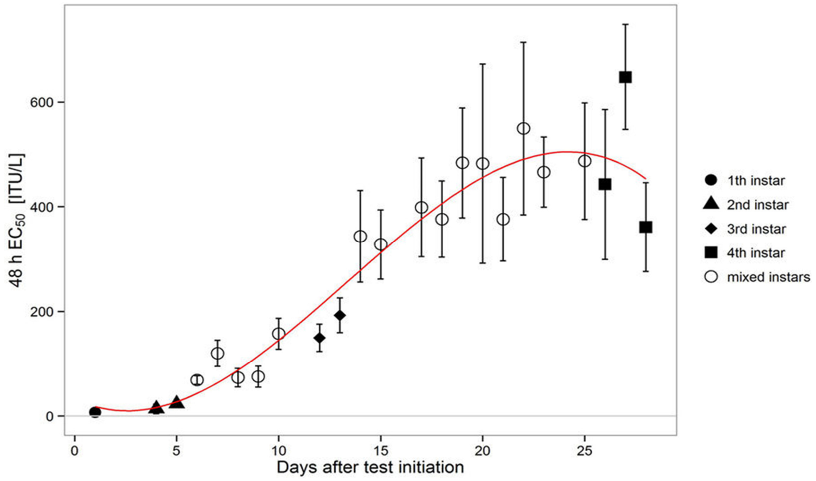

Figure 1: EC50 values with 95% Confidence Intervals (CI) on each test day during the 28 day test period. Days with more than 90% of the individuals attributed to a specific larval stage were assigned to first until fourth instars (filled symbols). The EC50 values where this criterion was not met are marked as mixed instars (unfilled symbol). Red line: curve fit of the EC50 values $f(x) = 37.2 - 20.1x + 4.1x^2 - 0.1x^3$ with adjusted $R^2 = 0.92$, p=1.165e-10. For each day 150 larvae were used.

For each of the four larval stages mean EC50 values were calculated and compared to each other. The mean EC50 values increase with increasing larval stage (Table 1). All mean EC50 values are statistically significant different from each other with p-values below 0.003 (Supplemental Information, Table S10) and separated by factors between 2.3 and 10.5. The highest increase is between second and third larval instar. First and second instar larvae show a high sensitivity to Bti compared to older larval stages.

Table 1: Mean EC50 values and 95% confidence intervals. All mean EC50 are statistically significant different from each other. Included test day (see main text) are provided.

Larval instar	Mean EC50 (ITU/L)	95% Confidence Interval (ITU/L)	Included test days
First	6.9	3.8-10.0	2
Second	16	13.6-18.4	4, 5
Third	168.7	147.9-189.4	12, 13
Fourth	485	416.6-553.3	26, 27, 28

The PEC/PNEC ratio for the biocide risk assessment was calculated with two different PECs. One was obtained from the risk assessment report of Bti Serotype H-14, strain AM65-52 (74 ITU/L)[26]. the other (1440 ITU/L) is the actual exposure concentration in surface water in mosquito control areas in the Upper Rhine Valley[10]. PNEC values are based on EC50 values of the different species obtained from literature (Supplemental Information, Table S3). For the lower exposure value four species exceed the trigger value 1 of the exposure/effect ratio (Fig. 2A). The most relevant value for the risk assessment is the EC50 of first instar larvae of *C. riparius*. Here the exposure/effect ratio is 105 (PEC = 74 ITU/L) or 2057 respectively (PEC = 1440 ITU/L). Both exceed the trigger value of 1 by several orders of magnitudes indicating an underestimated risk (Figure 2).

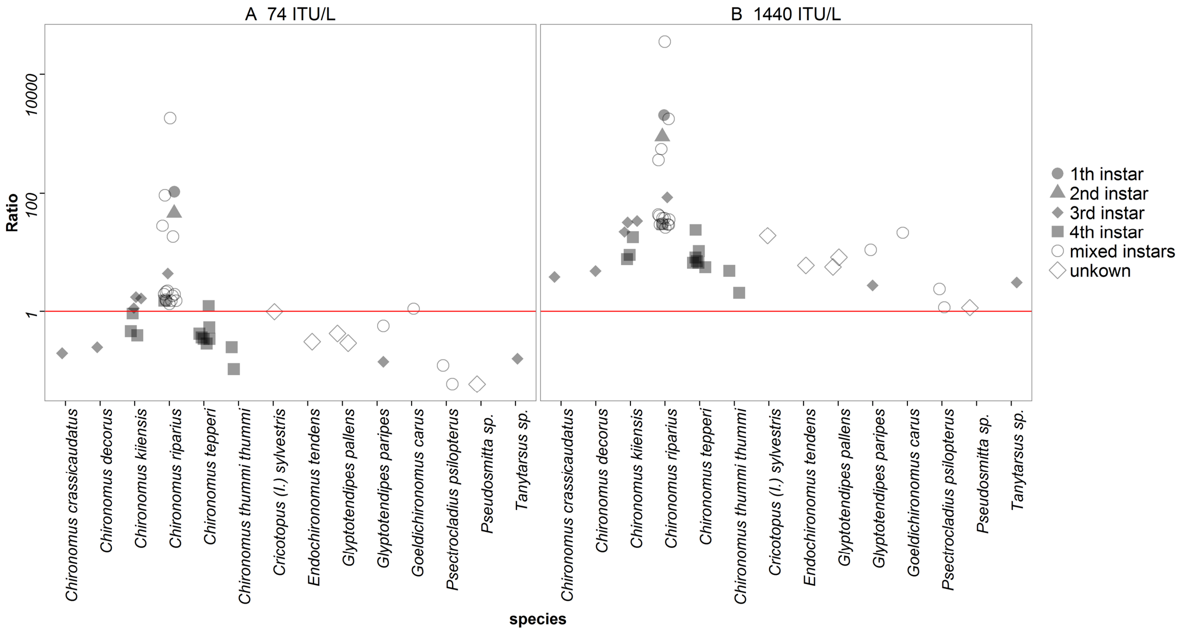

Figure 2: The calculated ratio of PEC/PNEC is shown. The ratio was calculated with a PEC of 74 ITU/L (A) respectively 1440 ITU/L which is the field rate (B). The red line marks the trigger value of the biocide guideline (PEC/PNEC=1).

Discussion

The actual assessment report on Bti for its registration in the EU did not include the most sensitive non-target organism – Chironomidae - but referred to the crustacean Daphnia magna instead[26]. Chironomids would be more suitable due to their close relationship to the target organism mosquito. They live in the targeted environment and the uptake and the mode of action of Bti is similar. Additionally midges are also recognized as central food resource in wetlands[30–33]. Following the Guideline 528/2012 the PEC/PNEC ratios exceed the trigger value for all reviewed chironomid species[25]. Most studies tested less sensitive instars, so the presumed safety of Bti for non-target Chironomidae is not given. In case of the first instar larvae of *C. riparius* the PEC/PNEC ratio is 2057 which is more than 2000 times higher than acceptable. Based on the violation of the PEC/PNEC ratio Bti and its formulated products need a reevaluation of the existing approval. Potential environmental harm is indicated by including our sensible and sensitive endpoint in the risk assessment.

The acute toxicity test is a worst case scenario for chironomid larvae. In the field the sensitivity of chironomids to Bti could be lower due to the presence of sediment, sunlight and other abiotic and biotic factors[34–36]. However, the exposure rates of 74 ITU/L and 1440 ITU/L used in our risk calculation are situated at the lower end of the existing range; field rates in Europe generally vary between 1,440 ITU/L and 3,198 ITU/L[10,31,37–39]. Field studies monitoring mosquito control in wetlands sometimes detected reductions of chironomid populations[37,40–42], others did not find any effects[38,43-45]. Community composition of chironomid species and larval instars in mosquito breeding sites with Bti application is often unknown. Field populations consist of a mixture of different larval instars[46] which lead to different sensitivity levels. Another possible explanation for the varying results in the field studies is the different species composition of the aquatic insect community which arises from different habitats like salt marshes, river floodplains and seasonal wetlands.

Chironomids represent non-target organisms in mosquito control scenarios[2,40]. Due to their ubiquitous occurrence and high numbers they are one of the most valuable food resources in temporary wetlands[14,47]. A decline of chironomids alongside with the removal of mosquito larvae leads to a reduction of available biomass for organisms at higher trophic levels and thus has implications for the entire food web[31,33,37,41]. Various predators feed directly and indirectly on chironomid larvae and imagines like dragonflies, spiders, amphibians and their larvae, fish, birds and bats[14,31,37,48–51]. Some studies exist on direct and indirect effects on higher trophic levels after Bti application[37,52] and only a thorough evaluation of their study designs and data analysis together with further coordinated research can help to come to a valid conclusion regarding potential food web deterioration due to Bti mosquito control. In Germany, in contrast to other countries as Sweden, USA or France no long-term environmental monitoring with control sites was established to allow a solid analysis[31,38,41,43].

The results from this laboratory study indicate that the risk for chironomids in the course of Bti-based mosquito control is underestimated. This could lead to disruptions on higher trophic levels within the wetland food web. As an environmental friendly alternative to other insecticides[3], in Germany Bti is also applied multiple times per season in nature conservation areas of European value with specific protected target species[10,31,40]. Currently the magnitude of Bti effects on wetland food webs is unknown and nature protection goals might be violated.

Material & Methods

Test organism

The test organism *C. riparius* Meigen 1804 (obtained from BayerCropScience AG, Monheim 2013) was kept in permanent culture within a climate controlled chamber (Weiss Environmental Technology Inc., Germany) at 20±1°C with a 16:8 light/dark regime with 800 – 1000 lux light intensity. Animals were cultured in M4 Medium[19] which was renewed once a week. The culture vessels with larvae were gently aerated and a layer of quartz sand (0.5 mm) was provided. Larvae were fed with grounded fish food (TetraMin, Germany).

Rearing the tested larvae

20 fertile egg ropes not older than 24 h were collected three days before test initiation and reared in separate culture vessel without any sediment but aeration. Ground fish food was added every two days. To reduce stress resulting from high density, larvae were randomly separated after five days into different vessels. Medium was renewed whenever necessary but latest after three days. The larvae were reared in the climatic chamber mentioned above.

Acute toxicity tests

The acute toxicity tests were conducted according to OECD guideline 235. Five larvae were exposed to five different test concentrations in 100 mL plastic beakers (Duny, Bramsche, Germany) filled with 50 mL test solution. Each of the five treatment concentrations (Supplemental Information, Table S1) and the M4 medium control consisted of five replicates. Individuals that did not move after a gentle stream produced with a pipette were considered as moribund. The number of immobile individuals was recorded after 24 h and 48 h. Individuals of the 4th instar larvae that started pupation during the test were excluded from the test[47]. Consequently the number of larvae per beaker deviated from five in the highest larval instar tests after 26 days. During the tests no food and aeration was provided. All tests ran in the climatic chamber as described previously. Oxygen content and pH was measured at the end of each test (after 48h) and was always found in agreement with the OECD Guideline 235.

Test substance and concentrations

The test substance VectoBac WDG (Valent BioSciences Corporation, USA) has the toxic potency of 3000 International Toxic Units (ITU) per mg. The active ingredient is *Bacillus thuringiensis israelensis* (strain AM 65-52). VectoBac WDG was sterilized by gamma radiation according to the standard procedure of the German Mosquito Control Association (KABS e.V.). Thereafter the potency of Vectobac WDG is reduced to approximately 2400 ITU/mg[53]. Due to larval development and decreasing sensitivity the test concentrations were adjusted during the test period of 28 days (further details in Supporting Information, Table S1 and S2). A solution with a certain amount of VectoBac WDG was prepared in M4 medium. The amount of Bti was weighed and a stock solution was prepared. The stock solution was diluted further using M4 medium until the desired test concentrations were achieved (details for preparation in Supplemental Information Table S2). To allow comparison with other studies the concentration is not given in mg VectoBac WDG/L but in ITU/L.

Determination of larval stages

Head capsule measurements were conducted to determine the larval stage of *C. riparius* since the age in days or body length is not a sufficient method to determine the larval instar [54]. Each day 10 to 20 randomly selected larvae were taken out of the rearing vessel, preserved in 70% Ethanol and head capsule width (HCW) and head capsule length (HCL) were determined using a binocular microscope (Leica CME, Leica Microsystems, Germany) fitted with a calibrated eyepiece micrometer. HCW and HCL of the selected individuals were analyzed with k-mean clustering (vegan package, R) and assigned to one of the four clusters corresponding to four larval instars. Percentages of the different larval instars were calculated daily (further details in Supplemental Information, Tables S1, S6 and Figure S7).

Risk assessment for biocides

The PEC/PNEC ratio was calculated following the Guideline EU 528/2012. PNEC is extrapolated from the EC50 value of the most sensitive organism, in this case first larvae of *C. riparius* and the assessment factor of 10. This leads to a PNEC of 0.69 (6.9 ITU/L: 10 = 0.69 ITU/L). The PNEC was calculated for all reported EC50 values. Differing test parameters of the nine evaluated studies were summarized (Supplemental Information, Table S3, Figure S6, Table S7). The PEC was derived from the assessment report of Italy and stated as 74 ITU/L, which is the lowest possible PEC in the report[26]. The resulting concentration after application to surface water in Germany is 1,440 ITU/L, and this was included as a realistic value in the analysis.

Statistical analyses

All statistical analyses were carried out using the statistical software R (version 3.1.0)[55]. The significance level to detect differences was set to $\alpha = 0.05$ for all tests. Dose-response models in the drc package[56] were fitted to the data and the daily 48 h EC50 with 95% Confidence Interval was calculated with the best model. Model fit was assessed using Akaike's information criterion. Tests were considered valid if the control mortality did not exceed 15% as recommended in the OECD Guideline 235 for acute toxicity tests[19]. As mentioned above larvae for headcapsule measurements were randomly selected every day. The headcapsule measurements of these larvae allowed conclusions about the instar stage. The percentage of larvae in respective larval instar was calculated for every test day (Supplemental Information,

Table S1). When more than 90% of the larvae were found to be in the same larval stage, the test on this day was assigned to this larval instar (Supplemental Information, Table S1). Test days which fulfilled the criterion of 90% larvae within the same instar, less than 15% control mortality and 100% mortality in the highest concentration were used for mean EC50 calculations. Data of every larval stage were fitted to a dose-response model (Supplemental Information, Figure S5, Table S3). Mean EC50 values were analysed for statistically significant differences among the four larval instars using confidence interval overlap testing (Supplemental Information, Table S10)[57]. To extract the most influential parameters of the EC50 values obtained from the literature review a linear model was calculated with the "car" package[58]. Different linear models were tested with ANOVA for significant difference to get the most parsimonious model (Supplemental Information, Figure S6, Table S7).

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Conflict of interest

The authors declare no conflict of interest.

Appendix II: Scientific publication II

Adverse effects of mosquito control using *Bacillus thuringiensis* var. *israelensis*: Reduced chironomid abundances in mesocosm, semi-field and field studies

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Authors' contribution:

Study design and supervision: SA, AK, CAB; Experimental work: AK, SA; Data analysis: SA; Manuscript writing: SA

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Abstract

The ecological consequences of mosquito control using the larvicide *Bacillus thuringiensis israelensis* (*Bti*) are still a matter of debate especially when it comes to adverse effects on non-target but susceptible non-biting midges (Chironomidae). Our work aimed to assess the effects of *Bti* applied in operational mosquito control rates on chironomid abundances in seasonal flooded freshwater wetlands. We assessed the invertebrate community alongside with aquatic insect emergence rates in studies with increasing ecotoxicological complexity, ranging from artificial mesocosms, over a semi-field approach using enclosures to natural conditions in field studies. Study sites represented different habitats (forest, meadow, floodplain) with regularly *Bti* treated and untreated temporal wetlands that are located in the Upper Rhine Valley, Germany. Larval chironomids were the most affected group of organism in the altered aquatic invertebrate communities of the *Bti* treated pond mesocosms. The larvae of the chironomid subfamilies Chironominae and Orthocladiinae were affected by *Bti* field concentrations while emergence rates of Tanypodinae did not change. The *Bti* treatment significantly reduced the targeted mosquitoes in the mesocosm and in the field studies. At the same time, however, the application of operational *Bti* field rates reduced overall chironomid emergence rates to about half of the control rates. The observed abundance reductions occurred independently of the ecological complexity in the study design in artificial mesocosms as well as realistic field conditions. The considerable reduction of the abundant non-target chironomids along with mosquitoes may subsequently lead to unwanted indirect negative effects for birds, bats and other aquatic organisms feeding on midges. Hence, large-scale applications of *Bti* for mosquito control in seasonal wetlands should be considered more carefully. This is of special importance when these wetlands are parts of national parks, nature reserves or Natura 2000 sites that were created for the protection of nature and environmental health.

Keywords: non-biting midge, non-target effect, insect emergence, biological insecticide

1. Introduction

Mosquitoes have affected human populations for centuries for two main reasons: mosquito-borne diseases and nuisance associated with mosquito bites (Becker et al., 2010). While vector control is performed locally around urban breeding sites by targeting adults or larvae of container-breeding mosquito species (van den Berg et al., 2012), nuisance control requires extensive treatments of wetlands that are regularly flooded. Flooding events trigger the simultaneous hatching of species like *Aedes vexans* and *Ae. sticticus* that subsequently pose an immense nuisance for the local population living next to these wetlands (Becker, 2006; Schäfer and Lundström, 2014).

The bacterium *Bacillus thuringiensis* var. *israelensis* (*Bti*) is seen as the most environmental friendly agent for the control of larval mosquitoes in artificial water bodies as well as natural wetlands. *Bti* is a specifically acting biopesticide that manifests its direct toxic effect in perforating the midgut epithelium of targeted Culicidae (mosquitoes) and several other nematocerous dipterans (Boisvert and Boisvert, 2000). The larvicidal activity relies on four pro-toxins produced at sporulation that get activated into toxins in the alkaline larval midgut after ingestion. The presence of specific receptor sites in the insect gut induces a perforation of the midgut epithelium which leads to a subsequent death (Boisvert and Boisvert, 2000; Bravo et al., 2007). In the short term, the use of *Bti* is considered environmentally safe with respect to most aquatic non-target organisms other than nematoceran larvae (Boisvert and Boisvert, 2000; Lacey and Merritt, 2003); nevertheless, long-term studies emphasize the lag-time of observing negative effects (Hershey et al., 1998). Above all, the significance and the extent of effects on the phylogenetically closely related non-biting midges (Chironomidae), particularly in the field, are still debated.

Since chironomids are characterized by a global ubiquity and high abundances in all kinds of freshwater environments, they generally serve as central food resource for a wide range of animals (Armitage et al., 1995). Considering this key position in wetland food-webs, a significant impairment of chironomid biomass in aquatic as well as terrestrial systems may lead to unwanted adverse effects on higher trophic levels. Such indirect effects propagated by decreased midge availability were shown in the French Camargue where dragonfly abundances decreased and birds showed a reduced breeding success (Jakob and Poulin, 2016; Poulin et al., 2010). However, observations of breeding success and bird communities in the US did not reveal such causal relationships to Bti treatments within a comparable sampling period (Hanowski et al., 1997).

Generally, chironomids showed a high susceptibility to *Bti* in laboratory studies (Boisvert and Boisvert, 2000). Effects were most pronounced in experiments that applied multiples of the recommended dosage for mosquito larval control (Boisvert and Boisvert, 2000) or were conducted under controlled conditions in artificial ponds or in situ enclosures (Charbonneau et al., 1994; Liber et al., 1998; Pont et al., 1999). Nevertheless, since most chironomids are assumed to react less sensitive than mosquitoes (Becker and Lüthy, 2017; Boisvert and Boisvert, 2000; Yiallouros et al., 1999) it is deduced that field populations are not impaired at regular mosquito control rates (Lundström et al., 2010a; WHO, 1999). However, a recent laboratory study highlighted the underestimated risk for freshly hatched chironomids in the field because the median lethal dose (LD50) for first instar larvae applied with *Bti* is about one two-hundredth of the operational application concentration in Germany (Kästel et al., 2017). In the field, biotic and abiotic environmental variables such as competition, sunlight, sediment, water depth and temperature could alleviate the toxic effects (Cao et al., 2012; Charbonneau et al., 1994). Natural soil reduces *Bti* efficacy since toxins might be absorbed by soil particles which makes them unavailable for ingestion by sediment-dwelling chironomids (Stevens et al., 2004).

Based on the prerequisites of the natural wetland, chironomid communities are characterized by diverse species compositions and age structures (Armitage et al., 1995) that vary in *Bti* susceptibilities between species (Liber et al., 1998; Yiallouros et al., 1999) and larval stages (Kästel et al., 2017). Besides, also local abiotic conditions, observation period, timing and dosage of the Bti application depict the major components when trying to predict *Bti*-related effects on chironomids in the field (Duchet et al., 2015; Hershey et al., 1998; Jakob and Poulin, 2016; Lagadic et al., 2016; Lundström et al., 2010b; Pont et al., 1999). Thus, field studies conducted in freshwater as well as brackish water environments yielded ambiguous results on side effects regarding chironomid abundances. Effects range from 60 to 80% decreased abundances (Hershey et al., 1998; Theissinger et al., 2018), over no impairment (Lagadic et al., 2014) to even higher species richness in Bti treated wetlands (Lundström et al., 2010a). Therefore, the generalization of potential impacts across wetland ecosystems is problematic.

While *Bti* is used in wetland mosquito control in more than 25 countries, the majority of studies on its environmental impact were conducted in France, Sweden and the US (Boisvert, 2007; Land and Miljand, 2014). In spite of the longstanding and extensive use of *Bti* in the Upper Rhine Valley (Germany), only two local studies specifically concentrated on chironomids (Fillinger, 1998; Theissinger et al., 2018). Both studies indicated considerable reductions of particularly aquatic chironomid species after applying *Bti* in different wetland habitats.

To reduce uncertainties associated with the aforementioned constrains, we assessed adverse effects of *Bti* on Chironomidae using studies of increasing ecological relevance and complexity. Study designs ranged from controlled conditions in an artificial mesocosm, over a semi-field approach with enclosures placed in an untreated wetland, to field studies in two different habitats that are subject to regular mosquito control. For all parts, *Bti* was applied in the operational control rates realistically used in the German mosquito control strategy of the Upper Rhine Valley. In the light of the high *Bti* sensitivity of aquatic chironomid midges, we expected that a successful mosquito control is accompanied by effects on overall chironomid abundances that are displayed across all study systems. Additionally, we hypothesized that these effects are most distinct in the artificial chironomid communities of the mesocosms since the reduced variation of environmental factors such as water turbidity, submersed vegetation and sediment. Due to the inclusion of habitat related factors in the field approaches like varying physico-chemical parameters, soil properties, UV-light, diverse invertebrate communities and species-rich natural chironomid community compositions, we assumed effects to be less pronounced in the different wetlands of the field studies. Although we focused our analysis on chironomids, we also assessed the entire aquatic invertebrate community in the mesocosms to investigate possible changes in the basic food resources available for potential predators.

2. Materials and methods

2.1 Mosquito control in the study area

Our investigations were performed in the Upper Rhine Valley, Germany where wetlands have been extensively treated with *Bti* for 40 years (Becker et al., 2018). Despite the protection through the registration as a Ramsar site (“Oberrhein/ Rhin supérieur”), more than 400.000 ha of wetlands along 350 km of the River Rhine are treated up to 11 times a year against floodwater and snowmelt mosquitoes (Becker et al., 2018). In contrast to other countries, *Bti* products applied in Germany need to be sterilized by gamma radiation before usage to expunge viable spores or cells (Becker et al., 2018). By doing so, the toxicity of the formulation decreases by about 20% (Becker, 2002). In general, the toxicity depends on the amount of endotoxins in Bti formulations and is specified in International Toxic Units (ITU) (Skovmand and Becker, 2000).

Two thirds of the application area in the Upper Rhine Valley were treated aerially by helicopters using *Bti* ice granules (IcyPearls®) while knapsack sprayers were deployed for ground application of the remaining area (Becker et al., 2018). IcyPearls® were manufactured with a suspension of VectoBac® WG which is converted to 4 mm grain sized granules that are stored frozen (Becker, 2003). VectoBac® WG (37.4% a.i., 3000 ITU/mg) is a water dispersible granule formulation produced by Valent BioSciences Corporation (Illinois, USA). Knapsack sprayers were filled with a suspension of either VectoBac® WG or VectoBac® 12AS (1200 ITU/mg). Due to varying water levels in the wetlands, recommended application rates are always stated as ITU/ha water body. In the German mosquito control strategy, the applied field rates depend on the degree of infestation of mosquito larvae and water quality (BAuA, 2018). Having low mosquito densities in wetlands, the Bti rate applied by helicopters is 0.6 kg VectoBac® WG/ha (1.44 x 10^9 ITU/ha) while ground application is performed using 0.5 kg VectoBac® WG/ha (1.2 x 10^9 ITU/ha). On occasion, these low application rates need to be increased to high application rates (1-1.2 kg VectoBac® WG/ha) to reach a sufficient treatment success (BAuA, 2018; Becker, 2003). This is routinely performed at sites with deeper water (> 10 cm), high mosquito populations densities and when older mosquitoes (3rd, 4th instar stages) are present, above all for the control of univoltine snowmelt mosquitoes (*Ochleratus communis, Oc. punctor, Ae. rusticus, Ae. cinereus)* (Becker, 2003; Schäfer et al., 1997).

2.2 Study sites

2.2.1 Mesocosm study

All study sites are located in the southwest of Germany (Fig. 1). The mesocosm study was performed at the stream mesocosm facility at the University of Koblenz-Landau, Campus Landau (49°12’13’’N, 8°06’18’’E).

2.2.2 Semi-field study

The semi-field study was conducted close to the River Rhine (48°99’47’’N, 8°27’36’’E) (Fig. 1). The site was part of a river floodplain with temporary flooded ponds located in a nature protection area (NSG “Stixwörth”). It is flooded from pressurized water during summer months and was never treated with *Bti* before since it is listed as off-limits zone for *Bti*-based mosquito control. Emergent vegetation is dominated by marsh iris (*Iris pseudocarus*). Besides deeper areas in the center (~2.5 m) the wetland is almost dry in autumn.

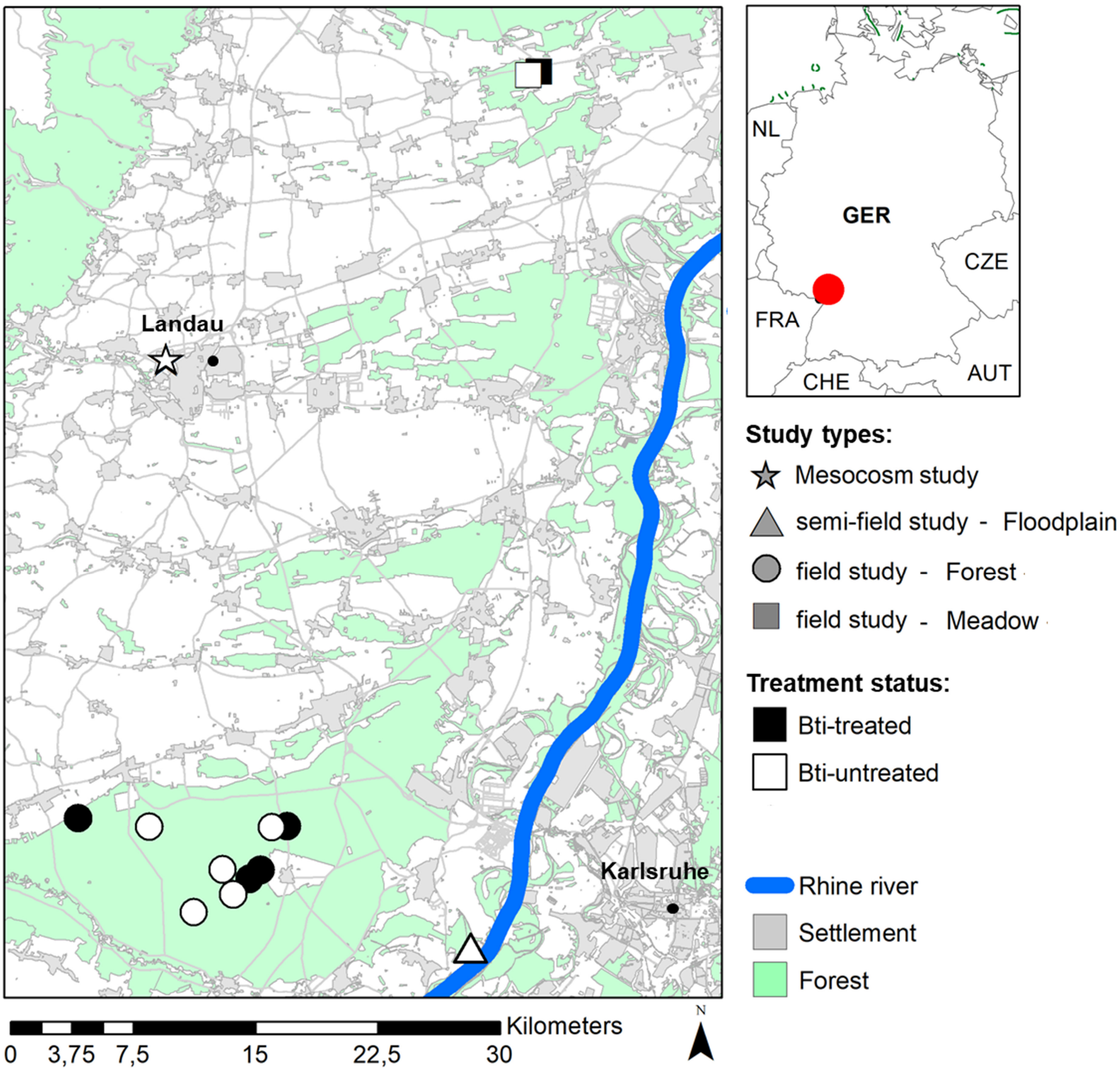

Figure 1: Study sites in the southwest of Germany. Semi-field study with enclosures located in an untreated temporary flooded pond next to the River Rhine (Δ). Field studies were conducted in a forest temporary wetland (○) with *Bti* treated (black, n = 4) and control ponds (white, n = 5) and a meadow temporary wetland (□) that was divided in one treated (black) and one untreated (white) part by split-design.

2.2.3 Field studies

Two field studies were conducted at sites that were subject of regular mosquito control activities. Study sites differed in structure and vegetation and can be categorized as forest and meadow temporary wetlands which are generally reflected in varying chironomid compositions (Brink et al., 1991).

The forest temporary wetlands (hereinafter referred to as forest) are part of the Bienwald (49°00’N, 8°15’E) (Fig. 1) which is a pine, oak and alder marsh forest located on the alluvial fan of the river Lauter. It is intertwined with small ponds and ponds which dry out during summer. Ponds around settlements were routinely treated with *Bti* against univoltine snowmelt mosquitoes while other areas of the forest serve as core zones for nature protection where *Bti* treatment is prohibited (off-limits zones). Ponds within this area served as controls for this study.

The meadow temporary wetland (hereinafter referred to as meadow) is located close to Neustadt/ Weinstraße (49°31'08''N, 8°31'19''E) (Fig.1). This study site included mainly flooded grassland with some small permanent water bodies that were regularly flooded in spring and dry during summer. The area was subject to mosquito control actions with one to two helicopter applications between March and June. In spring 2013, the study site was divided into two parts that are naturally separated by some trees and a path. While one part was still treated, the other part was left untreated for the first time for over 20 years. Emergence data of the first year after the implementation of the split field design was part of a study by Theissinger et al. (2018). This study was pursued to gain data on emergence after three consecutive years of this split field design.

2.3 Mesocosm study design

2.3.1 Study setup

We established aquatic model ecosystems in 16 artificial ponds (70 x 40 x 30 cm, 90L). These mesocosms were placed into tap water flow-through stream channels to avoid rapid heating of the water (Fig. A.2A). The control and *Bti* treatment were replicated eight times and randomly assigned to the pond mesocosms. The entire experimental phase lasted for seven weeks from May 8 to June 25, 2015. Seven weeks prior to the experiment start, mesocosms were filled with 30 L tap water and inoculated with 40 L of filtered (2 mm) water of the stream channels to provide near-natural assemblages of invertebrate fauna and algae. Each mesocosm contained a 1.5 cm deep layer of quartz sand (3.5 kg) and 30 g (wet weight) of a black alder/oak leaf mixture. Mesocosms were equipped with 60 cm *Elodea canadensis* and 30 cm *Myrophyllum spicatum* for oxygen supply. Aquatic invertebrates from local wetlands were continuously added to the mesocosms, starting from six weeks before the experimental phase. In total, 24 water lice (*Asselus aquaticus*) and 14 snails (Planorbidae, Physidae) were added to each mesocosm. Zooplankton was collected in local wetlands with a net (60 µm) once a week and homogenized with *Daphnia magna* from an in-house culture. Afterwards this medium was allocated into 16 equal volumes before addition to the mesocosms. As an initial food resource for daphnids 100 mL of diluted green algae *Desmodesmus* sp. was added to each mesocosm. Chironomidae were inserted as larvae (purchased from a pet shop; 90 mL) but also as eggs collected from an in-house culture (*Chironomus riparius*) and undefined species sampled in local wetlands. Chironomid larvae and eggs were portioned into aliquots mixed thoroughly and assigned randomly to the mesocosms once a week to ensure a diverse age structure and species mixture. Mosquito eggs and larvae were collected from the in-house culture (*Culex pipiens*) and multiple ponds and treated equally to chironomids. The mixtures of mosquito larvae and eggs were added to the mesocosm three and one week prior to experiment start.

Mesocosms were completely covered with an emergence trap (70 x 40 x 85 cm) to prevent colonization by external organisms and to collect emerged insects during the study. Collecting chambers on top of the traps were filled with glycol and a detergent. To sample emerging insects, traps were activated once a week for a period of three days. When inactivated, midges, as *C. riparius,* were allowed to swarm and potentially reproduce on the mesocosm waters (Pillot, 2014a). Mesocosms were covered with a shading net (reduction of solar radiation about 30%) to reduce high solar exposure, the subsequent rise in water temperature and to favor high abundances of chironomids and zooplankton (Mokany et al., 2008). Drainage holes close to the rim of the mesocosm containers prevented overflow after rainfall and whenever water evaporated mesocosms were refilled with tap water to ensure a volume of 70 L.

We treated the mesocosms on May 10, 2015 with VectoBac®WG granules that were applied directly on the water surface. The high water levels of 25 cm justified the application of the high Bti field rate (2.88 x 10^9 ITU/ha) (Becker, 2003) which also describes a realistic worst-case application rate. Including the surface area of the mesocosms (0.29 m²) this leads to the application of 34.8 mg VectoBac®WG/mesocosm which resulted in a concentration of about 1740 ITU/L.

2.3.2 Invertebrate sampling

The impact of *Bti* on chironomids and the invertebrate community was assessed sampling the water column and emergence. We sampled invertebrates two days prior the *Bti* application to check for an equal initial distribution of aquatic invertebrates and five times after adding the Bti treatment. Sampling days were assigned to weeks after application (WAA) resulting in WAA -1, 1, 2 3, 5 and 7. Invertebrates were sampled using a tube sampler (Ø 12 cm) with a Perspex plate as bottom lid. After gently mixing the mesocosm water and sand, the open tube was lowered quickly through the water column and the sand until it reached the ground of each mesocosm. To prevent tube content from flushing out the tube, its upper opening was tightly sealed with a suitable lid (Fig. A.3). Afterwards, the Perspex plate was carefully slid under the tube containing the sand and closed from the bottom. The content of the tube (1.5 L) was transferred into a bowl for manual identification and quantification. Organisms were identified to class (Ostracoda, Hirudinea), order (Copepoda), genus (Cladocera, Chaoboridae, Culicidae, Asellus, Hydra, Tubifex) or family (Physidae, Planorbidae, Chironomidae) immediately on site. After determination, samples were poured back into the respective mesocosm. Insect emergence was removed from the collecting chambers on WAA 0, 1, 2, 3, 5 and 7. Insects were preserved in 70% ethanol and determined to order level and the order Diptera to family level using a Leica M80 binocular with a 10x magnification. Chironomidae were determined to subfamily level with the help of the taxonomic key of Langton and Pinder (2007).

2.3.3 Semi-field study design

In total, 24 enclosures were installed on April 25 – 28, 2016 along the edge of the emergent vegetation zone of the wetland. Enclosures were constructed out of polyethylene barrels (72 x 57 x 84 cm; 300 L) that were driven ~15 cm into the sediment at a distance of 1 to 2 m of each other. Enclosures had a surface area of 0.4 m² and an average water depth of 60 ± 8 cm. After installation we removed all large predators (dragonfly larvae, newts, beetles) inhabiting the enclosures with dip-nets. For the time of the experiment, each enclosure was completely closed with an emergence trap to prevent further colonization and enable sampling of insect emergence (Fig. A.2B). Twelve replicated enclosures were assigned to *Bti* treatment and control, respectively, following a randomized design to compensate for possible habitat differences within the wetland. Emergence data was derived from 15 weekly collections from May 4 to August 17, 2016. For the comparison of the chironomid abundance in and outside of the enclosures we installed six floating traps next to the enclosures.

We treated the enclosures experimentally with *Bti* IcyPearls® on May 4 and May 24, 2016. Treatment dates were coordinated with actual application dates in nearby treatment areas. Application rate was adapted to a low application rate for IcyPearls® (1.44×10^9 ITU/ha). Including the surface area of the enclosure (0.4 m²) this resulted in the application of 61.5 mg IcyPearls®/enclosure.

2.4 Field study designs

2.4.1 Forest

We selected six pairs of neighboring ponds from a larger set of wetlands in the forest as study ponds. One half was located in the treated area around settlements and the other half in the control area (off-limits area). Study ponds met the following criteria: (1) treated and control ponds are located in the same forest section within a distance of <4km; (2) size of 0.5 to 1 ha; (3) mean water depth of <0.5 m; (4) presence of similar vegetation (mainly Carex sp.) and leaf litter. Insect emergence was collected with floating emergence traps (0.25 m^2 each, N = 3 per ditch, total: 36) according to Krell et al. (2015) (Fig. A.2C). Three traps were distributed over one pond in at a water level of at least 10 cm. When recognized that the traps went dry towards the end of the sampling period, they were relocated to deeper parts of the pond. Sampling was performed on a weekly basis over a period of five weeks from April 20 to May 26, 2015. Emergence per replicate presents the sum of insects in the three traps of one pond. *Bti* treatment of the ponds was performed by the local mosquito control operators according to the standard mosquito control protocols. Ponds were treated between March 20 – 27, 2015 with backpack sprayers applying 0.5 kg VectoBac® WG/ha (1.2 x 10^9 ITU/ha). To verify treatment status of the ponds we obtained treatment data from the local mosquito control operators. Due to uncertainties in the application status of three ponds, we focused further analyses on four treated and five control ponds.

2.4.2 Meadow

The treated and untreated part of the meadow was each equipped with twelve floating emergence traps (0.25 m^2) from the same type that were used in the forest. Traps were equally spread over the meadows at a distance of 1 to 2 m from the shore and an initial water level of 20 to 30 cm. Emergence was collected weekly starting two weeks prior to the *Bti* application to 13 weeks after the application (March 14 to June 26, 2016). *Bti* application on the treated part of the study sites was performed and verified by the local operator within the regular mosquito control actions in this region. *Bti* was applied by helicopter on March 29, 2016 using a high *Bti* application rate of 30 kg IcyPearls®/ha (2.88 x 10^9 ITU/ha).

2.5 Semi-field and field emergence

All emerged and preserved insects (70% ethanol) were counted per site, pond, trap and sampling week. Emergence was determined to order level in general and the order Diptera to family level using a Leica M80 binocular with a 10x magnification. Chironomids were not determined further since chironomid species composition is subsequently assessed using a metabarcoding approach as recently performed by Theissinger et al. (2018).

2.6 Water parameters

In the mesocosm study, measurements of pH, water temperature and dissolved oxygen concentration took place mid-morning of each invertebrate sampling day in a random design using a portable apparatus (WTW Multi 340i, WTW GmbH, Weilheim, Germany). In the semi-field and field studies, water temperature was measured weekly at each sampling day within each enclosure and next to each emergence trap. Water levels were measured to the nearest 1 mm using a folding rule in the semi-field and field studies. Mean depth was calculated as the mean of the deepest and lowest corner within the enclosures and underneath each emergence trap, respectively.

2.7 Data analysis

The effect of *Bti* treatment on the community structure in the mesocosms was analyzed using the principal response curve (PRC) method (Brink and Braak, 1999; Van den Brink et al., 2000) which is based on a redundancy analysis (RDA) of the communities over time. Taxa that made up $< 0.1\%$ of the sampled invertebrates were removed from the dataset and abundance data were $\ln(2x+1)$ transformed to avoid false discrepancies between zero abundances and low abundances (Van den Brink et al., 2000). The statistical significance of effects of the *Bti* treatment at different time points were tested using Monte Carlo permutations (999 permutations) (Legendre et al., 2011).

Abundance data of sampled organisms were compared between treated and control conditions and cumulative reductions were calculated over the entire sampling period. Comparisons were based on generalized linear mixed-effect models (GLMM) with treatment, weeks after application (WAA), and their interaction as fixed effects. We used a negative binomial error distribution for count data on mosquito and chironomid abundances. Since we had repeated measures we included mesocosm, enclosures, pond identity in the forest and the traps in the meadows respectively as random intercept term. The statistically significant difference in insect abundance was assessed on sampling dates where 95% confidence intervals of *Bti* treatment and control were not overlapping. A non-parametric Kruskal-Wallis test was used for not normally distributed data. Welch’s t-test was applied by normal distribution but unequal variances. Normality of data was examined using the Shapiro–Wilk test, as well as visual inspection. Homoscedasticity was tested with Levene’s test. Significant differences were denoted by asterisk in graphical representations of the data. All statistical analyses were conducted in the R computing environment (R version 3.3.2).

3. Results

3.1 Mesocosm study

3.1.1 Aquatic invertebrates

In total, 18 taxonomic groups of aquatic invertebrates were identified in the mesocosms for the whole study period with crustaceans (Cladocera (37%), Ostracoda (17%) and Copepoda (8%)) and chironomids (18%) as the most prevalent groups in the communities (Table A.1).

Table 1: Comparison of aquatic invertebrates and emergence between *Bti* treatment and control in the mesocosms, semi-field and field studies. Forest (-C5): Exclusion of very productive control pond. Statistically significant reductions due to the *Bti* treatment indicated in bold.

		sampling weeks	traps	overall organisms abundances	Culicidae		Chironomidae	
					cumulative abundance	reduction [%]	cumulative abundance	reduction [%]
mesocosm (aquatic invertebrates)								
	control	7		8797	27		1985	
	Bti treated	7		7363	4	**85.2**	928	**53.3**
mesocosm (emergence)								
	control	7	8	2282	21		2154	
	Bti treated	7	8	1213	2	**90.5**	1106	**48.7**
semi-field (emergence)								
	control	15	12	1473	17		923	
	Bti treated	15	12	1088	13	23.5	542	41.3
field (emergence)								
forest	control	6	15	5458	2978		1522	
	Bti treated	6	12	942	38	**98.7**	354	76.7
forest (-C5)	control	6	12	1424	1037		228	
	Bti treated	6	12	942	38	**96.3**	354	- 55.3
meadow	control	14	12	5489	165		3527	
	Bti treated	14	12	3436	13	**92.1**	1138	**67.7**

According to the PRC analysis, the invertebrate community structure differed between control and treated mesocosms due to the *Bti* treatment (*Bti*: F = 6.38, p = 0.001). Over the course of time, Monte Carlo permutation tests indicated that differences occurred at all sampling dates except the initial sampling (two days prior to the *Bti* application) and WAA 5 (Fig. A.8). Overall, time explained 35% of the total variance and 9% could be attributed to treatment, of which 65% was captured by the first PRC axis. According to the analysis of taxa weights, chironomid larvae contributed most to between-area differences, which is manifested in a

53% reduction in cumulative chironomid abundances in *Bti* treated mesocosms (GLMM: z = -2.24, p = 0.025, Table 1). Reductions in larval abundances were significant from WAA 2 to 5 (WAA 2: $X^2 = 11.36$, p < 0.001; WAA 3: $X^2 = 9.97$, p = 0.002; WAA 5: $X^2 = 7.24$, p = 0.007) and converged after seven weeks (Fig. 2). Although larval mosquitoes occurred in low abundances until WAA 3, *Bti* reduced the targeted organism significantly by 85% (GLMM: z = -3.19, p = 0.001, Table 1, Fig. A.9). Other aquatic invertebrates in the mesocosms were not significantly affected in their abundances as a result of the Bti treatment (Table A.1).

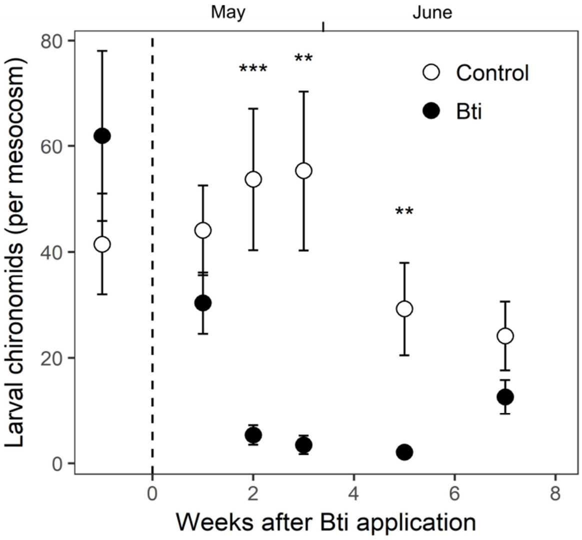

Figure 2: Change in mean abundances (± SE) of chironomid larvae in control and *Bti* treated mesocosms. Significant difference from control, **: p < 0.01; ***: p < 0.001.

3.1.2 Insect emergence

Chironomidae were by far the most abundant group of insects in the emergence traps during the entire sampling period (93%), followed by Chaoboridae (2%) and Culicidae (1%). During the observation period of seven weeks, chironomid midges were reduced by 49% in the *Bti* treated mesocosms compared to controls (GLMM: z = -3.23, p = 0.001, Table 1). One week after the application, *Bti* caused 59% reduction (t = 2.46, p = 0.04) followed by more than 80% in the two subsequent weeks (WAA 2: $X^2 = 6.98$, p = 0.008; WAA 3: $X^2 = 8.66$, p = 0.003) before abundances converged from WAA 5 onwards (Fig. 3A). However, emergence within the Chironomidae subfamilies Chironominae, Orthocladiinae and Tanypodinae was highly variable (Fig. 4, Table A.2). Chironominae and Orthocladiinae both comprised about half of all identifiable Chironomidae imagines (Chironominae: 46%; Orthocladiinae: 50%). The emergence rate of both subfamilies was reduced by 44% (Chironominae: GLMM: z = -4.58, p < 0.001) and 58% (Orthocladiinae; GLMM: z = -2.21, p = 0.03) in the *Bti* treated mesocosms. In the first two weeks after application, control emergence peaked for Orthocladiinae whereas *Bti* treatment emergence was two thirds lower (WAA 1: $X^2 = 4.42$, $p = 0.04$; WAA 2: $X^2 = 5.26$, $p = 0.02$). Peak emergence was recorded for Chironominae after around three weeks when *Bti* caused a reduction by 90% in comparison ($X^2 = 11.38$, $p < 0.001$). In contrast, *Bti* treatment did not affect emergence of Tanypodinae (GLMM: $z = 1.15$; $p = 0.25$) that comprised 4% of all identifiable Chironomidae. In a similar manner to mosquito larvae, *Bti* reduced adult mosquitoes (Culicidae) significantly by 90% (GLMM: $z = 3.04$, $p = 0.002$, Table 1, Fig. A.10A).

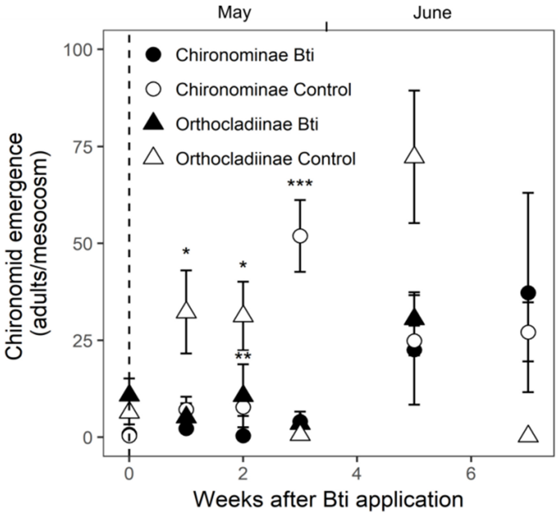

Figure 4: Change in mean emergence rates (± SE) of adult Chironomidae (○) and Orthocladiinae (∆) in control (white) and *Bti* treated (black) mesocosms. Significant difference from control; *: p < 0.05; **: p < 0.01; ***: p < 0.001.

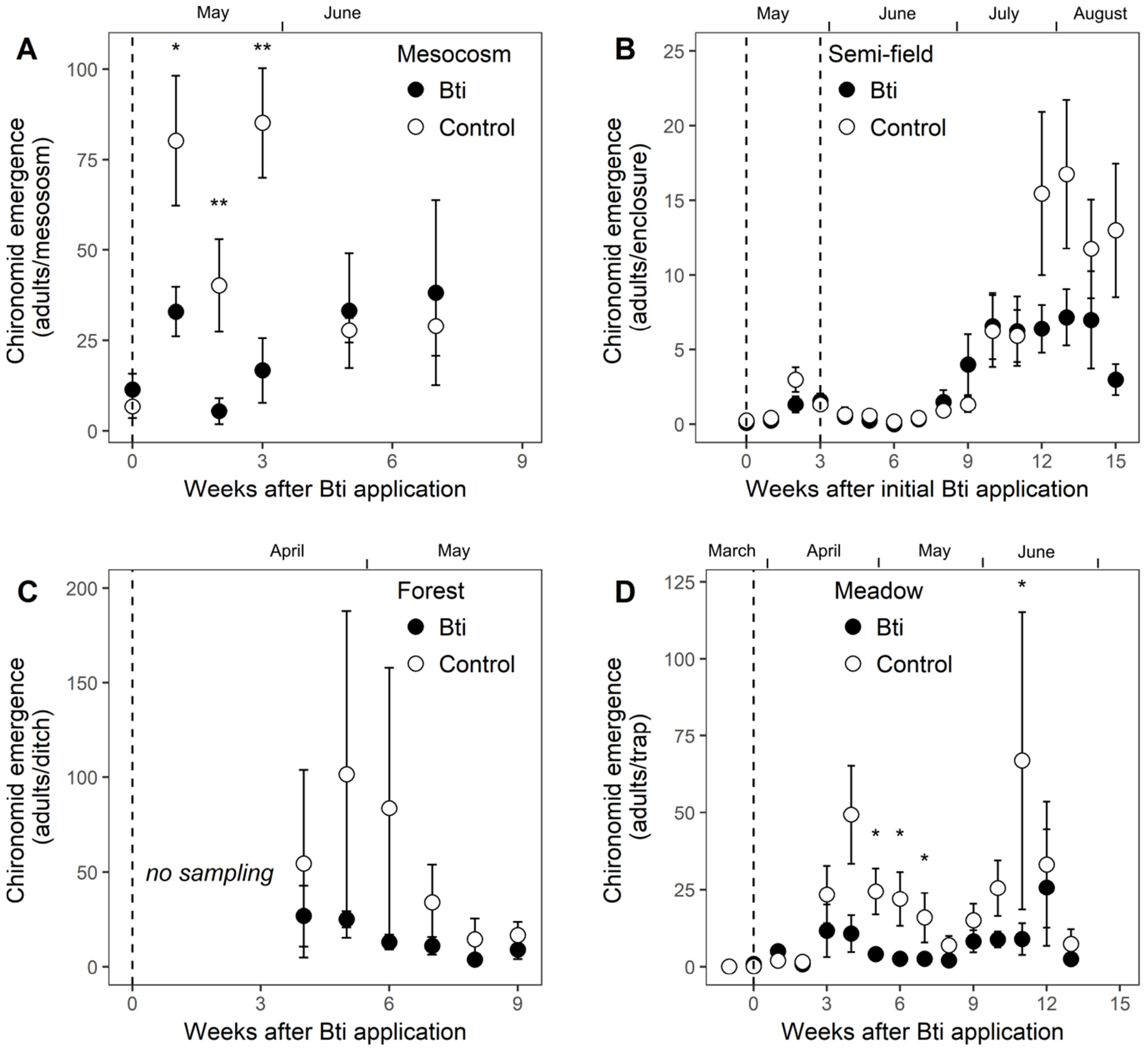

Figure 3: Comparison of mean chironomid emergence (±SE) between the control (white) and *Bti* treatments (black) in the A: mesocosms (n = 8), B: semi-field study (n = 12), C: forest (n = 4 resp. 5) and D: meadows (n = 12) after initial *Bti* application (dashed lines). Significantly different from control, *: $p < 0.05$; **: $p < 0.01$.

3.2 Semi-field: Insect emergence

In total, 2561 insects were sampled in the emergence traps of the enclosures with 73% belonging to the group of Nematocera. The most abundant taxa during the entire sampling period were Chironomidae which described more than half of all collected individuals (57%) followed by Chaoboridae (12%) and Brachycera (10%). Culicidae made up 1% of all organisms. Over the entire sampling period, adult chironomids were cumulatively reduced by 41% in the *Bti* treated enclosures which was not statistically significant (GLMM: $z = -1.56$, $p = 0.11$). While a small emergence peak occurred in mid-May, the majority (90%) of chironomids in both the control and the *Bti* treatment started to emerge eight weeks after the initial Bti application (WAA8 to 15) (Fig. 3B). From end of June (WAA8) onwards, *Bti* treatment affected chironomid emergence with proceeding time (GLMM (WAA**Bti*): $z = -2.73$, $p = 0.006$). Overall mosquito emergence were reduced by 24% but not statistically significantly affected looking at the entire sampling period (GLMM: $z = -0.60$, $p = 0.55$, Table 1, Fig. A.10B).

Floating traps surrounding the enclosures collected 1157 chironomids during the study time. The cumulative abundance was 20% higher than in the controls and twice as much as in the treated enclosures. The emergence peak was at around WAA 10 which is three weeks prior to the enclosure emergence peak (Fig. A.7A).

3.3 Field studies: Insect emergence

3.3.1 Forest

Within six weeks, 6400 organisms were collected: The majority belonged to the group of Nematocera (78%) with Culicidae (47%), Chironomidae (29%) and Brachycera (20%) describing the most dominant families in the samples. *Bti* treatment considerably reduced mosquito abundances by 99% over the entire sampling period (GLMM: $z = -2.75$, $p = 0.006$). The treatment effect can be seen between WAA 5 and 7 where no mosquito emergence peak occurred in the treated ponds compared to controls (Fig. A.10C). Chironomid emergence was highly variable within the control ponds, ranging from 0 to 277 individuals per trap and sampling date (Fig. 3C). Among these controls was one pond (C5) with high midge emergence rates (1941 mosquitoes and1294 chironomids). If this pond was included in the analysis, 77% less chironomids emerged from the *Bti* treated ponds during the entire sampling period (GLMM: $z = -0.6$, $p = 0.55$). When C5 is excluded from the analysis, chironomid abundances increased by 55% (GLMM: $z = 3.14$, $p = 0.26$). Both differences were statistically not significant (Table 1). The treatment effect on mosquitoes was unaffected by an exclusion of C5 (GLMM: $z = -3.15$, $p < 0.001$).

3.3.2 Meadow

Compared to semi-field and forest, a smaller proportion of 54% of the overall sampled 8925 individuals in the traps were nematocerans. Nevertheless, chironomids made up more than half (52%) of all collected insects. Chironomid abundance fluctuated over time showing one spring emergence peak at the end of April and another summer emergence peak in June (Fig. 3D). Both times, the *Bti* treated wetland produced less chironomids, resulting in a significant cumulative reduction of 68% during three months of sampling (GLMM: $z = -2.13$, $p = 0.03$). Mosquitoes occurred with a low relative abundance of 2% of all emerged individuals.

Nevertheless, *Bti* treatment significantly reduced mosquitoes by 92% (GLMM: $z = -3.66$, $p < 0.001$) which was most distinct from WAA 4 to 7 (Fig. A.10D).

4. Discussion

Effects of Bti treatments

Amongst all non-target aquatic invertebrates in the mesocosm communities, larval chironomids were the most affected group of organisms. While a few chironomid species are predatory the majority of species is described as (facultative) collector/gatherers, grazer or filter-feeder (Pillot, 2014a, 2014b) that may ingest *Bti* out of the water but also after deposition on the sediment. Additionally, first instar chironomids are often free-swimming (Armitage et al., 1995) which makes them equally exposed to *Bti* than mosquito larvae. As chironomid and mosquito larvae are physiologically similar they are affected by *Bti* with the same toxic mode of action (Ali, 1981). Moreover, acute effects due to increased oxygen consumption through the addition of the *Bti* formulation can be excluded since oxygen levels were consistent between treatments (Fig. A.4B). In this context, abundances of other abundant non-target invertebrates like zooplankton (Cladocera, Copepoda, Ostracoda), snails or water louse were not affected by the *Bti* treatment (Table A.1) which is in accordance with other studies on non-target organisms (Boisvert and Boisvert, 2000). However, the abundance of daphnids, for instance, was also not positively affected although they could have benefited from reduced competition as observed in field experiments (Kroeger et al., 2013).

Biotic and abiotic factors such as substrate, macrophytes, mixed larval instars and higher larval densities are known to diminish the larvicidal efficacy on benthic chironomids when compared to laboratory conditions (Cao et al., 2012; Stevens et al., 2004). Despite including these factors in the mesocosms, half the population of larval chironomids was adversely affected. The LC_{50} of the least sensitive fourth instar stage of *C. riparius* in the laboratory (Kästel et al., 2017) is approximately three times higher than the resulting *Bti* concentration in the mesocosms. Similar reductions of 50% were shown within a wetland enclosure study at 5.9 x 10^9 ITU/ha (VectoBac® G) (Liber et al., 1998) which is around twice the rate applied in our artificially created mesocosms. However, when comparing the toxicity of regular Bti applications, the high application rate of VectoBac® WG (2.88 x 10^9 ITU/ha) in the mesocosm equals operational treatments of VectoBac® G in the Swedish mosquito control (3 x 10^9 ITU/ha) and VectoBac® 12AS in the French Camargue (3.2 x 10^9 ITU/ha) (Jakob and Poulin, 2016; Lundström et al., 2010b).

The direct effect on larval chironomids in the mesocosms was reflected in half as much emerging chironomid imagines (Table 1). Overall emergence was characterized by high proportions of the subfamilies Chironominae and Orthocladiinae that are known to respond adversely to the *Bti* treatment (Boisvert and Boisvert, 2000; Liber et al., 1998). Their susceptibility towards *Bti* is explained by the herbivorous and detrivorous feeding strategy of most species within these subfamilies (Armitage et al., 1995). In general, the potential exposure to *Bti* via direct ingestion of the spores is higher for filter-feeders than predators such as most Tanypodinae. Tanypodinae were even not affected by concentrations exceeding the operational rate ten-fold in semi-field study (Liber et al., 1998). The low Tanypodinae abundances in our mesocosms (4%) is comparable to freshwater field studies where Tanypodinae occurred between 5 and 9% (Liber et al., 1998; Lundström et al., 2010b).

Effects on chironomid emergence were less pronounced in the semi-field approach, although a *Bti* related effect was evident with proceeding time when the majority of all chironomids emerged from July onwards (Fig. 3B). Interestingly, the enclosure community may have been slightly different to the one in the surrounding wetland indicated by a lower abundance of imagines in the enclosures compared to the free-floating emergence traps. Furthermore, the hatching peak in the enclosures was postponed by three weeks (Fig. 3B, Fig. A.7A). This shift can either be a result of delayed growth rates due to limited food resources within the enclosures or the prevention of re-colonization by aquatic bi-or multivoltine chironomids since April. *C. riparius*, for instance, is a *Bti* susceptible and multivoltine species that emerges and reproduces between March and November within 16 days under favorable conditions (Pillot, 2014a).Hence, a depleted community in terms of aquatic chironomids that generally show increased susceptibility towards *Bti* (Fillinger, 1998) may have diminished the effects compared to field studies.

While chironomids in the meadow were reduced by two-thirds after the Bti application (Table 1), the second field study, conducted in a forest, revealed inconsistent results that are driven by one control pond highly productive in nematocerans (Table 1). Overall, the forest emergence was dominated by mosquitoes which indicates that the composition of the insect community is different compared to our other study habitats. Additionally, low water levels (Fig. A.6B) and the emergence of Brachycera reflect that traps went dry at times. This could also be indicative of a different chironomid species composition with a larger proportion of less susceptible terrestrial chironomids (Fillinger, 1998) that moderated the impact of *Bti*.

Our study provides the first investigation of chironomids in artificially created and controlled freshwater pond mesocosms with *Bti* treatment as the only modified stressor. Hence, one of the main objectives of this study was to assess if *Bti* based chironomid effects found in these artificial systems can be transferred to ecologically more complex study systems under field conditions. Contrary to our assumption, overall chironomid emergence rates were comparatively reduced by about 50% throughout our study systems deploying increasing complexity (Table 1). Remarkably, the most distinct effect did not occur under controlled conditions but in the meadow field study where emergence of chironomids was reduced by two-thirds within three months after the *Bti* treatment.

Implications on wetland food webs

Seasonal wetlands subjected to mosquito control are occasionally classified as untypical chironomid habitats and less important in terms of chironomid productivity when being compared to permanent water bodies such as lakes (Becker and Margalit, 1993; Lundström et al., 2010b). Nevertheless, chironomids often represent the most dominant taxa constituting 42 to 93% of the invertebrate community (Hershey et al., 1998; Leeper and Taylor, 1998; Theissinger et al., 2018) which was also supported by data of our study wetlands. Thus, the observed significant reduction in chironomid abundances could have far-reaching implications on the ecological systems of temporary wetlands (Gopal and Junk, 2000). This is because chironomids play a key role in the trophic structure in these freshwater ecosystems due to their high protein content, digestibility and their ability to form large swarms over their breeding waters (Armitage et al., 1995; De La Noüe and Choubert, 1985). *Bti* treatments that almost completely eliminate mosquitoes (Becker et al., 2010) and concurrently reduce chironomids by 50% may ultimately evoke a significant loss of important food sources for aquatic and terrestrial predators. Indirect effects of *Bti* via the food chain were already demonstrated for dragonflies and birds in the Camargue (Jakob and Poulin, 2016; Poulin et al., 2010). Furthermore, changing populations of flying insects are also linked to the decline in aerial insectivorous birds which is recorded in North America (Nebel et al., 2010) but also in German wetlands that are more importantly subject to regular Bti treatments (Schrauth and Wink, 2018). Seasonal wetlands like the ones examined in our study are treated with *Bti* against univoltine snowmelt mosquitoes once or twice between March and May (Becker et al., 2010). However, in early spring, when terrestrial insect emergence is still reduced, chironomids are an abundant food source of particular importance. The diet of, for instance, bats that primarily hunt above wetlands was comprised out of chironomids to more than 80%

during springtime (Arnold et al., 2000). Considering the extend of the *Bti* treated area in the Upper Rhine Valley, a considerable reduction in overall midge abundances may in turn result in reduced food availability for many predators.

Chironomids in other Bti field studies

Generally, the extent of the observed effects on chironomids in our study is in agreement with an annual study in mosquito control areas in the salty marshes of the Camargue where 48% less adult chironomids occurred (Jakob and Poulin, 2016). Another study even found 94% less aquatic chironomids emerging after *Bti* field applications in a wetland in the Upper Rhine Valley comparable to the ones investigated in our study (Fillinger, 1998). Moreover, chironomid abundances decreased by 60 to 80% two and three years after the initiation of the *Bti* spraying in Minnesota (Hershey et al., 1998).

While an increasing number of studies on *Bti* have been conducted in brackish coastal wetlands or saltmarshes (Caquet et al., 2011; Duchet et al., 2015; Jakob and Poulin, 2016; Lagadic et al., 2014, 2016; Pont et al., 1999) field data on the impact of *Bti* on chironomids in freshwater ecosystems is rather scarce (Fillinger, 1998; Hershey et al., 1998; Lundström et al., 2010b). The latest study is a six-year monitoring of floodplains along River Dalälven in Sweden that did not record changes in chironomid production (Lundström et al., 2010a, 2010b) albeit annual sampling was performed between May and September. River floodplains treated against floodwater mosquitoes (*Ae. vexans*) are the most frequently treated wetland types in the Upper Rhine Valley. Nevertheless, transferring the results from Swedish floodplains must be considered with caution due to *Bti* application frequencies. While wetlands in Sweden experienced a maximum of five applications within six years (Lundström et al., 2010a, 2010b), floodplains along the River Rhine are treated on average five times per season (Becker et al., 2018).

Although habitat related and natural factors, above all the occurrence of flooding or drought, are suggested to be more important than *Bti* in explaining chironomid abundances particularly in long-term field investigations (Lagadic et al., 2016; Lundström et al., 2010b), conducting comparative studies in German floodplains is challenging. One reason is that it is hardly possible to find control areas that are often flooded but have not been treated with *Bti* for the last 20 years. Consequently, no long-term field monitoring data of ecological impacts at the Upper Rhine is available for the decision making progress in other areas such as along the Danube or German lakes where a *Bti* based mosquito control was frequently demanded by local inhabitants. Even though our results are referring to annual chironomid data, we are able to compare effects between the first and fourth year after the implementation of the split-field design in the meadows (Theissinger et al., 2018). Nevertheless, both examination years showed consistently reduced chironomid productions, namely, by two-thirds within three months after the *Bti* treatment (Theissinger et al., 2018).

Limitations of field studies

Reduced chironomid productivity in our natural study systems was especially apparent in narrowed emergence peaks between four and six (Fig. 3C, D) but also around twelve weeks after the initial *Bti* application (Fig. 3B, D). Since the larvicidal activity of *Bti* quickly decreases (Lacey, 2007; Sheeran and Fisher, 1992), the delayed effect on emergence is probably due to acute effects on larvae of species with different developmental duration, present at sensitive larval stages during the *Bti* application. With two to four months most chironomid species have a longer developmental time than mosquitoes that emerge in less than three weeks during summer months (Becker et al., 2010; Pillot, 2014a, 2014b). Hence, short-term studies running for two to a maximum of eight weeks revealing unaffected chironomid abundances (e.g. Charbonneau et al., 1994; Duchet et al., 2015; Liber et al., 1998; Pont et al., 1999) could also have missed *Bti*-related effects. Therefore, field approaches focusing on emergence should be performed over at least three months after an initial *Bti* application to assess comprehensive effects on several chironomid species.

Furthermore, unwanted effects on non-target organisms should always be evaluated in relation to the achieved effect on mosquitoes as targeted organism. It is important to stress that many semi-field and field studies that did not find adverse effects on chironomids under operational application rates do not provide explicit information on mosquito abundances (Caquet et al., 2011; Duchet et al., 2015; Lagadic et al., 2016, 2014; Liber et al., 1998; Pont et al., 1999). The absence of any effects on chironomids should only be seen as reliable prediction if mosquitoes are statistically significant reduced due to the *Bti* treatment at the same time. Otherwise it could also be possible that the treatment concentrations in the water were ineffective due to environmental conditions. *Bti* application rates in our mesocosm and field studies were sufficient to control mosquito emerging rates by more than 90% (Table 1) which is the desired efficacy of mosquito control programs (Becker et al., 2010). However, the effect on mosquitoes in our semi-field approach was very low (24%) indicating that the effect on chironomids may also be ranked as low. The application of a high application rate, justified by high enclosure water levels (42 to 75 cm) (Becker, 2003), could have led to significant mosquito control rates and more distinct effects on chironomids than observed.

5. Conclusion

The local and small-scale application of *Bti* to control container-breeding mosquito species is probably among the most environmental friendly methods. However, this study reveals evidence that the large-scale application of *Bti* for nuisance mosquito control in seasonal freshwater wetlands should be considered more carefully. We showed that chironomid abundances were reduced by 50% and more after field relevant *Bti* applications in studies of increasing ecological complexity. Based on the high abundances of chironomids in the wild a halving of non-biting midge numbers leads to a much larger loss in wetland biomass than an almost total elimination of mosquitoes. Thus, *Bti* induced reductions in midge availability can potentially impair wetland food webs and lead to unwanted indirect effects on predatory organisms of conservation interest. These relations should be of particular concern since large parts of *Bti* treated areas all across Europe are situated in national parks, nature reserves or Natura 2000 sites that intrinsically focus on the protection of nature and environmental health (Lagadic et al., 2014; Land and Miljand, 2014). Indeed, ecological consequences can vary in time and space which complicates efforts to generalize the environmental impact over the variety of wetlands where *Bti* is regularly applied. Since diverging environmental factors lead to wetland specific chironomid compositions, communities should be assessed further to possibly derive predictions over wetland types. Besides, studying mosquito abundances should be a key prerequisite for evaluating non-target effects of *Bti* since this is the indicator for both, successful mosquito control and the usage of an appropriate sampling scheme uncovering adverse effects. As chironomids can finally be considered the most susceptible non-target organism, appropriate endpoints should also be incorporated into the European risk assessment for *Bti* (European Comission, 2011).

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Declaration of interest

The authors declare that they have no competing interests.

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Appendix III: Scientific publication III

Using DNA metabarcoding for assessing chironomid diversity and community change in mosquito controlled temporary wetlands

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Authors' contribution:

Sampling: AK, SA; Laboratory work: AK, VE; Bioinformatic data analyses: VE; Biological data analyses: KT, AK, JM, SM; Multivariate statistics: SIS; Study design and supervision: KT, CB FL; Manuscript writing: KT, AK equal contribution.

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Abstract

The biocide *Bacillus thuringiensis* var. *israelensis* (Bti) is widely applied for mosquito control in temporary wetlands of the German Upper Rhine valley. Even though Bti is considered environmentally friendly, several studies have shown non-target effects on chironomids, a key food resource in wetland ecosystems. Chironomids have been proposed as important indicators for monitoring freshwater ecosystems, however, their morphological determination is very challenging. In this study, we investigated the effectiveness of metabarcoding for chironomid diversity assessment and tested the retrieved chironomid operational taxonomic units (OTUs) for possible changes in relative abundance and diversity in relation to mosquito control actions in four temporary wetlands. Three of these wetlands were, for the first time after 20 years of Bti treatment, partly left Bti-untreated in a split field design, and one wetland has never been treated with Bti. Our metabarcoding approach detected 54 chironomid OTUs across all study sites, of which almost 70% could be identified to species level by BLAST searches against the BOLD database. We could show that metabarcoding increased chironomid species determination by 70%. However, we found only minor significant effects of Bti on the chironomid community composition, even though Bti reduced chironomid emergence by 65%. This could be due to a time lag of chironomid recolonization, since the study year was the first year of Bti intermittence after about 20 years of Bti application in the study area. Subsequent studies will have to address if and how the chironomid community composition will recover further in the now Bti-untreated temporary wetlands to assess effects of Bti.

Keywords: *Bacillus thuringensis* var. *israelensis* (Bti), Barcode of Life Database (BOLD), cytochrome oxidase subunit I (COI), operational taxonomic units (OTU), macrozoobenthos emergence, chironomid saprobic index

Introduction

Since 1981 the biocide *Bacillus thuringiensis* var. *israelensis* (Bti) is widely applied for mosquito control (Culicidae, Diptera) in temporary wetlands of the German Upper Rhine valley to minimize nuisance of local residents (Becker 1998). Bti is considered as the most environmentally friendly alternative to chemical pesticides for mosquito control due to a supposedly high specificity to mosquito larvae and negligible non-target effects even on closely related dipterans (Boisvert and Boisvert 2000). This is important as large areas of both aquatic and terrestrial habitats of the Upper Rhine valley are protected (bird sanctuaries, nature reserves and Natura 2000 sites) and comprise of biodiversity hotspots (Biggs et al. 2005, Lukács et al. 2013).

However, several studies have shown that Bti non-target effects are possible (reviewed in Boisvert and Boisvert (2000)). Non-biting midges (Chironomidae, Diptera) are the most Bti-sensitive non-target family (Boisvert and Boisvert 2000). Controlled experiments revealed varying mortality rates on chironomid larvae with older larvae being typically less sensitive to Bti (Ali et al. 1981, Treverrow 1985, Ping et al. 2005). They also reported different sensitivities among species Yiallouros et al. 1999 and subfamilies Liber et al. 1998. A recent study found that first instar larvae of *Chironomus riparius* are highly susceptible to Bti treatment even at commonly used mosquito control application rates (Kästel et al. in press). Consequently, Bti application might overproportionately affect chironomid species in early larval stages at the time of application. So far, field studies have yielded ambiguous data on possible side effects of Bti on chironomid abundances. These range from positive effects on chironomid larvae richness possibly due to reduced mosquito competition (Lundström et al. 2010), over no effect on chironomid abundance (Lagadic et al. 2016), to a 35-80% reduction of chironomids abundances (Rodcharoen et al. 1991, Hershey et al. 1995, Vaughan et al. 2008, Poulin et al. 2010, Jakob and Poulin 2016).

Chironomids are a taxonomically and ecologically highly diverse group and often dominate all kinds of lotic and lentic ecosystems in terms of abundances (Ferrington 2008). With sometimes over 50% of the total macroinvertebrate fauna in aquatic ecosystems (Milošević et al. 2013, Puntí et al. 2009) chironomids are thus a key food resource in wetland ecosystems. They also constitute a central link between aquatic and terrestrial food webs as adult midges are prey for birds, bats, spiders and adult dragonflies (Niemi et al. 1999, Stav et al. 2005, Poulin et al. 2010, Pfitzner et al. 2015). Furthermore, temporal and spatial variability in chironomid community composition has been observed (Lindegaard and Brodersen 1995, Rossaro et al. 2006, Milošević et al. 2013) together with a high adaptability of the community to changing environmental conditions (Raunio et al. 2011). Given these particular ecological characteristics, chironomids have been proposed as important indicators for monitoring freshwater ecosystems (Moog 2002, Saether 1979). However, their morphological determination is very challenging and the taxonomic expertise needed for species identification of chironomids is often lacking (Batzer and Boix 2016). This makes it difficult to study changes in chironomid composition and utilize this as a monitoring tool. DNA-based determination approaches such as DNA barcoding seem therefore promising to support the taxonomic expertise for monitoring a more complete chironomid community composition.

During recent years, DNA metabarcoding of whole communities has become a new powerful tool for environmental monitoring of aquatic ecosystems (Hajibabaei et al. 2011, Carew et al. 2013, Gibson et al. 2015, Elbrecht and Leese 2015). The DNA-based assays to monitor species biodiversity proved to be a rapid and efficient tool that allows the recovery of a substantial amount of taxa (Sweeney et al. 2011, Taberlet et al. 2012, Yu et al. 2012, Carew et al. 2013, Elbrecht et al. 2017). For metabarcoding in animals, typically the mitochondrial cytochrome oxidase subunit I (COI) is used (Hebert et al. 2003). COI has a good taxonomic resolution and comparatively well-curated databases as reference for many taxa (Sweeney et al. 2011, Elbrecht et al. 2017). The species present in the sample are identified based on a comparison of retrieved COI sequences (summarized as operational taxonomic units; OTUs) with reference databases (e.g. NCBI or BOLD; Ratnasingham and Hebert 2007). Good species coverage in the database is necessary for retrieving species names from sequences, but the identification rate for the different taxa vary widely (Kwong et al. 2012). For Chironomidae only about 30% of the estimated 700 different species in Germany (Samietz 1996) have an entry in the databases BOLD (accessed on 14. July 2017). However, common taxa might be well represented.

1.1 Objectives, concept and approach

In this study DNA metabarcoding was applied to assess the distribution and species richness of chironomids in Bti treated vs. first year Bti untreated temporary wetlands in the Upper Rhine Valley. The study sites were part of a mosquito control area that has received regular Bti treatments for approximately 20 years (http://www.kabsev.de/1/1_2/1_2_3/index.php, accessed on 11 August 2017). Our first aim was to study the effectiveness of metabarcoding for chironomid diversity assessment as important and often overlooked freshwater bioindicator. We expected to obtain more species identifications based on molecular methods as compared to traditional taxonomic determination and used these data to calculate the saprobic index for the respective sites. Our second aim was to test for possible changes in the chironomid community composition of the temporary wetlands in response to mosquito control actions. Based on the above-mentioned studies we expected:

- an overall reduction in chironomid abundance at Bti-treated sites,
- a reduction in species richness of chironomids at Bti-treated sites, and therefore:
- an overall effect of Bti-treatment on chironomid community composition.

2. Methodology

2.1 Study sites

The study was conducted in Rhineland-Palatinate in southwest Germany close to Neustadt-Geinsheim (49°18'15.4"N 8°15'23.8"E, Fig. 1). The study sites are regularly flooded in spring and dry out in summer. Thus, the area can be classified as seasonal (= temporary) wetland, which is moreover partly protected as a key amphibian breeding area in the region (Williams 2006). The area has been subject to regular mosquito control management actions for over 20 years, with usually one to two helicopter-applications of Bti between March and June, depending on temperature and precipitation. The study sites Fig. 1 were: "Stiftungsfläche" (S): mainly flooded grassland with some small permanent water bodies, "Großwald" (G): alder carr with larger permanent water bodies, "Mitteltrumm" (M): alder /oak carr with some deeper trenches and ditches and flat sinks. Additionally, the site "Lachen-Speyerdorf" (CL; 49°20'10.0"N 8°12'18.8"E, see Fig. 1) served as control site and was located approximately 7 km away from the sites S, G and M. The site CL was dominated by open alder and pine forest with an abandoned river course.

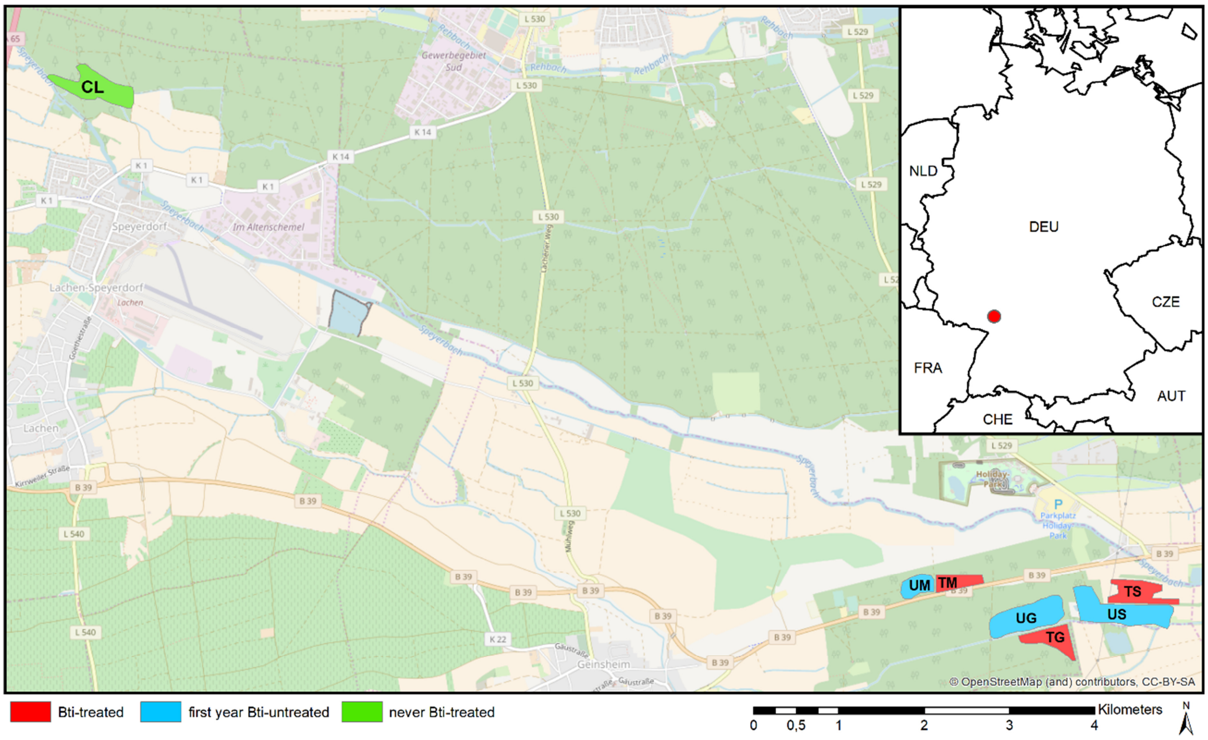

Figure 1: Study sites in southwest Germany close to Neustadt-Geinsheim. "Stiftungsfläche" (S), "Großwald" (G), "Mitteltrumm" (M) "Lachen-Speyerdorf" (CL). S, G and M were divided into Bti-treated (T; 20 years treated) and Bti–untreated (U; first season untreated) site pairs, and CL served as control site never been treated with Bti.

Study sites in southwest Germany close to Neustadt-Geinsheim. "Stiftungsfläche" (S), "Großwald" (G), "Mitteltrumm" (M) "Lachen-Speyerdorf" (CL). S, G and M were divided into Bti-treated (T; 20 years treated) and Bti–untreated (U; first season untreated) site pairs, and CL served as control site never been treated with Bti.

For the first time after 20 years of regular Bti treatment, parts of the study area were left Bti-untreated in spring 2013 allowing for a split field design. Accordingly, S, G and M were divided into Bti-treated (T; 20 years treated) and –untreated (U; first season untreated) site pairs , and CL served as control site never been treated with Bti. The helicopter application took place on April 10, 2013 using IcyPearls (Vectobac WG®, ValentBiosciences) at a concentration of 1.44 x 10^9 ITU/ ha.

2.2 Emergence data

Insect imagines were collected weekly with emergence traps (N = 5 per site, in total 35 traps, 0.25 m^2 area each) over a period of four months (April – July 2013) for 13 weeks after application (WAA; WAA 1 – WAA 13) of Bti. All specimens were conserved in 70% ethanol. The total emergence was determined to order level and the order Diptera to family level using a Leica M80 microscope and a 10x magnification and counted per trap and sampling week. All chironomid specimens were selected for further analyses.

Chironomid samples of all emergence traps per WAA were pooled for Bti-treated and Bti-untreated sites. For specific emergence peaks (N = 18, see Fig. 2 and text in results) these pooled samples were selected for metabarcoding to determine whether abundance differences can be attributed to a shift in species community composition.

Chironomid mean abundances of all traps at the four sampling locations, M, G, S, and CL (control site) across the whole sampling period until 13 weeks after application. Filled symbols indicate pooled emergence peak samples (N = 18) used for metabarcoding.

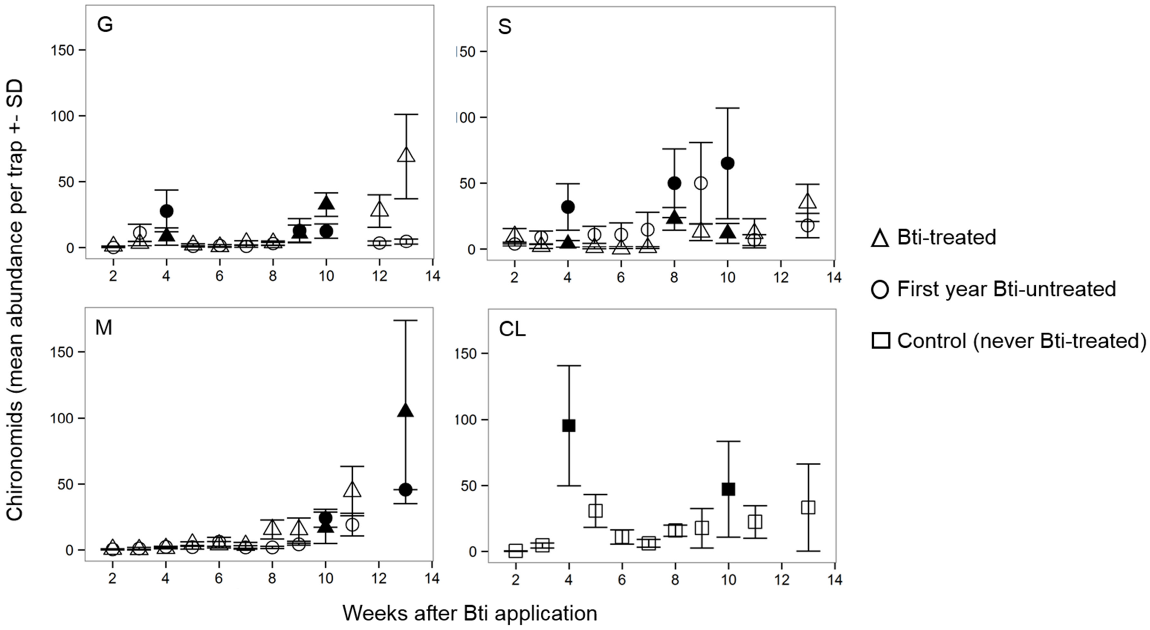

Figure 2. Chironomid mean abundances of all traps at the four sampling locations, M, G, S, and CL (control site) across the whole sampling period until 13 weeks after application. Filled symbols indicate pooled emergence peak samples (N = 18) used for metabarcoding.

2.3 Laboratory methods

Chironomids were sampled at emergence peaks (N = 18) and dried overnight at 60 °C. The specimens of each sample were grinded three times using a Tissue Lyser II (Quiagen, Hilden, Germany) at 30 Hz for 3 x 1 min with two sterile metal beads (3 mm, Hobbyfix, Opitec, Giebelstadt) with a brief centrifugation in between. DNA was extracted from each sample following a high salt DNA extraction protocol after (Aljanabi and Martinez 1997). Extraction success was verified using a Nanodrop (ND-1000 Spectrophotometer, Wilmington, USA). 50 µL of DNA were extracted from each sample and treated with 1.1 µL RNase (10 mg/mL, Roth, Karlsruhe, Germany) at 37°C for 30 min, followed by purification using a MinElute

Reaction Clean up Kit (Qiagen, Hilden, Germany). The DNA concentration after clean-up was again measured using the Nanodrop and DNA concentrations of all samples were adjusted to 25 ng/µL.

A 322 bp fragment of the mitochondrial COI gene was amplified using the BF2 + BR1 primer set (Elbrecht et al. 2017). The used fusion primers included Illumina adapter tails for sequencing (P5 or P7) and inline barcodes of different length for sampling multiplexing (see Elbrecht and Leese 2015). For each of the 18 samples two PCRs were conducted using the same primer pair but switching P5 and P7 Illumina adapters for half of the samples (Elbrecht and Leese 2015, Suppl. material 1). PCR reactions consisted of 1× PCR buffer (including 2.5 mM Mg^{2+}), 0.2 mM dNTPs, 0.5 µM of each primer, 0.025 U/µL of HotMaster Taq (5Prime, Gaithersburg, MD, USA), 25 ng DNA, and HPLC H_2O to a total volume of 50 µL. The PCR program included the following steps: 94 °C for 3 minutes, 35 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds, 65 °C for 120 seconds and ended with 65 °C for 5 minutes. PCR success was checked on a 1% TAE agarose gel. Since some samples exhibited low DNA quantity (Qubit 2.0, Life Technologies, Carlsbad, CA, USA; measured concentration below 1 ng/µL), PCR for the respective samples was repeated with cycle number increased to 40 (Suppl. material 1). Amplicons were purified and size selected (retaining fragments of >300 bp) with a left-sided size selection using magnetic beads (SpriSelect, Beckmann Coulter, Bread, CA, USA, ratio: 0.76x). The DNA concentration was quantified using the Qubit and a high sensitivity (HS) Assay Kit. Purified PCR products were pooled proportionately according to the number of specimens used in the extraction into a library to ensure all specimens are sequenced with the same sequencing depth. After pooling, the library was sent to an external laboratory (Macrogen, Seoul, Korea) for 300 bp paired-end sequencing on a MiSeq Illumina system (v3) run.

2.4 Bioinformatic analysis

Following the bioinformatic pipeline as previously described in Elbrecht and Leese 2017, the sequence data were processed as follows. In brief, after demultiplexing using a custom R script, paired-end reads were merged to one sequence (Usearch version 8.8.1756; Edgar 2010). Primer sequences were removed via cutadapt (version 1.9.1). Singletons in each sample were removed before clustering OTUs with the cluster_otus command at 97% identity Edgar 2013. Only OTUs occurring in both replicates were considered and their read abundance had to be at least 0.003% to be used for further analysis. All samples (including singletons) were matched against the OTUs (Usearch). To enhance data reliability, sequences matched to the respective OTU had to occur in both replicates and exceed the 0.003% threshold sequence abundance to being considered in downstream analysis. Finally, the obtained OTU sequences were matched against the BOLD database to retrieve taxon identification.

2.5 Using metabarcoding data for chironomid diversity assessment

The retrieved chironomid species list was checked for biogeographical and ecologically plausibility, i.e., if the species names were listed for Germany and are representative for temporary wetlands. If more than one species name per OTU was retrieved from BOLD with over 98% identity, we carefully examined the resulting hit table. From the most hits, we then selected the biogeographically plausible species for our study region, based on its known biogeographical distribution and chironomid expert knowledge, for further ecological interpretations. If no clear decision could be made together with expert taxonomists, we followed the conservative approach to select the species name already represented in our data by another OTU. The species names retrieved in that way were categorized into morphological determination “possible”, “difficult” and “impossible” based on larval morphology. With this, we aimed to elucidate which proportion of the chironomid species pool is neglected in standard water quality assessments, where only the clearly determinable chironomid larvae are considered. We then calculated the percentage of species retrieved via metabarcoding (here emergence data) in relation to species, which would have also been possible to determine morphologically in standard water quality assessments (usually larval data). Based on available saprobic indices of the chironomid species (Moog 1995, Moog 2002) we also calculated the chironomid saprobic index (SI; Moog 2002) exemplarily for the four study sites across all WAA. When the same species name was retrieved from more than one OTU the respective abundances were summed up.

2.6 Bti effects on chironomid community composition

To test whether the abundance data of emerged chironomids differed among Bti-treated and Bti-untreated sites (including the control site) for pooled samples of WAA 1 - WAA 4 (first emergence peak) and WAA 1 - WAA 13 (whole sampling period) a generalized linear mixed effect model (GLMM) (packages "nlme" v. 3.1-117, Pinheiro et al. 2016, and "MASS" v. 7.3-31, Venables and Ripley 2002) was implemented. As error structure the quasi Poisson family was chosen, where “study site” was implemented as random factor.

To test for differences in chironomid species richness between Bti-treated and Bti-untreated sites (including the control site), a Welch test was applied to the total number of OTUs and based on retrieved species names.

To test the hypothesis that the chironomid community composition differed between treatments, an adonis analysis (nonmetrical permutational MANOVA equivalent, Anderson 2001) was performed. For this, the Bray-Curtis distances between the Hellinger-transformed (see below) OTU read abundance assemblages per sites were calculated between pairs of sites, and these pairwise distances between sites were combined to a distance matrix of all sites, using the command 'vegdist' in the package "vegan" v. 2.4-1 (Oksanen et al. 2016). Within this distance matrix the nonmetrical permutational MANOVA equivalent was calculated using "Bti-treatment" as the distinguishing factor, with the command 'adonis' from the vegan package. We then assumed that time (WAA) would have a dominant effect on chironomid communities, but that Bti-treatment would alter the community as well. Therefore, also the interaction time * Bti-treatment was tested. Due to low number of replicates, the samples from the sites M and CL of WAA 8 and 9 were combined to belong to the same time period. For the site S and G only WAA 8 and WAA 9, respectively, was available.

All analyses were conducted in R (https://www.R-project.org). For all multivariate analyses, the Hellinger transformation was chosen to give less weight to the few high abundant OTUs, since the abundance data were highly left-skewed with few taxa reaching abundances several orders of magnitude higher than those of the less abundant species (Legendre and Legendre 2012).

3. Results

3.1 Emergence data

In total, 11,589 emerged insects were collected, comprising of 17 taxa groups (Chironomidae: 78%; Culicidae: 14%; Trichoptera: 4%; Chaoboridae: 2%; Brachycera: 1%, other: 1%). On the Bti-treated sites 27 mosquito individuals were collected in the emergence traps, while on the Bti-untreated sites 1,006 mosquitoes emerged. Based on morphological identification 9,033 adult chironomids were collected. The number of chironomid specimens per emergence trap across all sites varied from 1 (Bti-treated site) to 1,239 (first year Bti-untreated site).

Emergence of chironomids fluctuated over time with varying emergence peaks at the Bti-untreated sites (Fig. 2). In particular, we detected one spring emergence peak (WAA 4) and two summer emergence peaks (WAA 9 + 10) at site G. At site S one spring peak (WAA 4) and two summer peaks (WAA 8 + 10) were detected. At site M, two summer peaks (WAA 10 + 13) were identified. A spring peak (WAA 4) and a summer peak (WAA 10) were identified at the never Bti-treated site CL. Specifically pooled emergence peak samples (N = 18, Figure 2) were selected for metabarcoding to investigate, if the abundance difference between Bti treated and untreated site pairs can be attributed to a shift in the chironomid community composition. The amount of individuals per pooled emergence peak sample varied from 22 to 541 (Suppl. material 1).

3.2 Bioinformatic analyses

In total 20,805,626 raw reads were generated by the MiSeq run with good read quality (Q30 ≥ 76.7% of reads). Raw data are available at NCBI SRA archive (accession number SRR4244505). After demultiplexing, merging and trimming of PCR primers 8,869,048 sequences were used for further analysis. The number of sequences in each sample was significantly correlated ($p < 0.001$, adj. $R^2=0.942$) with the abundance of specimens per sample (Suppl. material 2).

Bioinformatic analysis resulted in 442 OTUs. After application of the previously defined quality standards (0.003% minimum abundance) 89 OTUs were retained and used for subsequent analyses. The BOLD database searches identified 54 of the 89 OTUs (60.7%) as chironomids (Table Appendix 3). Of those, 38 OTUs (68.5%) could be assigned a species identification with 98 - 100% similarity, leaving 17 OTUs (31.5%) without species identification (Suppl. material 3).

3.3 Using metabarcoding data for chironomid diversity assessment

In total 30 chironomid species were detected in the metabarcoding data set, with six species being assigned to 2-3 OTUs respectively (Table 1). For 11 OTUs we retrieved more than one species name with a sequence similarity of 97.82–100% (Suppl. material 3). For those OTUs, we were able to select the only biogeographically plausible species for our study region, based on biogeography and chironomid expert knowledge, for further ecological interpretations (underlined species names in Suppl. material 3). Only for OTU_12 two species names were biogeographically plausible, namely Chironimus luridus and *C. riparius*. Here, we selected the latter species as *C. riparius* was also characterized by other OTUs in our data, whereas *C. luridus* was not represented otherwise.

Table 1. Retrieved chironomid species names out of 54 obtained chironomid OTUs based on BOLD database searches. Given are OTU number(s), species names and the classification of the species determination based on larval morphology as routinely possible, difficult and impossible. Species names indicated with * are questionable.

		morphological larvae determination		
OTU	Species names	possible	difficult	impossible
OTU_15	*Ablabesmyia monilis* (Linnaeus)		x	
OTU_73	*Acricotopus lucens* (Zetterstedt)	x		
OTU_11	*Chironomus annularius* (Meigen)			x
OTU_29	*Chironomus curabilis** (Bel. et.al.)			x
OTU_24	*Chironomus melanescens* (Keyl)			x
OTU_5	*Chironomus dorsalis* (Meigen)			x
OTU_13 + OTU_25 + OTU_12	*Chironomus riparius* (Meigen)	x		
OTU_42	*Corynoneura scutellata* (Winnertz)			x
OTU_68	*Corynoneura coronata* (Edwards)			x
OTU_35	*Cricotopus sylvestris* (Fabricius)			x
OTU_40	*Dicrotendipes lobiger* (Kieffer)	x		
OTU_75	*Limnophyes minimus* (Meigen)			x
OTU_18 + OTU_64	*Limnophyes pentaplastus* (Kieffer)			x
OTU_34	*Monopelopia tenuicalcar* (Kieffer)			x
OTU_47	*Parachironomus parilis* (Walker)			x
OTU_4	*Paralimnophyes longiseta* (Thienemann)			x
OTU_33	*Paratanytarsus grimmii* (Schneider)			x
OTU_51	*Paratanytarsus tenellulus* (Goetghebuer)			x
OTU_77	*Paratendipes albimanus* (Meigen)	x		
OTU_1	*Polypedilum uncinatum* (Goetghebuer)			x
OTU_45	*Procladius* cf. *fuscus** (Brundin)			x
OTU_19 + OTU_28 + OTU_97	*Psectrocladius limbatellus* (Holmgren)			x
OTU_32	*Psectrotanypus varius* (Fabricius)	x		
OTU_66	*Rheocricotopus fuscipes* (Kieffer)			x
OTU_60 + OTU_264	*Tanytarsus heusdensis* (Goetghebuer)			x
OTU_48	*Tanytarsus pallidicornis* (Walker)			x
OTU_14 + OTU_137	*Tanytarsus usmaensis* (Pagast)			x

OTU_7	*Telmatopelopia nemorum* (Goetghebuer)	x		
OTU_26	*Xenopelopia nigricans* (Fittkau)			x
OTU_41 + OTU_79	*Zavrelimyia barbatipes* (Kieffer)			x
N = 38	N = 30	N = 6	N = 1	N = 23

Retrieved chironomid species names out of 54 obtained chironomid OTUs based on BOLD database searches. Given are OTU number(s), species names and the classification of the species determination based on larval morphology as routinely possible, difficult and impossible. Species names indicated with * are questionable.

Of the 30 retrieved species, seven species can be routinely determined based on larval morphology in ecological water assessments, whereas the remaining species are difficult (N = 2) or impossible (N = 21) to determine based on larval morphology Table 1. This resulted in a 70% increase of retrieved chironomid species names based on metabarcoding (using emergence data) in relation to morphological larvae determination.

The chironomid SI was calculated based on the available saprobic value for 14 chironomid species (45.2%) of our data set Table 2. The saprobic value per detected species ranged from 0.8 (*Monopelopia tenuicalcar, Xenopelopia nigricans*) up to 3.5 (*C. riparius*). For our study sites the chironomid SI ranged from 0.8 to 2.3 (Table 2).

Table 2. Saprobic Index (SI) calculations per site (N = 7) across the whole sampling period based on 14 species retrieved from our data set for which the SI is available. Given are the species saprobic values (s), weights (w) as well as the species sequence frequencies (h) summed over all traps and sampling time points.

Species names	s	w	h [G]	h [S]	h [M]	h [CL]
Ablabesmyia monilis (Linnaeus)	2.3	2	13	100,034	269	6
Chironomus riparius (Meigen)	3.5	3	9	42,808	1	75
Corynoneura scutellata (Winnertz)	1.7	2	80	17	2,622	237
Cricotopus sylvestris (Fabricius)	2.6	2	6	13,553	7	11
Limnophyes pentaplastus (Kieffer)	1.3	2	17	800	3	54,508
Monopelopia tenuicalcar (Kieffer)	0.8	4	8	6,828	0	0
Paratendipes albimanus (Meigen)	2.3	2	0	136	0	0
Psectrocladius limbatellus (Holmgren)	1.8	3	2,588	65,009	18	1,331
Psectrotanypus varius (Fabricius)	2.8	1	2,767	2,156	4,273	0
Rheocricotopus fuscipes (Kieffer)	2.2	3	0	0	0	120
Tanytarsus heusdensis (Goetghebuer)	1.4	1	4	857	0	0

Tanytarsus pallidicornis (Walker)	1.8	1	2	1,426	0	0
Xenopelopia nigricans (Fittkau)	0.8	2	403	114	16,913	0
Zavrelimyia barbatipes (Kieffer)	1.0	3	1	157	3,266	0
SI			**0.8**	**2.3**	**1.1**	**1.3**

Saprobic Index (SI) calculations per site (N = 7) across the whole sampling period based on 14 species retrieved from our data set for which the SI is available. Given are the species saprobic values (s), weights (w) as well as the species sequence frequencies (h) summed over all traps and sampling time points.

3.4 Bti effects on chironomid community composition

The abundance of emergent chironomids until WAA 4 at the Bti-treated sites was reduced by 64.99% compared to the abundance in the Bti-untreated sites (t = 11.29, p = 0.008, df = 2, GLMM). After WAA 13, slightly more chironomids hatched at the Bti-treated vs. Bti-untreated sites (2,132 vs 1,800 individuals, respectively). However, this difference was not statistically significant (t-value = -0.239884, df = 2, p-value 0.833, GLMM).

Neither the number of OTU per sample (Welch two sample t test, t = 1.33, p-value = 0.20) nor the number of species assigned from the OTU based on the data base (Welch two sample t test, t = 1.45, p-value = 0.17) were significantly different between Bti-treated and Bti-untreated site pairs.

The adonis model of crossed Bti-treatment * site effects (with the variation from time implemented as groups (strata) within which permutations are constrained) explained 51% of the variation of the multivariate chironomid community composition, 34% of which were due to the differences within sites (Table 3). The effect from Bti treatment suggests a statistically significant, but only minor component explaining 12% of the variation (p = 0.02; Table 3).

Results from the adonis analysis on the effect of treatment over time. The variation due to differences between sampling events was taken into account by the ”strata = time” argument in the model. Df = degrees of freedom; F model = F statistic of the respective sub model.

Table 3. Results from the adonis analysis on the effect of treatment over time. The variation due to differences between sampling events was taken into account by the ”strata = time” argument in the model. Df = degrees of freedom; F model = F statistic of the respective sub model.

	Df	Sums of Squares	F Model	R^2	Pr(>F)	
Site (time)	3	0.82	1.89	0.34	0.003	***
Bti Treatment	1	0.30	2.05	0.12	0.02	*
Site (time) : Bti-Treatment	2	0.13	0.90	0.05	0.45	
Residuals	8	1.15		0.48		
Total	13	2.40		1.00		

4. Discussion

In this study we investigated the effectiveness of metabarcoding for chironomid diversity assessment and tested the retrieved chironomid OTUs for possible changes in relative abundance and diversity in relation to mosquito control actions in the temporary wetlands.

4.1 Using metabarcoding data for chironomid diversity assessment

With our metabarcoding approach we detected 54 chironomid OTUs across all study sites, of which almost 70% could be identified to species level using the BOLD database. Even though we did not have a specific reference database for our study system (e.g. Carew et al. 2013), we have mainly extracted biogeographically and ecologically meaningful species names as many of these species are frequently found in periodically desiccative ponds as euryoecious ubiquists (e.g., *Ablabesmyia monilis, Acricotopus lucens, Chironomus riparius, Corynoneura scutellata, Cricotopus sylvestris, Limnophyes pentaplastus, Paratendipes albimanus, Polypedilum uncinatum, Psectrotanypus varius and Tanytarsus pallidicornis*). Despite selecting only chironomid specimens for metabarcoding, also non-chironomid OTUs (N = 35; 39.3%) were detected (Suppl. material 3). These records included other Dipterans (N = 14; 15.7%), Trichopterans (N = 2; 2.2%), Lepidopterans (N = 2; 2.2%), Arachnids (Pionidae: N = 6; 6.7%), Fungi (Sporidiobolale, N = 2, Eurotiales, N = 1, Tremellales, N = 1 and Microstromatares, N = 1; 5.6% in total) and Bacteria (Rickettsia: N =1; 1.1%). In addition, OTUs without hit in the BOLD-database (N = 6, 6.7%) were detected. However, these non-chironomid OTUs were only present in the data set with low abundancies due to residual tissue or DNA traces from the original sample.

For 11 OTUs we retrieved more than one species name with a sequence similarity of 97.82–100% (Suppl. material 3). This could indicate 1) limited taxonomic resolution of the short fragment amplified by the used primer set, producing only 322 bp COI fragments opposed to the 658 bp COI fragments using the classical Folmer primers (Folmer 1994); 2) different taxonomic keys used thus having different synonyms included; or 3) potential taxonomic misidentification which are also discussed in (Elbrecht et al. 2017) who recommend better data curation in taxonomic databases. In contrast, six chironomid species names were retrieved from two or three different OTUs, respectively, and two OTUs (45, 29) were assigned to questionable species names due to their biogeography (*Procladius cf. fuscus* and *C. curabilis*, Table 1). This could suggest cryptic intraspecific diversity, as we used a species divergence rate of 3% which might be too low for some species (Carew et al. 2013). As little is known about the genetic lineage of Chironomidae, cryptic species or variable phenotypes could be possible and cumber the correct identification by taxonomist (Carew et al. 2007).

The information benefit of metabarcoding by obtaining species names strongly depends on the quality of the database. For 17 chironomid OTUs (31.5%) no species identification could be obtained (Suppl. material 3). BOLD holds 270,292 published records of Chironomidae forming 5,540 BINs (clusters) with specimens from 49 countries. Of these records (accessed on 14.07.2017) , 100,231 have species names, and represent 1,233 species for an estimated species diversity of 15,000 worldwide (Armitage et al. 1995). For Germany, with an estimated species richness of approx. 700 different Chironomidae (Samietz 1996), BOLD has 3,706 published records forming 217 BINs (clusters). Of these records (accessed on 14.07.2017), 3,683 have species names, representing only 208 species (around 30%). Metabarcoding can only be as good as the database on which it relies for OTU matching to species identifications. We thus encourage experienced chironomid taxonomists to increase the number chironomid species in the BOLD database to even improve the effectiveness of metabarcoding for chironomid diversity assessments.

By applying metabarcoding we obtained 70% more chironomid species identifications than would have been possible based on traditional taxonomic determination of larval samples (Table 1), thus proving the usefulness of metabarcoding for chironomid diversity assessment. Some of our retrieved species are indicators for high water quality and were previously detected in spring biotopes, such as *Acricotopus lucens, Chironomus luridus, Dicrotendipes* *lobiger, Limnophyes minimus, Limnophyes pentaplastus, Psectrocladius limbatellus, Psectrotanypus varius* and *Tanytarsus usmaensis* (Reiff et al. 2015), suggesting a general good water quality of our study sites. Only seven of those species can be determined based on larval morphology (Table 1). Even though various determination keys for larval and adult chironomids exist, not all taxa can be determined to species level even by experts (Kranzfelder et al. 2016). Especially larvae and female midges are almost impossible to determine morphologically, since often male genitals are necessary to distinguish species. Some chironomid species have a parthenogenetic life cycle (e.g. *Paratanytarsus grimmi,* Langton 1988), so only females occur especially in temporary wetlands (Dettinger-Klemm 2003). Without determination of all occurring chironomids, including females, around 27% of the species diversity could be lost (Ekrem et al. 2010). Even if morphology enables the determination down to genus level, an ecological interpretation is difficult since chironomid species of the same genus might have very dissimilar ecological preferences Milošević et al. 2013.

The saprobic index per site based on 14 chironomid species and their sequence abundancies ranged from 1.3 (control site) to 2.7 in one of the untreated sites (Table 2). Due to the demanding morphological chironomid species determination, it is common practise to exclude chironomids from bioassessment programs (Milošević et al. 2013). However, in standard water quality assessments in Germany sometimes only all red chironomid larvae are counted, summarized as Chironomus spec. and included in the saprobic index with a value around 3.5. Considering the high chironomid diversity and the range of saprobic values for chironomids between 0.8 (very good water quality) and 3.5 (bad water quality), the standard water quality assessment would have resulted in a severe underestimation of the studied water bodies due to the presence of *C. riparius*. In addition to the difficult morphological determination, small chironomid larvae (< 1 mm) from freshly hatched species can be easily overlooked by larvae picking. Thus, a metabarcoding approach based on water and homogenized sediment samples could be highly useful for future application in water quality assessments by increasing the chironomid diversity in a sample without specialised taxonomic expertise needed.

The advantages of metabarcoding over traditional monitoring for water quality assessments is gaining increasing attention. Since Haase et al. 2010) postulated the overlooking of many taxa in traditional stream monitoring programs, many studies proved that metabarcoding can provide higher numbers and more accurate taxonomic identifications than morphology-based methods for many freshwater macroinvertebrates (Hajibabaei et al. 2011, Elbrecht et al. 2017, Carew et al. 2013Hajibabaei et al. 2011, Elbrecht et al. 2017, Carew et al. 2013Elbrecht and Leese 2015). Moreover, barcoding has been promoted for rapid biodiversity assessment and biomonitoring for many terrestrial taxa (Yu et al. 2012, Taberlet et al. 2012, Brehm et al. 2013. Ji et al. 2013) compared metabarcoded samples of arthropods and birds with standard biodiversity data sets, and found that the genetic data sets were taxonomically more comprehensive, quicker to produce and less reliant on taxonomic expertise. Cristescu (2014) raised the urge for a coordinated progression of species barcoding that integrates taxonomic expertise and genetic data. For the family Chironomidae an extended and reliably curated barcode database (analogous to the Trichoptera Barcode of Life Database, Zhou et al. 2016) would be highly useful for integrating chironomids in standard freshwater biomonitoring which enhance water quality assessments and might lead to better management of aquatic ecosystems.

4.2 Bti effects on chironomid community composition

In our study sites we could show that a considerable number of chironomids live in these wetlands subject to mosquito control. The uptake and the mode of action of Bti is similar for mosquitos and chironomids (Ali et al. 1981). Regarding potential effects of mosquito control actions using the biocide Bti we expected an overall reduction in chironomid abundance in the Bti-treated sites as well as a reduction in species richness and resulting community composition changes.

The chironomid abundance until WAA 4 was significantly reduced by almost 65% in the Bti-treated sites compared to the Bti-untreated sites, including the never Bti-treated control site. At the control site this spring peak was especially pronounced, indicating that WAA 4 (here: begin of May) is a key time period for the overall chironomid emergence in this area. The observed abundance reduction can be explained by the recent Bti treatment, which killed not only the mosquito larvae but also the chironomid larvae, predominantly affecting freshly hatched larvae. Especially first instar larvae of *C. riparius* were shown to be highly affected by Bti in laboratory experiments while older larvae were less sensitive (Kästel et al. in press). Until WAA 13 there was a non-significant trend towards more chironomids in the Bti-treated sites, which could be due to a reduced mosquito competition (Lundström et al. 2010) and subsequently chironomids with a second reproductive cycle in the same year had better conditions (more food resources available) to reproduce. Moreover, species have different egg laying and hatching times, and Bti does not affect eggs but only hatched individuals (Boisvert and Boisvert 2000). The species richness, however, was not significantly different between Bti-treated and untreated sites, neither on OTU-level, accounting for potential cryptic species diversity, nor on species level. The Bti-treatment thus seems to have a mainly quantitative effect on the abundance of the species present in the communities, which is stronger shortly after application during the main chironomid emergence peak in spring.

The adonis analysis corroborated the assumption that site and time (seasonality) had a dominant effect on chironomid communities (Table 3). The predominant effect of site can be explained by the study sites different vegetation (grassland, alder carr, oak carr and pine forest) which influences species composition by varying substrate availability, water chemistry, and the availability of nutritional resources (Van Den Brink and Van Der Velde 1991). Thus, it is not feasible to directly compare the chironomid species composition on the never Bti-treated control site (CL) and the Bti-treated sites (S, G and M) among each other since the vegetational surroundings are quite different and so is the species composition (see Table 2). Note, however, that it is hardly possible to find a "true" control site in the Upper Rhine Valley, i.e., a wetland which has never been treated with Bti next to wetlands subject to mosquito control. In this study we have therefore compared four different study sites, three of which have been subject to mosquito control through Bti.

Even though site and time influenced the species composition the most, the first year of Bti intermittence significantly altered the chironomid community as well. This Bti effect was rather low (12%, Table 3). However, considering the more than 20 years of continued Bti application in the study area each spring (in some years even several applications per season) and the proven toxic effect of Bti on chironomid first instar larvae (Kästel et al. in press), we can assume a more or less depleted community in terms of chironomid diversity. As dispersal for adult chironomids from the next Bti-untreated areas might take too long given the reduced flight capacities (Armitage et al. 1995), a recolonization of univoltine species would probably need longer than one season of intermitting Bti treatment. Therefore, resilience in terms of significantly increased species richness may even only be expected after several seasons intermitting of the Bti treatment. This highlights the importance for follow-up studies at the sites.

Conclusions

We could show the effectiveness of metabarcoding for chironomid diversity assessments, which led to a 70% increase in species determination compared to determination based on larval morphology. Thus, metabarcoding improves data quality by generating taxonomic resolution. Regarding the question of non-target effects of Bti on the chironomid community, our study found only minor significant effects even though Bti reduced the chironomid emergence by 65%. This could be due to a time lag of chironomid recolonization, since the study year was the first year of Bti intermittence after around 20 years of Bti application in the study area. A follow-up study after a few years of Bti intermittence could result in a more obvious recovery of the chironomid community composition in the Bti-untreated temporary wetlands.

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The authors declare that there is no conflict of interest.

Appendix IV: Scientific publication IV

Mosquito control actions affect chironomid diversity in temporary wetlands of the Upper Rhine Valley

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Authors' contribution:

Sampling: AF, SA; Laboratory work: NR, AB; Bioinformatic data analyses: NR; KT; Biological data analyses: KT, NR, SA, SM; Multivariate statistics: NR; Study design and supervision: KT, CB, KS; Manuscript writing: KT

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Abstract

The Upper Rhine Valley, a Hotspot of Biodiversity in Germany, has been treated with the biocide *Bacillus thuringiensis* var. *israelensis* (Bti) for mosquito control for decades. Previous studies discovered Bti non-target effects in terms of severe chironomid abundance reductions. In this study, we investigated the impact of Bti on species level and addressed the community composition of the non-target family Chironomidae by use of community metabarcoding. Chironomid emergence data were collected in three mosquito-control relevant wetland types in the Upper Rhine Valley. For all three sites the chironomid species composition, based on operational taxonomic units (OTUs), was different to varying degrees in the Bti-treated samples *vs*. control samples, ranging from a significant 63% OTU reduction to an OTU replacement. We assumed that predatory chironomids are less prone to Bti than filter feeders, as the latter feed on floating particles leading to direct ingestion of Bti. However, a comparable percentage of predators and filter feeders (63% and 65%, respectively) was reduced in the Bti samples, suggesting that the feeding strategy is not the main driver for Bti sensitivity in chironomids. Finally, our data was compared to a three year old data set, indicating possible chironomid community recovery due to species recolonization a few years after the last Bti application. Considering the currently discussed worldwide insect decline we recommend a rethinking of the usage of the biocide Bti, and to prevent its ongoing application especially in nature protection reserves to enhance ecological resilience and to prevent boosting the current biodiversity loss.

1 Introduction

The Upper Rhine Valley is one of 30 "Hotspots of Biodiversity" in Germany (Ackermann et al., 2012). The river Rhine, being the biggest river in Germany, regularly breaks its banks in spring and summer time and creates temporary wetlands with an exceptional high floral and faunal biodiversity. Temporary wetlands are often protected areas in Natura 2000 networks (Lukacs et al., 2013), as they comprise both aquatic and terrestrial habitats and are characterized by water bodies with high numbers of mainly macroinvertebrate species (Biggs, Williams, Whitfield, Nicolet, & Weatherby, 2005; Brooks 2000; Lukacs et al., 2013), and also rare species (Biggs et al., 2005; Williams et al., 2004). The macroinvertebrate community composition depends on the vegetation in the wetlands and the number and duration of dry periods (Brooks, 2000; Batzer & Wissinger, 1996).

Among macroinvertebrates, non-biting midges (Diptera: Chironomidae) are one of the most dominant taxa (Puntí, Rieradevall & Prat, 2009; Milošević et al., 2013), showing high species richness and ecological diversity in all kind of lotic and lentic systems (Ferrington, 2008). Temporal and spatial variability in the chironomid community composition has been observed (Rossaro, Lencioni, Boggero & Marziali, 2006; Lindegaard & Brodersen, 1995; Milošević et al., 2013) together with a high adaptability of the community for changing environmental conditions (Raunio, Heino, Paasivirta, 2011). The high chironomid biomass is an important food resource, serving as prey for both aquatic (fish, amphibians, dragonfly larvae) and terrestrial (birds, bats, spiders, dragonfly imagines) predators (Niemi et al. 1999; Stav, Blaustein & Margalit, 2005; Poulin, Lefebre, Paz, 2010; Pfitzner, Beck, Weitzel, Becker, 2015). Thus chironomids represent important links between the aquatic and the terrestrial food web and reductions in abundance may result in severe negative effects on the wetland food web community (Poulin et al., 2010).

Recent field studies in the Upper Rhine Valley demonstrated for three temporal wetland types that chironomid abundances were significantly reduced by 41-68% due to mosquito control actions with the biocide *Bacillus thuringiensis* var. *israelensis* (Bti) (Theissinger et al., 2018; Allgeier, Kästel & Brühl, 2019). Even though Bti is considered environmentally friendly, the non-biting midges are the most Bti-sensitive non-target family (Boisvert & Boisvert, 2000). Filter-feeding chironomid and mosquito larvae both feed on floating particles leading to a direct ingestion of Bti (Ali, Baggs & Stewart, 1981). Bti activates its toxicity in the alkaline milieu of the midgut by forming pores in the epithelium, resulting in a disruption of the midgut cells and finally to death of the larvae (Bravo, Gill &

Soberon, 2007; Bravo & Likitvivatanavong, 2011). Several other studies have previously shown Bti non-target effects on chironomids with abundance reductions ranging from 35-80% (Rodcharoen, Mulla & Chaney, 1991; Hershey, Shannon, Axler, Ernst, & Mickelson, 1995; Liber, Schmude & Rau, 1998; Vaughan, Newberry, Hall, Liggett & Ormerod, 2008; Poulin et al., 2010; Jakob & Poulin, 2016). However, also no effect (Lagadic et al., 2016; Wolfram, Wenzl & Jerrentrup, 2018) and even positive effects on chironomid species richness (Lundström et al., 2010) were reported in the context of Bti application, although the data sets in these studies were small or Bti effects could not be demonstrated even for target taxa.

Chironomid communities of different wetland types can be highly diverse in terms of species compositions and age structures (Armitage, Cranston & Pinder, 1995), with younger larvae being more sensitive to Bti (Ali et al., 1981; Treverrow, 1985; Ping, Wen-Ming, Shui-Yun, Jin-Song, & Li-Jun, 2005; Kästel, Allgeier & Brühl, 2017), and with different sensitivities among species (Yillarous et al., 1999). In a mesocosm study with seminatural conditions Liber et al. (1998) discovered a difference in Bti sensitivity among the three chironomid subfamilies, with significant reductions due to Bti treatment in Chironominae and Orthocladinae (comprising mainly filter feeding species) and no effect in Tanypodinae (mainly predartory species). Hence, a Bti-induced reduction in abundance can possibly lead to a change in chironomid community composition (species turnover or reduction). To further assess potential ecological consequences of the Bti-induced chironomid abundance reduction in three temporal wetlands of the Upper Rhine Valley (Allgeier et al., 2019), the chironomid communities need to be assessed with higher taxonomic resolution. Chironomid taxonomy based on morphology is often subject to misclassification, but community metabarcoding has been proven to be an efficient tool to assess chironomid species diversity (Carew, Pettigrove, Metzeling & Hoffmann, 2013; Theissinger et al., 2018; Beermann et al., 2018).

In this study we applied state of the art DNA metabarcoding on the chironomid emergence collection from Allgeier et al. (2019) to assess qualitative changes in the chironomid species composition under Bti influence. Our chironomid emergence data is comprised of three mosquito control relevant temporary wetland types (meadow; floodplain; forest). All study sites were very different in terms of hydraulic conditions (i.e., connection to permanent water bodies, springs or ground water). The forest site is characterized by many little temporary ponds, which are not connected to permanent springs or other waterbodies and thus often fall dry. In contrast, the meadow and floodplain sites are permanently connected to nearby persistent water bodies and therefore the soil is still moist even when the wetland has dried out. We thus hypothesized (i) that chironomid species composition differs significantly among study sites. Considering that chironomid species have very different developmental times and that smaller larvae are more susceptible to Bti than bigger larvae, we hypothesized (ii) that Bti-treated and control samples are significantly different in chironomid species composition at all three sites. Specifically, we expected (iii) that species with filter-feeding strategy are more reduced at the Bti-treated sites than predatory species, as the latter were shown to be less susceptible to Bti (Liber et al., 1998). The meadow site has been left Bti-untreated in a split field design since 2013, after 20 years of Bti treatment with one or two Bti applications per year. The site was also part of a study, which investigated the chironomid community resilience effects after one year of Bti intermittence (Theissinger et al., 2018). Here we discovered already minor but significant effects of Bti on the chironomid community composition. Thus, we hypothesized (iv) that ongoing (fourth year) Bti intermittence in the meadow temporary wetland results in an increased chironomid species diversity compared to three years before, as predicted in the respective pilot study by Theissinger et al. (2018).

2 Materials and Methods

2.1 Study design

The field studies were conducted by Allgeier et al. (2019) at three different mosquito control relevant temporary wetland types along the Upper Rhine Valley in Rhineland Palatinate, Germany, each site with different Bti application histories:

1) a meadow temporary wetland (meadow) close to Geinsheim (49°18'36.4"N 8°18'43.4"E) was sampled in spring and summer 2016 in the fourth year of Bti intermittence after 20 years of regular Bti treatment with one or two Bti applications per year. Since 2013, half of this meadow wetland has been left Bti-untreated, considered as control site in a split field design (Theissinger et al., 2018). In 2016 Bti, measured in International Toxic Units (ITU), was applied once by helicopter using ice granules with 2.88×10^9 ITU/ha;

2) a river floodplain (floodplain) close to Hagenbach (48°59'41.1"N, 8°16'25.3"E) sampled in spring and summer 2016. This floodplain is listed as off-limits zone for Bti application by the local authorities. Within this study, parts of the site were treated with Bti for the first time in replicated enclosures, i.e., polyethylene barrels driven into the sediment. Half of these enclosures were randomly left Bti-untreated as control sites. Bti was applied as ice granules twice with 1.44×10^9 ITU/ha;

3) forest temporary wetlands (forest) within the Bienwald (49°00’N, 8°15’E) sampled in spring 2016. Here, 12 temporary ponds were sampled, of which some have been regularly

treated with Bti for 20 years (N = 4), whereas others have never received Bti treatment (N = 5) serving as control sites, and three ponds had an unknown Bti application status (see Allgeier et al., 2019) and were not considered for further analyses. Bti was applied once as a liquid using backpack sprayers with 1.44 x 10^9 ITU/ha.

For all study sites aquatic emergence was collected with floating emergence traps (meadow: N = 24; forest: N = 36, three per pond) or fixed enclosure emergence traps (floodplain: N = 24) with 0.25 m^2 surface coverage each. At the floodplain site, in addition to the fixed enclosure emergence traps, also floating emergence traps (N = 6) outside the enclosures were used to assess potential effects of the enclosures on the sampled aquatic community (samples not used to evaluate Bti effects). Emergence was collected weekly over a duration of 14 (meadow), 15 (floodplain) and six (forest) weeks. Samples were stored in 96% ethanol. Emergence was determined to subfamily level by Allgeier et al. (2019) and all chironomids were stored for subsequent metabarcoding to identify species. Further details on study site description, Bti application and emergence sampling procedure can be found in Allgeier et al. (2019).

To condense the number of separate samples for the sites meadow and floodplain, chironomid samples were pooled over time, keeping traps separate to retain replication, but split in two time periods (early: week 1-7; late: week 8-14/15, respectively) to test for potential Bti-induced chironomid community changes in spring *vs*. summer. However, these early and late sample groups did not result in any significant difference regarding a potential Bti effect on the chironomid community (data not shown). Consequently, we decided post-hoc to combine both sampling time periods for all subsequent analyses.

At the floodplain site, the six floating emergence trap samples were pooled over the entire sampling time. This resulted in 48 and 54 metabarcoding samples for meadow and floodplain, respectively (see Table 1, Supporting Information 1). For the forest site, the chironomid samples were pooled over the six sampling weeks and also for the three traps per pond, as the ponds can be referred to as true biological replicates. In one of the forest control ponds an incomparably high number of 1270 chironomids had been collected across six weeks (Allgeier et al., 2019). Therefore this sample was kept separate by weeks, i.e. split in six subsamples due to technical reasons during DNA isolation (B - 65, Supporting Information 1). Consequently, this resulted in 17 forest samples for metabarcoding. A detailed list of all samples per site and number of individuals pooled for metabarcoding can be found in Supporting Information 1. A summary of the study design per site is shown in Table 1.

Table 1 Summary of the study design per site of the field data collected by Allgeier et al. (2019) applied for the subsequent chironomid metabarcoding in this study. Given is per site and treatment (Bti *vs*. control) information on the Bti application history, number of emergence trap replicates, number of sampling weeks, the cumulative chironomid abundances, and the number of samples for subsequent metabarcoding.

	meadow		**floodplain**		**forest**		
	Bti	control	Bti	control	Bti	control	unknown
Bti history	20 years	4 y. ago[†]	first year	never	20 years	never	NA
N ponds	1	1	1	1	4	5	3
N emergence traps	12	12	12	18[‡]	12	15	9
chironomid abundance	1138	3527	542	923	354	1522	134
sampling weeks	14	14	15	15	6	6	6
Time periods	2	2	2	2	1	1	1
metabarcoding samples	24	24	24	30[‡]	4	10[§]	3

[†] four years since first Bti intermittence after 20 years of continuous Bti application

[‡] including floating emergence traps (N = 6) not included in Bti effect analyses

[§] including one sample that was split in six subsamples

2.3 Laboratory methods

Pooled chironomid samples for all sites and treatments (N = 119) were dried for at least 24 h at 60°C. Specimens were grinded using the Tissue Lyser II (Quiagen, Hilden, Germany) at 30 Hz for 3 x 1 min using two metal beads (3 mm, Hobbyfix, Opitec, Giebelstadt) with a brief centrifugation in between. DNA was extracted from each sample with two technical replicates (N_{total} = 238) following a high salt DNA extraction protocol after Aljanabi and Martinez (1997). Extraction blanks were included to ensure data reliability. 50 µL of DNA extract were treated with 0.55 µL RNAse (10mg/mL, Roth, Karlsruhe, Germany) and incubated at 37°C for 30 min followed by purification using a MinElute Reaction Clean up Kit (Qiagen, Hilden, Germany) according to manufacturer´s instructions. The DNA concentration was measured using Nanodrop spectroscopy and concentrations of all samples were adjusted to approx. 20 ng DNA/µL. For DNA concentrations per technical replicate see Supporting Information 1.

A 421 bp COI fragment was amplified using the BF2/BR2 primer set (Elbrecht & Leese, 2017) in a two-step PCR reaction. The initial PCR amplifies the target fragment with standard BF2/BR2 primers. In the second PCR using the product of PCR 1 as template, fusion primers of the same primer sets were applied, including Illumina adapters for sequencing (P5 or P7) and inline barcodes of different length for an upscaled sampling multiplexing (Elbrecht & Steinke, 2019). PCR for 238 samples plus 36 negative and three positive controls was conducted in 25 µL reaction volume using 1× Buffer, 0.2 mM dNTPs, 0.5 µM of each primer, 0.025 U/µL 5Prime HotMaster Taq DNA Polymerase (Quantabio, Beverly, USA), 1 µL DNA/amplicon template under the following cycling profile: 94°C for 3 minutes, 25 cycles (15 cycles in second PCR) of 94°C for 30 seconds, 50 °C for 30 seconds, 65 °C for 120 seconds and ended with 65°C for 5 minutes. PCR success was checked on a 1% TBE agarose gel. The DNA concentration was quantified using a Fragment Analyzer (Standard Sensitivity NGS Fragment Analysis Kit; Advanced Analytical, Ankeny, USA). The library was purified and size selected (retaining fragments of >300 bp) with left size selection of magnetic beads (SpriSelect, Beckmann Coulter, Bread, CA, USA, ratio: 0.76x). Purified PCR products were pooled into a library proportional to the number of specimens in each sample (see Supporting Information 1) to ensure all specimens are sequenced with comparable sequencing depth. The library was sent to an external laboratory (GATC, Konstanz, Germany) for 2 x 250 bp Paired-end sequencing on a MiSeq Illumina system (v2) run with 5% PhiX spike to increase sequence diversity. The 12 different inline barcodes and parallel sequencing in forward and reverse direction enabled us to process all samples including technical replicates (N = 238) as well as extraction blanks and PCR negative controls (N = 44) on a single Ilumina Miseq run according to the upscaled metabarcoding procedure proposed by Elbrecht and Steinke (2019).

2.4 Bioinformatic analysis

Raw data were processed with R JAMP (https://github.com/VascoElbrecht/JAMP, last accessed on 06/08/18, R script available in Supporting Information 2). After demultiplexing (removal of barcode- and adapter sequences) using the module Demultiplexing_shifted, we used Usearch (v10.0.240; Edgar, 2013) for paired-end merging. Primer sequences were removed via cutadapt (version 1.9.1; Martin, 2011). For OTU-clustering a 3% error rate, accounting for 1-2% sequencing error rate and 1% intraspecific variation, was accepted. Removal of chimeric sequences was conducted to eliminate the sequencing artefacts. All sequences (including singletons) were matched against the OTUs in Usearch. The obtained OTUs were taxonomically assigned using the Barcode of Life identification engine (BOLD, Ratnasingham & Hebert, 2007; last accessed on 06/08/2018) by querying against the full reference database of animal COI barcodes. Subsequently, the BOLD_web_hack module of the JAMP pipeline was used, where the 20 best matches (i.e. BOLD sequences with the highest similarity) per OTU were considered. Genus and species of an OTU were determined according to the most frequent taxon above a predefined similarity threshold (95% and 97% similarity for genus and species, respectively). The most frequent taxon (JAMP approach) was compared to the best match taxon (i.e., the species assignment with highest similarity) and, if different, both species were considered possible. All taxon assignments were then checked and conservatively selected based on biogeographical and ecological plausibility, equivalent to Theissinger et al. (2018).

2.5 Statistical analyses

The raw reads of the technical replicates per sample were checked for consistency, i.e. whether the number of reads ranged in the same order of magnitude, to evaluate the technical success of the metabarcoding approach. To enhance data reliability, sequences matched to the respective OTU had to occur in both technical replicates and exceed the 0.003% threshold sequence abundance for being considered in downstream analysis. The maximum number of reads per OTU from all negative controls was subtracted from the reads per sample (as suggested by Elbrecht & Steinke, 2019) to reduce the effect of low abundance tag switching, i.e. false combinations of used tags (Schnell et al. 2015). The subsamples were combined per sample across the whole sampling weeks. The raw data table was then transformed in presence/absence data for subsequent analyses (Supporting Information 3). To estimate whether the read depth was sufficient to cover all chironomid OTUs in our samples we calculated octave plots according to Edgar and Flyvbjerg (2018), where the binned read abundances were plotted against the number of OTUs (for more details see Supporting Information 3). All statistical analyses were conducted in R (R Core Team 2017).

2.5.1 Chironomid community composition at different study sites

For comparing the chironomid community composition among the three sites both Bti-treated and control samples were combined. A Venn diagram was calculated for all chironomid OTUs across all samples. The floating emergence trap samples at the floodplain site as well as the three undefined samples for the forest site were also included in this analysis. We determined the most frequent OTUs per site based on the OTUs with more than 50% presence records across samples. To assess whether our sampling was exhaustive enough to evaluate the chironomid community composition for all sites separated by treatment (Bti and control), exact site-based species accumulation curves (based on OTUs) and bootstrap estimates of the extrapolated species richness were calculated by the specaccum and the specpool function of the R package vegan v. 2.5-2 (Oksanen et al., 2017).

2.5.2 Bti effects on chironomid community composition

To compare species compositions between Bti and control sites we excluded three samples of the forest site due to unknown application status (see Allgeier et al., 2019) and the samples of the floating emergence traps at the floodplain site as those were not regarded as comparable control sites.

We calculated Venn diagrams for all site pairs based on the detected OTUs per site. To plot the site and treatment specific differences in chironomid species composition a correspondence analysis was conducted as ordination tool for presence/absence data without pre-transformation, as this analysis is not influenced by double zeros (Borcard, Gillet & Legendre, 2011), using the R package vegan v. 2.5-2 with the function cca (Oksanen et al., 2017).

The OTU presence or absence in pooled Bti *vs*. control samples for all sites was used to calculate species dissimilarity rates per site using the function beta.pair in the R package betapart (Baselga & Orme, 2012). The Sørensen dissimilarity index (sor) measures the overall beta diversity, *i.e.* the variation in OTU composition, among a pair of samples (here: Bti-treated *vs*. control) and is defined between 0 and 1, where a higher number indicates a greater variation among samples. This variation in OTU composition can either result from a OTU replacement, measured with the Simpson dissimilarity index (sim) as the OTU turnover component of the Sørensen dissimilarity, or from a OTU reduction, measured with the nestedness-resultant fraction of the Sørensen dissimilarity (sne) (Baselga & Orme, 2012).

To test the hypothesis that the chironomid species richness differed between Bti and control samples at the three different sites, a Wilcoxon rank sum test was performed comparing the number of OTUs detected in each sample per site and treatment. Moreover, a PERMANOVA analysis (nonmetrical permutational MANOVA equivalent; Anderson, 2001) was performed on the matrix of Jaccard distances between samples and 999 permutations, using the command adonis in the R package vegan v. 2.5-2 (Oksanen et al., 2017). To further evaluate whether the filter feeding taxa were more affected by the Bti treatment than predatory taxa, we compared the presence records across samples per site and treatment and categorized a change in OTU presence (P) of predatory and filterer taxa (feeding type indicated in Supporting Information 4) at Bti-treated *vs*. control sites as higher ($P_{Bti} > P_{control}$), equal ($P_{Bti} = P_{control}$) or lower ($P_{Bti} < P_{control}$). Finally, we compared the retrieved chironomid OTU list from the meadow collected in 2013 (Theissinger et al., 2018; OTU list updated in BOLD on 10/10/18) with the OTU list obtained in this study, to evaluate the chironomid community resilience effect after three consecutive years of Bti intermittence.

3 Results

3.1 Bioinformatic analyses

In total, 18,991,507 raw reads for each forward and reverse sequencing run were generated with good read quality (Q30 ≥ 78.2% and 71.8% of reads, respectively). After demultiplexing, merging and trimming of PCR primers 9,847,457 sequences were used for downstream analysis. Bioinformatic analysis resulted in 344 OTUs. After application of the previously defined quality standards (0.003% minimum abundance) 280 OTUs were retained and used for subsequent analyses. The BOLD database searches identified 108 of the 280 OTUs (38.6%) belonging to the family Chironomidae, corresponding to 83.5% of all reads (Supporting Information 3). The octave plot (Figure SI3, Supporting Information 3) indicates a sufficient read depth to detect all chironomid OTUs present in our samples. All other OTUs were identified as belonging to phyla other than Arthropoda (76 OTUs), classes other than Insecta (29 OTUs), orders other than Diptera (15 OTUs), families other than Chironomidae (47 OTUs) or they could not be assigned at all (5 OTUs). Technical replicate read abundances were in the same order of magnitude for all samples (Supporting Information 3), indicating reliable results. Negative controls showed only few reads in some samples for especially high abundant OTUs (Supporting Information 3) and thus potential contamination or tag switching was not considered as an issue in our study.

3.2 Species identifications

Of the 108 detected chironomid OTUs, 75 (69.4%) could be assigned to a species with 97 - 100% sequence similarity to a reference sequence in BOLD. The remaining 33 OTUs could only be assigned to a genus because i) similarities were < 97% to the best matching BOLD sequences; ii) only the genus was provided in BOLD; or iii) the suggested species name was not plausible (e.g., we excluded *C. curabilis*, *C. sollicitus* and *M. klinki* as, to our knowledge, these species do not occur in Germany). This resulted in 63 different species names (Table 2, Supporting Information 4). Ten species names comprised of two or three different OTUs, namely: *Polypedilum uncinatum*: OTU_1 + 312; *P. cultellatum:* OTU_128 + 135; *P. tritum*: OTU_116 + 296; *Chironomus dorsalis*: OTU_10 + 89 + 307; *C. pseudothummi*: OTU_95 + 198; *Tanytarsus usmaensis*: OTU_17 + 82 + 270; *Procladius fuscus:* OTU_48 + 88; *Paratanytarsus lauterborni*: OTU_55 + 250; *Zavrelimyia barbatipes*: OTU_74 + 150; *Parachironomus parilis*: OTU_37 + 336 (Table 2, Supporting Information 4). Of the 108 chironomid OTUs, 19 OTUs (17.6%) belonged to the subfamily Tanypodinae, 28 OTUs (25.9%) to the subfamily Orthocladiinae and 61 OTUs (56.5%) to the subfamily Chironominae (Supporting Information 4). In total, 19 OTUs were identified as predatory taxa, 26 OTUs as (facultative) filter feeders, 49 OTUs as (facultative) detritivorous taxa and 27 OTUs as (facultative) grazers (Supporting Information 4) (Moog, 1995; 2002).

Table 2. OTU presence at different sites (meadow, floodplain, forest) and treatments (Bti *vs*. control) across 57 samples. Given are OTU numbers, genus, species (if available) and the percent [%] of presence records (read abundance >0) across *N* samples for Bti treated and control sites. Color intensity corresponds to the frequency of an OTU across *N* samples.

OTU	**Genus**	**Species**	**meadow**		**floodplain**		**forest**	
			Bti	control	Bti	control	Bti	control
			N=12	N=12	N=12	N=12	N=4	N=5
OTU_1†	*Polypedilum*	*uncinatum*	100	100	8	0	100	100
OTU_2	*Chironomus*	sp. TE11	17	0	0	0	100	80
OTU_3	*Telmatopelopia*	*nemorum*	17	67	0	0	100	80
OTU_4	*Xenopelopia*	*falcigera*	17	42	92	92	0	0
OTU_5†	*Chironomus*	NA	0	17	50	58	50	0
OTU_6	*Chironomus*	NA	8	83	0	8	0	0
OTU_7†	*Chironomus*	*aprilinus*	0	25	25	75	25	0
OTU_8	*Dicrotendipes*	*lobiger*	0	8	75	67	0	0
OTU_10	*Chironomus*	*dorsalis*	25	58	8	8	0	0
OTU_12†	*Xenopelopia*	*nigricans*	25	58	67	67	25	20
OTU_13	*Chironomus*	*melanotus*	0	8	42	83	0	0
OTU_15‡	*Chironomus*	NA	0	0	0	0	0	40
OTU_17	*Tanytarsus*	*usmaensis*	0	17	25	17	0	0
OTU_18	*Trissocladius*	*brevipalpis*	58	25	0	8	0	0
OTU_20	*Monopelopia*	*tenuicalcar*	17	17	50	58	0	0
OTU_21	*Chironomus*	*nuditarsis*	0	17	25	42	0	0
OTU_24	*Phaenopsectra*	*punctipes*	0	0	8	17	0	0
OTU_25	*Paralimnophyes*	*longiseta*	50	67	0	0	50	60
OTU_26	*Endochironomus*	*tendens*	0	0	17	8	0	20

OTU_28‡	*Diplocladius*	*cultriger*	0	8	0	8	0	0
OTU_29	*Tanytarsus*	*pallidicornis*	0	8	8	8	0	0
OTU_30	*Chironomus*	NA	8	75	0	8	0	0
OTU_35	*Corynoneura*	*scutellata*	50	42	33	8	0	0
OTU_37	*Parachironomus*	*parilis*	8	33	33	25	0	0
OTU_39	*Guttipelopia*	*guttipennis*	0	0	33	17	0	0
OTU_41	*Ablabesmyia*	*monilis*	8	67	0	0	0	0
OTU_42	*Zavrelimyia*	*schineri*	0	8	8	0	0	0
OTU_44†	*Limnophyes*	*minimus*	0	8	8	8	100	80
OTU_46	*Paratanytarsus*	*tenellulus*	0	17	50	25	0	0
OTU_48‡	*Procladius*	*fuscus*	0	17	0	0	0	0
OTU_49‡	*Limnophyes*	NA	0	17	0	0	0	0
OTU_50†	*Limnophyes*	sp. 14ES	50	83	42	33	75	40
OTU_51‡	*Acricotopus*	*lucens*	0	0	0	17	0	0
OTU_52	*Psectrocladius*	*limbatellus*	42	58	0	0	0	0
OTU_54	*Procladius*	NA	0	25	17	0	0	0
OTU_55	*Paratanytarsus*	*lauterborni*	0	0	8	0	0	0
OTU_60	*Conchapelopia*	*melanops*	0	17	0	0	0	0
OTU_61‡	*Procladius*	sp. ES02	0	17	0	0	0	0
OTU_66	*Paratanytarsus*	*grimmii*	0	0	25	25	0	0
OTU_67†	*Limnophyes*	*asquamatus*	8	0	0	8	75	40
OTU_68	*Limnophyes*	NA	0	33	0	0	0	0
OTU_69	*Pseudosmittia*	BOLD:AAG6458	0	0	0	0	50	0
OTU_70	*Chironomus*	*acidophilus*	0	0	8	0	0	0
OTU_74‡	*Zavrelimyia*	*barbatipes*	0	17	0	0	0	0
OTU_76‡	*Chironomus*	*melanescens*	0	0	8	0	0	0
OTU_77†	*Kiefferulus*	*tedipediformis*	0	8	17	17	0	20
OTU_78‡	*Limnophyes*	NA	8	8	0	0	0	0
OTU_79	*Corynoneura*	*carriana*	8	17	0	0	0	0
OTU_80‡	*Micropsectra*	NA	0	17	0	0	0	0
OTU_82	*Tanytarsus*	*usmaensis*	0	17	25	0	0	0
OTU_84‡	*Limnophyes*	*pentaplastus*	0	8	0	0	0	20
OTU_85	*Cricotopus*	*sylvestris*	33	25	8	17	0	0
OTU_88‡	*Procladius*	*fuscus*	0	0	8	0	0	0
OTU_89	*Chironomus*	*dorsalis*	0	0	25	33	0	0
OTU_94	*Corynoneura*	sp. 16ES	8	8	17	17	0	0
OTU_95	*Chironomus*	*pseudothummi*	0	17	0	17	0	0

OTU_97‡	*Corynoneura*	*coronata*	8	0	0	0	0	0
OTU_99	*Psectrotanypus*	*varius*	17	25	0	0	0	0
OTU_105‡	*Pseudosmittia*	BOLD:AAM6263	0	0	0	0	25	0
OTU_106	*Cricotopus*	*reversus*	0	0	33	8	0	0
OTU_111	*Zavreliella*	*marmorata*	0	0	0	25	0	0
OTU_113	*Psectrocladius*	*schlienzi*	0	33	0	0	0	0
OTU_115‡	*Paratendipes*	*albimanus*	0	0	8	0	0	0
OTU_116	*Polypedilum*	*tritum*	0	8	8	8	0	0
OTU_119‡	*Tanytarsus*	*heusdensis*	0	8	0	0	0	0
OTU_122	*Chironomus*	*pseudothummi*	0	0	0	0	0	0
OTU_126‡	*Synendotendipes*	*impar*	0	0	8	0	0	0
OTU_128‡	*Polypedilum*	*cultellatum*	0	8	0	0	0	0
OTU_129‡	*Paratanytarsus*	*dissimilis*	0	8	0	0	0	0
OTU_132‡	*Micropsectra*	NA	0	8	0	0	25	0
OTU_133	*Microtendipes*	*chloris*	0	17	0	0	0	0
OTU_134‡	*Macropelopia*	*nebulosa*	0	8	0	0	0	0
OTU_135‡	*Polypedilum*	*cultellatum*	0	8	0	0	0	0
OTU_136‡	*Glyptotendipes*	sp. 2sc	0	0	0	0	0	0
OTU_137‡	*Phaenopsectra*	*flavipes*	0	8	0	0	0	0
OTU_138	*Micropsectra*	*atrofasciata*	0	0	0	0	0	40
OTU_140‡	*Metriocnemus*	*eurynotus*	0	8	0	0	0	0
OTU_141‡	*Micropsectra*	*lindrothi*	0	8	0	0	0	0
OTU_150‡	*Zavrelimyia*	*barbatipes*	0	8	0	0	0	0
OTU_156‡	*Polypedilum*	NA	0	8	0	0	0	0
OTU_157‡	*Tanytarsus*	*eminulus*	0	8	0	0	0	0
OTU_158‡	*Georthocladius*	BOLD:ACD9509	0	0	0	0	0	20
OTU_160‡	*Polypedilum*	NA	0	8	0	0	0	0
OTU_171	*Xenopelopia*	NA	8	8	0	0	0	20
OTU_178‡	*Procladius*	NA	0	17	0	0	0	0
OTU_181‡	*Paratanytarsus*	*laccophilus*	0	8	0	0	0	0
OTU_184‡	*Limnophyes*	*natalensis*	0	0	0	0	25	0
OTU_188‡	*Tanytarsus*	*volgensis*	0	0	8	0	0	0
OTU_198‡	*Chironomus*	*pseudothummi*	0	0	0	0	0	20
OTU_205‡	*Smittia*	*edwardsi*	0	0	0	17	0	0
OTU_206‡	*Smittia*	NA	0	0	8	0	0	0
OTU_220	*Smittia*	sp. 8ES	0	0	0	0	0	40
OTU_233‡	*Polypedilum*	NA	0	8	0	0	0	0

OTU_237†	*Limnophyes*	sp. 14ES	33	58	8	25	25	0
OTU_250ǂ	*Paratanytarsus*	*lauterborni*	0	0	8	0	0	0
OTU_262	*Polypedilum*	NA	8	58	0	0	0	0
OTU_270	*Tanytarsus*	*usmaensis*	0	17	0	0	0	0
OTU_272	*Tanytarsus*	NA	0	8	0	0	0	0
OTU_281	*Chironomus*	NA	0	0	17	42	0	0
OTU_283ǂ	*Polypedilum*	NA	0	0	0	0	0	40
OTU_295ǂ	*Chironomus*	NA	0	8	0	0	0	0
OTU_296†	*Polypedilum*	*tritum*	42	83	8	0	50	60
OTU_298ǂ	*Endochironomus*	*albipennis*	0	0	8	8	0	0
OTU_307	*Chironomus*	*dorsalis*	0	25	0	0	0	0
OTU_312	*Polypedilum*	*uncinatum*	17	58	0	0	0	20
OTU_317ǂ	*Procladius*	NA	0	8	0	0	0	0
OTU_326	*Chironomus*	NA	0	0	0	8	0	0
OTU_336	*Parachironomus*	*parilis*	0	8	17	8	0	0

† OTUs shared among all three sites

ǂ rare OTUs: present in only one or two samples

3.3 Chironomid community composition at different study sites

At the meadow site we detected 76 OTUs, while 63 OTUs were detected at the floodplain site and 30 OTUs at the forest site (Figure 1). Overall, the three study sites shared 11 OTUs (10.2%), namely *P. uncinatum* (OTU_1), *Chironomus* spec. (OTU_5), *C. aprilinus* (OTU_7), *Xenopelopia nigricans* (OTU_12), *Limnophyes minimus* (OTU_44), *Limnophyes* spec. (OTU_50 + 237), *L. asquamatus* (OTU_67), *Kiefferulus tedipediformis* (OTU_77) and *P. tritum* (OTU_296) (Table 2). 27 OTUs were only detected at the meadow site, 22 OTUs only at the floodplain site and nine OTUs were solely discovered at the forest site (Figure 1).

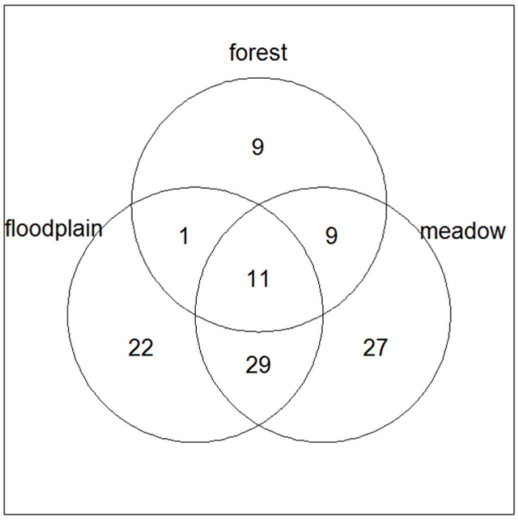

Figure 1. Venn diagram showing number of detected and shared OTUs per site across all samples analysed.

The most frequent OTUs per site and treatment are shown in Figure 2. For the meadow site we detected 14 OTUs which were present in at least 50% of the samples, for floodplain seven and for forest six OTUs. None of these most frequent OTUs was present in all three study sites (Figure 2). One OTU (OTU_12: *X. nigricans*) was shared between the sites meadow and floodplain, four OTUs (OTU_1: *P. uncinatum*; OTU_3: *T. nemorum*; OTU_25: *P. uncinatum*; OTU_296: *Polypedilum* spec.) were shared between meadow and floodplain and no OTU was shared between floodplain and forest (Figure 2). Comparing the sampling strategies at the floodplain site, we found that the untreated fixed enclosures (N = 12) and floating traps (N = 6) shared 26 OTUs. Additionally, 15 OTUs were collected in the floating emergence traps which were not discovered in the controls of the fixed enclosure traps, while 14 OTUs were only found in the latter.

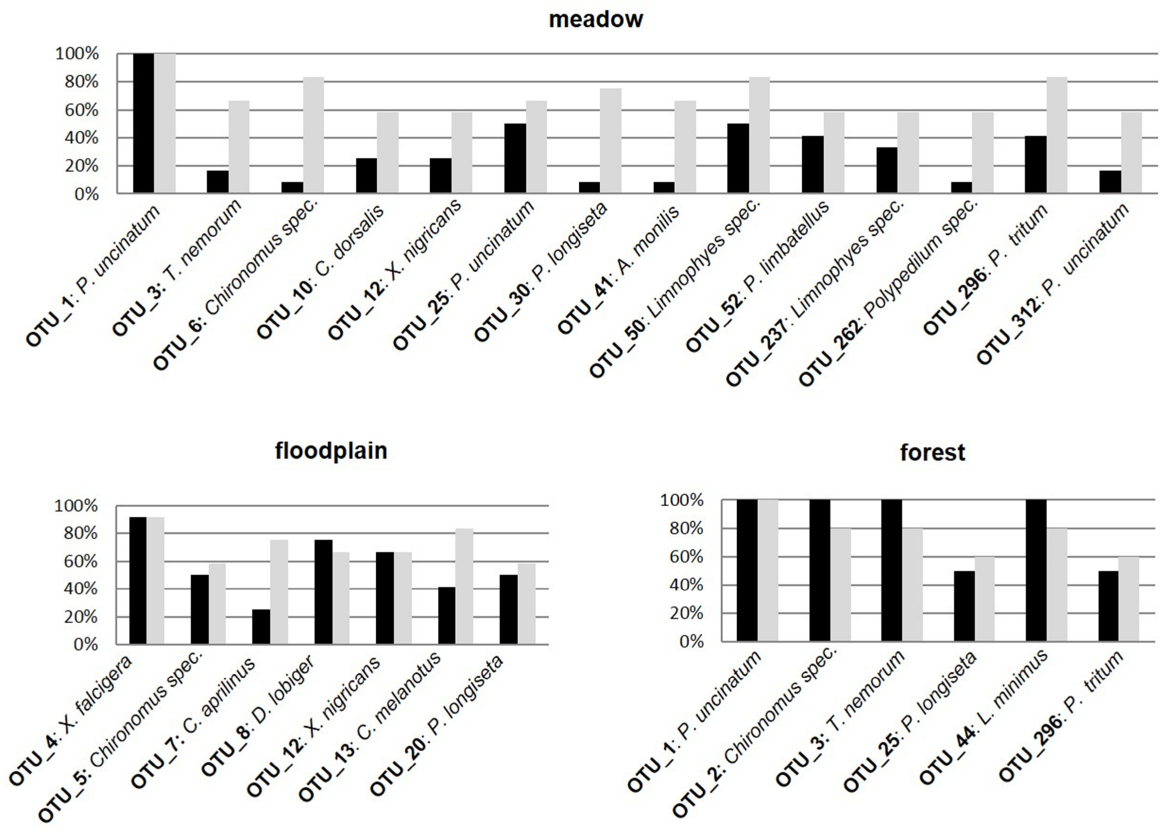

Figure 2. Most frequent OTUs per site and treatment (Bti-treated: black; control: light grey). Given is the OTU presence across all samples in [%].

OTU_1, corresponding to the species *P. uncinatum,* was detected in 34 of in total 57 samples and is with 35% of all chironomid reads the most dominant species in the meadow and the forest site (Table 2; Supporting Information 3). In contrast, out of the 108 chironomid OTUs, 44 OTUs were recorded in only one or two of all samples (Table 2; Supporting Information 3), and thus 40.7% of the detected OTUs can be classified as rare taxa in this study. There was also a high spatial heterogeneity between the traps within each site, i.e. there were many rare taxa among samples within a site. Estimates of the extrapolated species richness (Figure 3) showed that the number of detected OTUs was close to (meadow and floodplain) or even within (forest) the expected range (bootstrap +/- SE). By comparing only the control samples among sites, the extrapolated species richness increased by factor two in the forest (N = 20) to floodplain (N = 40) and meadow (N = 73).

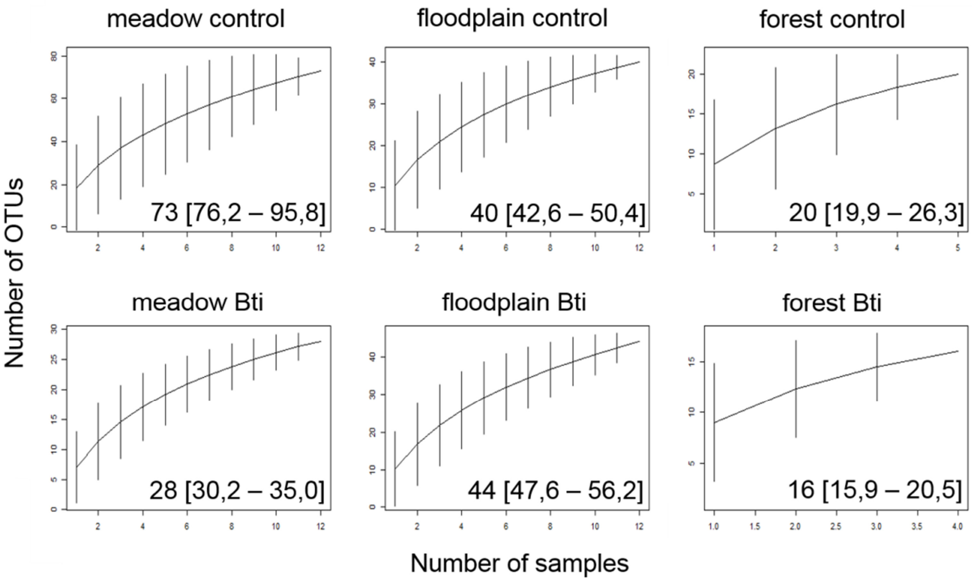

Figure 3: Exact site-based species accumulation curves based on OTUs for all sites separated by treatment. Given are the number of detected OTUs and the expected range of OTU numbers (bootstrap value +/- SE) per site.

3.4 Bti effects on chironomid community composition

For the meadow site we detected overall 76 OTUs, of which 48 OTUs (65.8%) were solely found in the control samples, three OTUs (4.1%) were only detected in the Bti-treated samples and 25 OTUs (34.2%) occurred in both sample types (Figure 4, Table 2). For the sites floodplain and forest the number of OTUs found solely in either Bti-treated or control samples was 14 *vs*. 10 and 7 *vs*. 11, respectively (Figure 4, Table 2). At the floodplain site more but different OTUs were detected in the Bti-treated samples than in the control samples (44 *vs*. 40, respectively). At the forest site 16 OTUs were detected in the Bti-treated samples and 20 in the control samples (Figure 4, Table 2).

The correspondence analysis (Figure 5) depicts the constrained ordination of the community composition in terms of OTU distribution for the three sites and treatments (Bti *vs*. control). The model, with a total explained variation of 16%, showed that there was a significantly different distribution of OTUs across sampling sites (envfit: $R^2 = 0.89$; $p = 0.001$). There was a slight ellipsoid overlap across sites, and a stronger overlap between Bti and control samples per site (Figure 5).

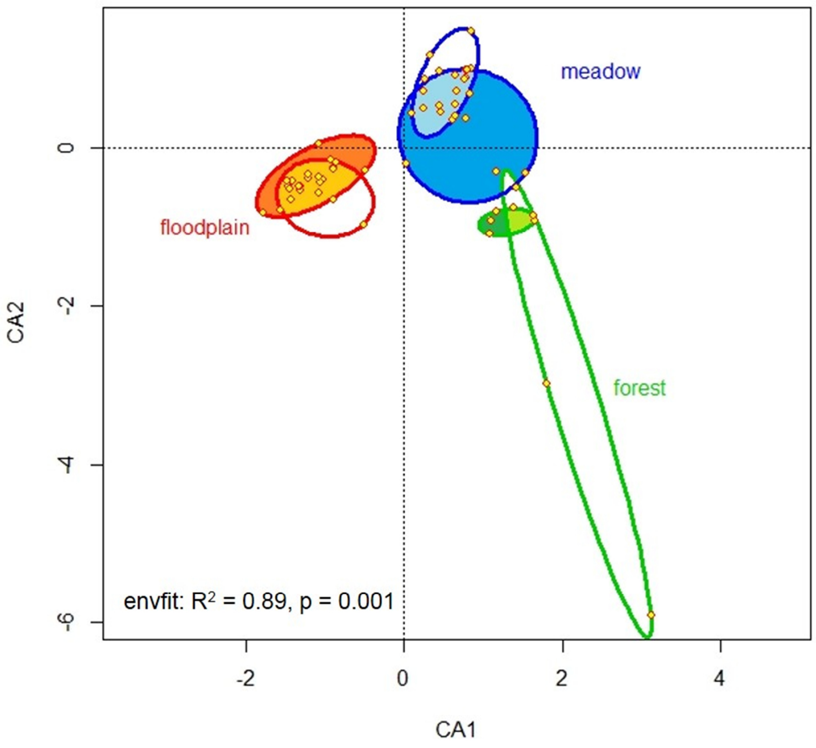

Figure 5: Correspondence analysis. Dots represent samples, ellipsoids represent the significant best fit of OTU composition on the environmental samples and enclose all points in the group. Filled ellipsoids: Bti-treated samples; transparent ellipsoids: control samples.

The Sørensen pair-wise dissimilarity based on pooled communities per site and treatment was higher for the meadow and the forest site pairs (sor = 0.5) than for the floodplain site pair (sor = 0.3). For the meadow site pair the Simpson dissimilarity was lower (sim = 0.1) than the nestedness-resultant fraction of the Sørensen dissimilarity (sne = 0.4). For both the floodplain and the forest site pairs the Simpson dissimilarity was higher than the nestedness component (floodplain: sim = 0.3, sne = 0.0; forest: sim = 0.4, sne = 0.1).

The Wilcoxon rank sum test exhibited a significant difference regarding the detected number of OTUs between Bti-treated and control samples for the meadow site (p = 0.0009) but not for floodplain (p = 1.0) and forest (p = 0.9013) (Supporting Information 3). The PERMANOVA (Table 3) showed that Bti treatment explained 12.6% (meadow), 5.4% (floodplain) and 12.4% (forest) of the variation in the chironomid community composition. However, this effect was only significant at the meadow site (p = 0.002).

Table 3: Results from the PERMANOVA on the effect of treatment at the three study sites. Df = degrees of freedom; F model = F statistic of the respective sub model.

	DF	Sum of squares	mean squares	F Model	R^2	p
meadow						
Treatment	1	0.892	0.892	3.179	0.126	0.002**
Residuals	22	6.171	0.281		0.874	
Total	23	7.062			1.000	
floodplain						
Treatment	1	0.367	0.367	1.236	0.054	0.224
Residuals	22	6.527	0.297		0.947	
Total	23	6.893			1.000	
forest						
Treatment	1	0.204	0.204	0.986	0.124	0.450
Residuals	7	1.450	0.207		0.877	
Total	8	1.655			1.000	

When focusing on the feeding strategy of the species, the OTU presence of the 19 predatory taxa (Supporting Information 4) was 17 times lower (63.0% of all detections, Table 4) and four times higher (14.8% of all detections, Table 4) at Bti-treated *vs.* the respective control samples across all study sites. Similarily, of the 26 filterer taxa the OTU presence was 24 times lower (64.9%) and eight times higher (21.6%) (Table 4).

Table 4: OTU presence of predatory and filter feeding taxa at Bti-treated samples as compared to controls across all samples (P) per site. higher: $P_{Bti} > P_{control}$; equal: $P_{Bti} = P_{control}$; lower: $P_{Bti} < P_{control}$; absent: OTU not present at this site.

OTU	**Species**	**meadow**	**floodplain**	**forest**
	Predators			
OTU_3	*Telmatopelopia nemorum*	lower	absent	equal
OTU_4	*Xenopelopia falcigera*	lower	equal	absent
OTU_12	*Xenopelopia nigricans*	lower	equal	equal
OTU_20	*Monopelopia tenuicalcar*	equal	lower	absent
OTU_39	*Guttipelopia guttipennis*	absent	higher	absent
OTU_41	*Ablabesmyia monilis*	lower	absent	absent
OTU_42	*Zavrelimyia schineri*	lower	higher	absent
OTU_48	*Procladius fuscus*	lower	absent	absent
OTU_54	*Procladius spec.*	lower	higher	absent
OTU_60	*Conchapelopia melanops*	lower	absent	absent

OTU_61	*Procladius spec.*	lower	absent	absent
OTU_74	*Zavrelimyia barbatipes*	lower	absent	absent
OTU_88	*Procladius fuscus*	absent	higher	absent
OTU_99	*Psectrotanypus varius*	lower	absent	absent
OTU_134	*Macropelopia nebulosa*	lower	absent	absent
OTU_150	*Zavrelimyia barbatipes*	lower	absent	absent
OTU_171	*Xenopelopia spec.*	equal	absent	lower
OTU_178	*Procladius spec.*	lower	absent	absent
OTU_317	*Procladius spec.*	lower	absent	absent
	Filter feeder			
OTU_1	*Polypedilum uncinatum*	equal	higher	lower
OTU_8	*Dicrotendipes lobiger*	lower	higher	absent
OTU_24	*Phaenopsectra punctipes*	absent	lower	absent
OTU_26	*Endochironomus tendens*	absent	higher	lower
OTU_28	*Diplocladius cultriger*	lower	lower	absent
OTU_46	*Paratanytarsus tenellulus*	lower	higher	absent
OTU_52	*Psectrocladius limbatellus*	lower	absent	absent
OTU_55	*Paratanytarsus lauterborni*	absent	higher	absent
OTU_66	*Paratanytarsus grimmii*	absent	equal	absent
OTU_77	*Kiefferulus tedipediformis*	lower	equal	lower
OTU_116	*Polypedilum tritum*	lower	equal	absent
OTU_126	*Synendotendipes impar*	absent	higher	absent
OTU_128	*Polypedilum cultellatum*	lower	absent	absent
OTU_129	*Paratanytarsus dissimilis*	lower	absent	absent
OTU_133	*Microtendipes chloris*	lower	absent	absent
OTU_135	*Polypedilum cultellatum*	lower	absent	absent
OTU_136	*Glyptotendipes spec.*	absent	absent	absent
OTU_137	*Phaenopsectra flavipes*	lower	absent	absent
OTU_181	*Paratanytarsus laccophilus*	lower	absent	absent
OTU_233	*Polypedilum spec.*	lower	absent	absent
OTU_250	*Paratanytarsus lauterborni*	absent	higher	absent
OTU_262	*Polypedilum spec.*	lower	absent	absent
OTU_283	*Polypedilum spec.*	absent	absent	lower
OTU_296	*Polypedilum tritum*	reduced	higher	lower
OTU_298	*Endochironomus albipennis*	absent	equal	absent
OTU_312	*Polypedilum uncinatum*	lower	absent	lower

Across all sites, the OTU presence per sample was lower in the Bti treated samples in 99 comparisons (OTU presence in Bti *vs*. control samples, Table 2). This became especially apperent in the rare OTUs with only one or two presence records (Table 2). However, in 19 comparisons the OTU presence was not affected by Bti treatment and in 39 comparisons the OTU presence was higher in the Bti samples (Table 2). Moreover, of all OTUs occurring at more than one site, 11 OTUs showed the same response to Bti treatment, while another 30 OTUs showed a reverse trend (Table 2).

When comparing the chironomid taxa composition at the meadow site from 2016 (this study) with the chironomid taxa composition at the same meadow site from 2013 (Theissinger et al., 2018) we found some differences (Table 5). In 2013, a total 29 chironomid species were found, of which 14 (48.3%) were detected solely in the control samples, two (6.8%) solely in the Bti-treated samples and eight species (27.6%) were present in both sample types. In this study, with more traps and over a longer sampling period, we detected overall 45 species. Of those, 18 species were found in both study years. Six species were detected in 2013, which were not detected in 2016. On the other hand, 27 species were only found in the 2016 data set from this study. Out of these 27 newly discovered species, 21 (77.8%) were only detected in the control samples, six (22.2%) were found in both treatments and one species (3.7%) was found only in the Bti-treated samples (Table 5).

Table 5: Comparison of species presence at the meadow site in the metabarcoding study from 2013 (Theissinger et al. 2018) to this study with data collected in 2016. It is indicated whether the discovered species were detected solely in the control samples (C), solely in Bti-treated samples (B), or both (C + B). Species that were not detected across all samples per sampling year are indicated with NA (not available).

2016			**2013**		
Genus	**Species**	**Bti / control?**	**Genus**	**Species**	**Bti / control?**
Ablabesmyia	*monilis*	C + B	*Ablabesmyia*	*monilis*	C + B
Chironomus	*cf. aprilinus*	C	NA		
Chironomus	*dorsalis*	C + B	*Chironomus*	*dorsalis*	C + B
Chironomus	*melanotus*	C	NA		
Chironomus	*nuditarsis*	C	NA		
Chironomus	*pseudothummi*	C	NA		
NA			*Chironomus*	*riparius*	C
NA			*Chironomus*	*annularis*	C
NA			*Chironomus*	*curabilis*	C
NA			*Chironomus*	*acidophilus*	C + B
NA			*Chironomus*	*sollicitus*	C + B

Conchapelopia	*melanops*	C	NA		
Corynoneura	*carriana*	C + B	NA		
Corynoneura	*coronata*	C + B	*Corynoneura*	*coronata*	C
Corynoneura	*scutellata*	C + B	NA		
Cricotopus	*sylvestris*	C + B	*Cricotopus*	*sylvestris*	B
Dicrotendipes	*lobiger*	C	*Dicrotendipes*	*lobiger*	B
Diplocladius	*cultriger*	C	NA		
Kiefferulus	*tedipediformis*	C	NA		
Limnophyes	*asquamatus*	B	NA		
Limnophyes	*minimus*	C	NA		
Limnophyes	*pentaplastus*	C	*Limnophyes*	*pentaplastus*	C
Macropelopia	*nebulosa*	C	NA		
Metriocnemus	*eurynotus*	C	NA		
Micropsectra	*lindrothi*	C	NA		
Microtendipes	*chloris*	C	NA		
Monopelopia	*tenuicalcar*	C + B	*Monopelopia*	*tenuicalcar*	C
Parachironomus	*parilis*	C + B	NA		
Paralimnophyes	*longiseta*	C + B	*Paralimnophyes*	*longiseta*	C + B
Paratanytarsus	*laccophilus*	C	NA		
Paratanytarsus	*tenellulus*	C	NA		
Paratanytarsus	*dissimilis*	C	NA		
NA			*Paratendipes*	*albimanus*	C
Phaenopsectra	*flavipes*	C	NA		
Polypedilum	*cultellatum*	C	NA		
Polypedilum	*tritum*	C + B [†]	NA		
Polypedilum	*uncinatum*	C + B	*Polypedilum*	*uncinatum*	C + B
Procladius	*uscus*	C	*Procladius*	*fuscus*	C
Psectrocladius	*limbatellus*	C + B	*Psectrocladius*	*limbatellus*	C + B
Psectrocladius	*schlienzi*	C	NA		
Psectrotanypus	*varius*	C + B	*Psectrotanypus*	*varius*	C
Tanytarsus	*eminulus*	C	NA		
Tanytarsus	*heusdensis*	C	*Tanytarsus*	*heusdensis*	C
Tanytarsus	*pallidicornis*	C	*Tanytarsus*	*pallidicornis*	C
Tanytarsus	*usmaensis*	C	*Tanytarsus*	*usmaensis*	C
Telmatopelopia	*nemorum*	C + B	*Telmatopelopia*	*nemorum*	C
Trissocladius	*brevipalpis*	C + B	NA		
Xenopelopia	*falcigera*	C + B	*Xenopelopia*	*falcigera*	C + B
Xenopelopia	*nigricans*	C + B	*Xenopelopia*	*nigricans*	C
Zavrelimyia	*barbatipes*	C	NA		
Zavrelimyia	*schineri*	C	NA		

†) Species was present in the data set with two OTUs: OTU_116 was only present in C, OTU_296 was present in C + B.

4. Discussion

In this study, we investigated the impact of mosquito control actions with the biocide Bti on the community composition of the non-target family Chironomidae using state of the art metabarcoding. Technical sample replication and numerous negative controls demonstrate the high reliability of our results, according to the claim by Zinger, Bonin, Alsos, Bálint, Bik, et al. (2019) for robust experimental design to draw ecological conclusions. Moreover, the extrapolated species richness based on OTUs (Figure 3) showed that the biological study design (see Table 1) was exhaustive enough to sample a substantial proportion of the chironomid community. By focusing on the chironomid emergence across several weeks after Bti application we also sampled species, which were 1st or 2nd instar larvae at the time point of Bti application. These species would have been neglected by picking larvae from sediment (Wolfram et al., 2018) or by sampling the emergence only few days after Bti application. This highlights the necessity of investigating the long-term community effects (i.e., across several weeks) to assess the total chironomid community composition under Bti influence.

4.1 Chironomid community composition at different study sites

At the floodplain site floating emergence traps had been installed to account for the influence of fixed enclosures on the chironomid communities. Allgeier et al. (2019) already showed that the mean chironomid abundance in the floating emergence traps (N = 6) was 2.5 times higher compared to the mean of the fixed control emergence traps (N = 12), and the time of chironomid peak emergence was three weeks earlier for the floating traps as compared to the fixed traps. They concluded that this could be due to altered biotic and abiotic conditions in the polyethylene barrels as compared to the outside environment, with delayed growth rates due to limited food resources and/or the prevention of re-colonization of multivoltine chironomid species, potentially resulting in a depleted chironomid community (Allgeier et al., 2019). In this study, we could confirm that the fixed enclosures had a strong influence on the sampled chironomid community (compare Supporting Information 3). Out of the 55 chironomid OTUs detected in the floodplain control samples, we found 47% in both sampling types, while 27% were only detected in the floating emergence traps, probably due to the lower area of sediment encompassed by the fixed emergence traps hampering recolonization by additional species. In contrast, 25% of the detected OTUs were only found in the fixed emergence traps, possibly due to favourable microclimatic habitats and missing predators within the barrels. Hence, the community diversity sampled with the fixed traps was not depleted but rather shifted as compared to the floating emergence traps.

As hypothesized, the three sites meadow, floodplain and forest differed significantly in their chironomid community composition, with only 10% of shared OTUs (Figure 1, Table 2). Also the correspondence analysis (Figure 5) showed that ellipsoids, enclosing all points of a group, do not substantially overlap among sites, indicating the relatively little congruence in chironomid species composition of the three different habitats. Communities were characterised by few highly dominant taxa (e.g., *P. uncinatum, L. minimum, L. asquamatum, C. dorsalis, T. nemorum, X. falcigera, X. nigricans, D. lobiger)* and many rare taxa (41%) (Table 2). Especially *P. tritum* and *P. uncinatum* as well as species of the genus *Limnophyes* are typical generalists for temporary wetlands, which can survive dry periods in moist soil in a larval diapause (Dettinger-Klemm, 2003). We discovered a very high spatial heterogeneity between the traps within each site, which was most likely due to the high numbers of rare species and the patchy and random deposition of chironomid egg clutches within a water body. Nevertheless, the comparison of the extrapolated species richness revealed that our sampling was exhaustive enough to evaluate the chironomid community composition in the three study sites.

All study sites were very different in terms of hydraulic conditions (i.e., connection to permanent water bodies, springs or ground water), which we regard as the main reason for the very different chironomid communities. The forest site is characterized by many little temporary ponds, which are not connected to permanent springs. Therefore, they can periodically dry out, depending on the ground water level, leading to terrestrial or semi-terrestrial habitats. This can result in highly variable habitats with differing moisture parameters. At the forest site the chironomid community was thus mainly comprised of species typical for temporary ponds, whereas ubiquitous species typical for persistent water bodies were missing. In contrast, the meadow and floodplain sites are connected to nearby persistent water bodies and inhabit a more diverse range of chironomid species and also ubiquitous species. Even though real biological replication of sites with the same Bti treatment history was not feasible, because it is hardly possible to find Bti-untreated wetlands within the Upper Rhine Valley, the different diversities across the three study sites provided a good basis for testing potential Bti-induced non-target effects across a wide range of chironomid OTUs in all three mosquito-control relevant wetland types.

4.2 Bti effects on chironomid community composition

For all three sites and Bti application histories the chironomid OTU composition was different to varying degrees in the Bti-treated samples *vs*. control samples (Figure 4). The correspondence analyses (Figure 5) showed that the sites, including hydraulic and other abiotic differences, had the biggest influence on species composition. However, Bti treatment also might have an effect as illustrated by the little overlap of ellipsoids for Bti-treated and control samples. The pairwise OTU dissimilarity analyses among pooled communities per site and treatment suggested that the Bti induced variation in OTU composition is more pronounced among the meadow and the forest site pairs than within the floodplain site. At the meadow site, the low Simpson dissimilarity index, accounting for the species turnover component, and the higher nestedness-resultant fraction of the Sørensen dissimilarity indicate, that the difference in OTU composition between Bti-treated and control samples is due to a significant OTU reduction (supporting information 3, Wilcoxon rank sum test), with 63% chironomid diversity loss in the Bti-treated samples (Figure 4). The PERMANOVA further showed that the Bti treatment had a 12% significant effect on the community composition (Table 3). In contrast, at the sites floodplain and forest the pairwise species dissimilarity analysis indicates an OTU turnover with species numbers in Bti-treated and control samples being quite similar (Figure 4, supporting information 3). This species turnover within the chironomid community might also have cryptic effects on ecosystem functioning through altered trophic interactions (Benke, 1998).

We assumed that predatory chironomids, feeding mostly on living benthic larvae, are less prone to Bti than filter species feeding on floating particles leading to direct ingestion of Bti (Liber et al., 1998). However, a comparable percentage of predatory and filter taxa was reduced in the Bti samples across all sites (63% and 65%, respectively, Table 4), indicating that the feeding strategy is not the main driver for Bti effects in chironomids (Kondo, Ohba & Ishii, 1995). Despite the fact that the predatory subfamily Tanypodinae was not affected by Bti in some mesocosm studies (Liber et al., 1998; Allgeier et al., 2019) it is conceivable that predatory chironomids might be both directly and indirectly affected through the food chain: Tanypodinae in the 1st instar larval stage show a planktonic mode of life and feed on diatoms and monocellular algae (Vallenduuk & Moller Pillot, 2007). During this developmental stage ingestion of Bti is also possible, and direct Bti effects on these 1st instar larvae can be assumed due to probably the same Bti receptors as in the digestive system of Tanypondinae. As 2nd instar larvae Tanypodinae then switch to the predatory feeding type and feed on 1st and 2nd instar chironomid larvae as well as oligochaetes, because those taxa are small and immobile enough to be caught (Vallenduuk & Moller Pillot, 2007). If this prey is reduced due to high sensitivity to Bti treatment the survival of the 2nd instar Tanypodinae larvae might also be indirectly affected through Bti. Additionally, by feeding on Bti-contaminated prey (i.e., larvae that have ingested a sublethal Bti dose) the toxic Bti crystals produced during sporulation (Boisvert & Boisvert, 2000; Bravo et al., 2007) could be recycled into the digestive system of the predator (Khawaled, Ben-Dov, Zaritsky & Barak, 1990) leading to direct Bti exposure and subsequent death of Tanypodinae larvae. This prey-mediated Bti effect was already demonstrated for a stonefly predator feeding on Bti-contaminated mosquito larvae (Hilbeck, Moar, Pusztai-Carey, Filippini & Bigler, 1999). However, experiments on Bti induced direct and indirect effects particularly on predatory chironomids are to our knowledge still pending.

Our data showed that the Bti effect can be highly variable across sites with different Bti application modes (Table 2, Figure 2). Considering the Bti sensitivity of *C. riparius* under laboratory conditions, 2nd instar larvae are half as sensitive compared to the most sensitive 1st instar larvae (Kästel et al., 2017). If this result is also applicable to other species, an increased Bti dose might not only severely affect the youngest but also older larvae and thus potentially influence a wider range of species at the application time point. Due to the different habitats among sites the Bti application doses cannot be compared directly as they were each applied to the field relevant dose (compare Allgeier et al., 2019). However, at the meadow site the very strong OTU reduction of 63% could be explained by a very effective Bti application in terms of the applied toxicity amount. Here, the nominal Bti rate was doubled compared to the floodplain and the forest site in order to reach a sufficient mosquito reduction (Allgeier et al., 2019).

At the meadow site we detected more chironomid species in the control samples as compared to the same study site three years earlier (Theissinger et al., 2018). Of all detected chironomid species at the meadow site, only 47% were detected in 2013 and 88% in 2016 (Table 5), where the sampling effort was higher (24 traps over 14 weeks in 2016 compared to 10 traps over 13 weeks in 2013). A statistical comparison of the species compositions of 2013 and 2016 was not possible due to the different sampling designs. However, the descriptive comparison showed that of the 27 newly detected species in 2016 almost 80% were solely found in the control samples (Table 5). This suggests that a recolonization by new chironomid species had happened on the sites with continued (fourth year) Bti intermittence. Since it is difficult to find true Bti control sites, i.e., regularly flooded areas within the Upper Rhine Valley that have never received Bti treatment, the indicated resilience effect at the meadow site is a valuable finding and implies that a stop of mosquito control with large-scale biocidal Bti applications has a positive effect on the biodiversity of non-target species within temporary wetland ecosystems.

The Bti-induced quantitative (abundance, Allgeier et al., 2019) and qualitative (species composition, this study) alterations on chironomid communities might have severe consequences for the wetland ecosystems. Because chironomids serve as important food resource for many aquatic and terrestrial species (Armitage et al., 1995) an abundance reduction can lead to bottom-up effects in the food chain, resulting in, e.g., reduced breeding success in birds and dragonflies (Poulin et al., 2010; Jakob & Poulin, 2016). Moreover, also a qualitative change in the chironomid community due to species turnover or species reduction could potentially lead to altered trophic interactions (Benke, 1998). The family Chironomidae is an ecologically highly diverse group, reflected in the broad range of feeding types and life cycles (Ferrington, 2008) as well as in the different sensitivity to varying anthropogenic stressors (Cranston, 2000; Pettigrove & Hoffmann, 2005; Marzalli et al., 2010; Carew, Pettigrove, Cox & Hoffmann, 2007; 2013; Nicacio & Juen, 2015). Thus, chironomid communities are generally characterized by a high adaptability for changing environmental conditions (Raunio et al., 2011). The loss of especially the rare species could lead to undesirable homogeneous biotic communities hampering this adaptive potential.

To conclude, our study demonstrates that the application of the biocide Bti can result in a biodiversity loss and species turnover in temporary wetlands of the Upper Rhine Valley. Moreover, we show the importance of continued sampling across several weeks after Bti application to more comprehensively investigate Bti effects on the chironomid community composition. Considering the very diverse chironomid communities in terms of species composition and age structures at different wetland types the Bti effect can be highly variable, depending also on time and mode of the Bti application. Potential direct and indirect food chain effects on predatory chironomids as well as top-down (e.g. on algal community) or bottom-up (e.g. on amphibians or fish) effects of the chironomid community shift into the aquatic or terrestrial food web requires further laboratory or mesocosm research. Finally, our data indicate a possible community recovery due to species recolonization a few years after the last Bti application. Considering the currently discussed global insect decline (Sánchez-Bayo & Wyckhuys, 2019) we recommend a re-evaluation of the usage of the biocide Bti in mosquito control and suggest avoiding applications especially in nature protection reserves to enhance ecological resilience and prevent an ongoing biodiversity loss.

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The authors declare that there is no conflict of interest concerning this study.

Data accessibility:

The raw sequence reads are deposited in the NCBI Sequence read archive (SRA) under the accession number SRP159056.

Supporting Information

Supporting Information 1: Detailed study design for field samples and replicated samples for metabarcoding per site. Given are the number of collected individuals from Allgeier et al. (2019), the DNA concentration per technical replicate and the library input as well as the accession numbers for each metabarcoding sample.

Supporting Information 2: R script used within the JAMP pipeline (https://github.com/VascoElbrecht/JAMP) for raw data processing and OUT clustering.

Supporting Information 3: List of 108 chironomid OTUs with sequence and read numbers per sample as well as transformed presence/absence table of 57 samples used for statistical Bti effect analyses.

Supporting Information 4: Taxonomic identification of 108 chironomid OTUs as extracted from BOLD. Provided is information on subfamily, genus and, where possible, a species name based on >97% similarity. For all taxa with species identification some ecological traits are listed.

Appendix V: Scientific publication V

European common frog Rana temporaria (Anura: Ranidae) larvae show subcellular responses under field-relevant *Bacillus thuringiensis var. israelensis* (Bti) exposure levels

Stefanie Allgeier, Bianca Frombold, Valentin Mingo, Carsten A. Brühl

Authors' contribution:

Study design and supervision: SA, CB; Experimental work: BF, SA; Data analysis: SA; Biomarker analysis: VM; Manuscript writing: SA

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Highlights:

- VectoBac®12AS and ®WG causes no mortality in *Rana temporaria* tadpoles.
- Bti in field relevant rates increases the activity of GST, GR and AChE enzymes.
- Alterations in enzymes are independent of application rate and Bti formulation.
- Repeated Bti applications seem to increase the risk for biochemical alterations.

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Abstract

Bacillus thuringiensis var. *israelensis* (Bti) is presumed to be an environmental friendly agent for use in either health-related mosquito control or the reduction of nuisance associated with mosquitoes from seasonal wetlands. Amphibians inhabiting such valuable wetlands may be exposed to Bti products several times during their breeding season. Up until now, information regarding effects on the non-targeted group of amphibians has to be considered rather inconsistent. On this account, we evaluated how three repeated exposures to frequently used Bti formulations (VectoBac®12AS, VectoBac®WG) in field-relevant rates affect European common frog (*Rana temporaria*) larvae. In a laboratory approach, we assessed potential effects with regard to enzymatic biomarkers (glutathione-S-transferase (GST), glutathione reductase (GR), acetylcholine esterase (AChE)), development, body condition and survival until the end of metamorphosis. Although survival and time to metamorphosis were not significantly affected, larval development tended to be shortened in the Bti treated water phase. Furthermore, exposure to Bti induced significant increases of GST (37 - 550%), GR (5 - 140%) and AChE (38 – 137%) irrespectively of the applied formulation, indicating detoxification, antioxidant responses as well as an alteration of neuronal activity. GST activity increased twice as much after two repeatedly executed Bti applications within a time period of 6 days. The examination of several biochemical markers is needed to fully evaluate the ecotoxicological risk of Bti for amphibian populations, especially in the context of worldwide amphibian declines. Nevertheless, following the precautionary principle, it may be advisable to implement certain thresholds for application numbers and intervals in order to ensure environmentally friendly mosquito control programs, especially in areas designated for nature conservation.

Keywords: amphibians, biocide, biomarker, mosquito control, non-target organism, sublethal effects

1. Introduction

In mosquito control, the widespread use of synthetic insecticides like organophosphates and pyrethroids had several downsides such as the development of insect resistances or adverse effects on environment and human health (Hemingway and Ranson, 2000). Consequently, the usage of more specifically acting bio-pesticides increased substantially over the last decades. Above all, commercial formulations containing the active ingredient *Bacillus thuringiensis* serotype *israelensis* (Bti) represent one of the main bacterial insecticides for the control of larval mosquitoes, blackflies and chironomids (Becker, 2006; Lacey and Merritt, 2003) with global application amounts of 70-300 tons of formulated product per year (van den Berg et al., 2012). Comparatively, the output quantity of organophosphates amounts to 163 tons per year (van den Berg et al., 2012).

On a global scale, Bti is largely applied for human health issues by controlling vector-borne diseases in subtropical and tropical urban breeding sites (van den Berg et al., 2012). However, temperate regions such as the Upper Rhine Valley in Germany look back on more than 40 years of Bti treatments in river floodplains with the objective of reducing nuisance for the local population (Becker, 2006). To this end, more than 30.000 ha wetlands along the river Rhine are periodically treated against floodwater and snowmelt mosquitoes (KABS e.V., 2016). Noteworthy, the majority of treated wetlands is protected by the EU's Natura 2000 network (KABS e.V., unpublished; Swedish Chemicals Agency, 2015). Bti is generally considered environmentally safe in regard to non-target aquatic organisms due to its specific mode of action (Boisvert and Boisvert, 2000). The driver of toxicity are endotoxins (Cry-toxins) that get activated after ingestion and bind to specific receptor sites in the midgut epithelium of the target organism. The preceding activation depends on several factors such as the alkaline condition and the number of receptors in the midgut (Bravo et al., 2007). Nevertheless, during the last years, some studies revealed uncertainties about the environmental compatibility of ordinary Bti applications. Contrasting results can be found especially when it comes to adverse effects on chironomids (Kästel et al., 2017; Lagadic et al., 2016) that may be propagated upward in wetland food chains (Jakob and Poulin, 2016; Poulin et al., 2010) affecting environmental health.

In addition to the mass occurrence of larval mosquitoes, temporary flooded wetlands also offer suitable breeding sites for many other aquatic organisms, including amphibians (Blaustein and Margalit, 1996). The latter are currently considered the most globally threatened group of vertebrates and their populations are declining worldwide at alarming rates (Stuart et al., 2004). One of the reasons held responsible for the dramatic population decline is the growing rate of human-induced environmental contamination, most notably the influence of pesticides (Sparling et al., 2001). In contrast to chemical pesticides that largely reach the water body indirectly through spray drift, run-off or atmospheric transport (Mackay et al., 2014; Schulz, 2001), Bti reaches amphibian habitats by a direct application to the water surface (Becker, 2006) during the spawning season of many amphibian species. Consequently, amphibian larvae may be exposed to Bti during multiple instances during their development.

Despite of the potential exposure risk for amphibians, (eco-) toxicological research on Bti and amphibians has been quite scarce especially compared to studies on agricultural pesticides. Basic knowledge was gained through direct toxicity studies conducted in the 1980s and ‘90s that indicated mortalities in anurans at high dosages of several Bti products or self-produced bacterial laboratory cultures (Channing, 1998; Morawcsik, 1983; Paulov, 1985). A recent study found intestine damage and increasing glutathione-S-transferase and catalase activity levels after the exposure of a tropical frog species to sublethal Bti concentrations of a commercial Bti formulation (Introban®) (Lajmanovich et al., 2015). Induced enzyme activities were directly linked to lethal effects at high Bti concentrations, resulting in a LC_{50} at 22.45 mg Bti/L (Lajmanovich et al., 2015) which is comparable to actual application rates in the Upper Rhine Valley, particularly when older mosquito larvae are present (Becker, 1998). However, toxic effects might not be caused entirely or at all by the active ingredient Bti: for pesticides, it has been shown that additives in commercial formulations can potentiate amphibian toxicity (Puglis and Boone, 2011; Relyea and Jones, 2009) or, in other cases, have been shown to be the main cause of toxicity (Cox and Surgan, 2006; Wagner et al., 2013). The effects caused by Bti formulation additives were so far not considered in scientific studies or the environmental risk assessment. In addition, the German Mosquito Control Association (GMCA, Speyer, Germany) even applies different delivery forms of commercially available Bti VectoBac® formulations, depending on application type, habitat accessibility and wetland size (KABS e.V., 2016) which may also change the toxic properties of the final product. Considering that amphibians are key components for energy transfers between aquatic and terrestrial habitats (Gibbons et al., 2006), highlights the need of ecotoxicological data in order to ensure environmental health of wetland ecosystems.

The goal of the present study was to examine the effects of three Bti delivery forms on the common frog *Rana temporaria* which is widely distributed throughout Europe. Its spawning habitats range from stagnant shallow to temporary ponds (Schlüpmann and Günther, 2004) co-occurring with mosquito larvae. We simulated the current practice of mosquito control using environmentally relevant rates (Table 1) and frequencies of Bti adapted to the control program in the Upper Rhine Valley, where smallest temporary wetlands are treated several times a year in short intervals. By doing so, tadpoles were exposed to three consecutive Bti applications with three common Bti formulations at three different stages during their larval development, in a fully crossed design. Based on the findings by Lajmanovich et al. (2015) and the scanty toxicity information on VectoBac® formulations in the pesticide risk assessment (European Food Safety Authority, 2013), we hypothesized that consecutive applications of Bti formulations would affect survival, physiological fitness (judged by growth) and developmental time of tadpoles depending on the application rate. Moreover, we selected three well studied biomarkers of effect in anuran larvae to examine sublethal effects: glutathione-S-transferase (GST), glutathione reductase (GR) and acetylcholine esterase (AChE) (Venturino and D'Angelo, 2005). We expected subcellular alterations in the enzymatic activity rates of GST and the antioxidant enzyme GR after each Bti application at sublethal concentrations similarly to the effects of Introban®. Furthermore, we assumed the absence of any neurotoxic effects (AChE) due to the specific mode of action of Bti.

2. Material and methods

2.1 Tadpole collection and animal husbandry

To ensure the lack of previous exposure and a high genetic variability of tested individuals, six freshly laid (up to 3 days old) *R. temporaria* egg clutches were collected from a pristine pond in the Bienwald forest, Rhineland-Palatinate, Germany (49°01'19.2'' N, 8°10'46.1'' E) in March 2016. Egg clutches were randomly assigned to aerated 30 liter glass tanks (50 x 30 x 20 cm) filled with filtered tap water (0.2 µm Supor, Pall Corporation, Port Washington) until embryos developed to freely swimming tadpoles which were used in the following experiment. Tadpoles were fed with commercially available rearing food for aquarium animals (Sera Micron, Sera GmbH, Heinsberg) three times a week during the renewal of water. Housing, rearing and all experimental procedures took place at 18 – 24°C and a 16/8-h light/dark cycle. All experimental procedures in our study were evaluated and approved by the Institutional Animal Care and Use Committee at the University Koblenz-Landau and the federal investigation office (Landesuntersuchungsamt – LUA, NTP-ID: 00008349-1-2). All animals not used in the experiment were euthanized using 0.1% MS-222. Tadpole development stages (GS) were determined using a binocular (Leica KL300 LED, Wetzlar, Germany) according to Gosner (1960).

2.2 Bti formulations

Bti formulations were chosen according to the application practice in the German mosquito control program. Two commercially available formulations containing the active ingredient Bti (strain AM 65-52) are used in Germany: VectoBac®WG and VectoBac®12AS. VectoBac®WG is a water dispersible granule formulation (37.4% a.i. 3000 ITU/mg) whereas VectoBac®12AS is an aqueous suspension (11.6% a.i. 1200 ITU/mg) (Valent BioSciences Corporation, Illinois, USA). No further information on other ingredients is provided by the manufacturer. The GMCA uses VectoBac®WG and VectoBac®12AS as a basis for the preparation of three different delivery forms: ice-pellets, sand-granule and liquid. Ice-pellets are manufactured with a suspension of VectoBac®WG that is converted to 4 mm grain sized granules with the help of liquid nitrogen (Becker, 2003). In case of Bti sand-granule, the respective amount of VectoBac®WG granules is bound to coarse sand as a mineral carrier with the use of vegetable oil. The liquid formulation is prepared as a 1:10 solution of VectoBac®12AS and tap water. All formulations were obtained from stock material of the GMCA. In the following, ice-pellets are referred to as formulation “Ice”, sand-granule as formulation “Sand” and the liquid formulation as “Liquid”.

2.3 Exposure conditions

To adequately simulate realistic exposure conditions, three concentrations according to the actual applied field rates were used (KABS e.V., 2016): the nominal field rate (1x), twice (2x) and tenfold (10x) the nominal field rate (FR). According to the control strategy of the GMCA, field rates depend on the age structure of the mosquito larvae population, water depth and temperature. In wetlands with deep-water areas and older mosquito larvae (2nd, 3rd stages) the nominal field rate is routinely doubled in order to reach a sufficient treatment success (Becker, 1998, 2003). The tenfold field rate was employed both to assess dose-dependent effects and to include a worst-case exposure. In mosquito control programs, Bti is applied repeatedly whenever mosquito populations start to develop in monitored wetlands. A mean application frequency of three was chosen related to typical Bti application numbers in wetlands in the Upper Rhine Valley within the last ten years between the months March and September Formulations were directly applied on the water surface without further mixing to recreate realistic exposure conditions.

2.4 Experimental design

2.4.1 Multiple exposure experiment

When tadpoles developed gill buds at GS 19, they were transferred into 100 separate plastic aquaria (7 x 16 x 22 cm) in which the experiments took place. Each aquarium contained five *R. temporaria* individuals in 1.7 liters of filtered (0.2 µm Supor, Pall Corporation, Port Washington) and aerated tap water. Overall, Bti was applied three times, referred to as application frequency, at three time points in a fully crossed design: three formulations x three field rates. Each treatment and one Bti-free control was replicated ten times (n=10). The applied volumes of the respective formulation were calculated based on the surface area of the aquaria (Table 1).

Table 1: Application rates of the three formulations (Ice, Liquid, Sand) based on the nominal (1x), doubled (2x) and tenfold (10x) field rate (FR) used in the German mosquito control strategy (KABS e.V., 2016). Aquarium surface area = 0.046 m^2; Volume = 1.7/L.

	Ice				**Liquid**				**Sand**			
	FR		Application rate		FR		Application rate		Field rate		Application rate	
	[kg/ha]	[10^9 ITU/ha]	[mg/L]	[ITU/L]	[L/ha]	[10^9 ITU/ha]	[µl/L]	[ITU/L]	[kg/ha]	[10^9 ITU/ha]	[mg/L]	[ITU/L]
1x FR	15	1.44	40.56	3,900	2	1.92	5.41	6,494	25	1.2	67.61	3,247
2x FR	30	2.88	81.13	7,800	4	3.84	10.82	12,988	50	2.4	135.21	6,494
10x FR	150	14	405.65	39,000	20	19.2	54.12	64,940	250	12	676.06	32,470

ITU = International Toxic Unit

The first Bti application took place 3 days after test start when tadpoles reached GS 21 to 23 and larvae start to feed autonomously (Fig. 1). Embryos were not included in the experiment since the most likely way of tadpole exposure towards Bti is orally via food intake (Mokany and Shine, 2003). The second application was conducted after additional 6 days when the external gills receded at GS 24 to 28. Finally, a third application took place after a further 32 days, before the forelimbs started to become visible at GS 36 to 40. The determination of GS took place on a randomized basis prior to applications. Tadpoles used for biomarker analyses were assigned to GS 23, 25 and 39 and sampled 48 h after each application (n=10). Euthanasia took place using 0.1% MS-222, after which individuals were shock-frozen in liquid nitrogen and individually stored at -80°C until further analyses. After the third application (GS 39), five randomly chosen individuals were sampled for biomarker analysis at 2x and 10x FR, since remaining individuals were used for establishing the biomarker assay.

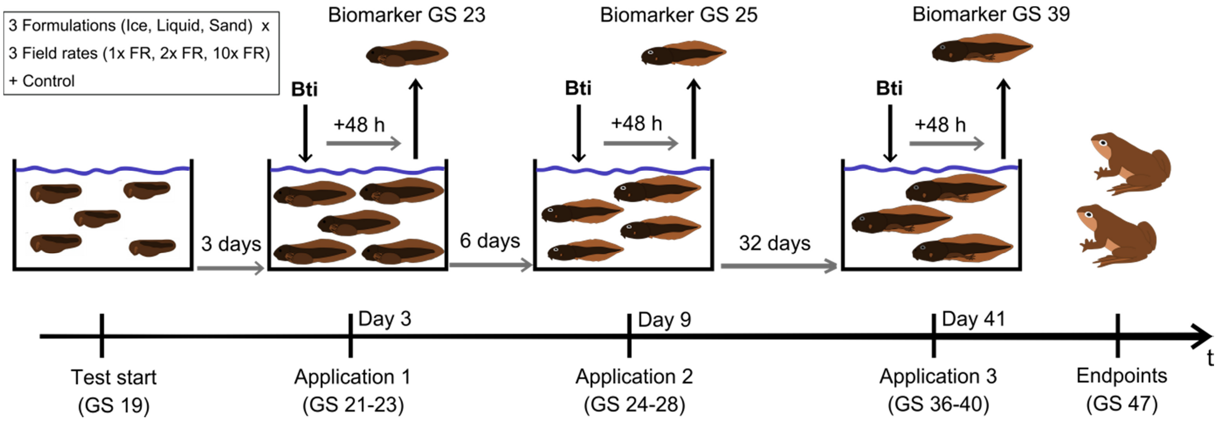

Figure 1: Schematic overview of the experimental design of the early exposure experiment using *R. temporaria* tadpoles. Three formulations (Ice, Liquid, Sand) at three field rates (FR) (1x, 2x, 10x) were applied three times (Application 1-3) at three consecutive time points (Day 3, 9, 41) after the test start at Gosner stage (GS) 19 until recording of endpoints (mortality, physiological parameters) at GS 47 (n=10). Tadpoles for biomarker analysis were sampled 48 h after each application having GS 23, 25 and 39 (n=10).

Larval mortality was evaluated every second day and additionally 24 h after each application. During the experiment, tadpoles were fed ad libitum with rearing food for aquarium animals (Sera Micron, Sera GmbH, Heinsberg), while half of the water was replaced every second day. As soon as the forelimbs of one froglet became visible, the aquaria were placed in an inclined position in order to offer dry areas to avoid drowning after completion of metamorphosis. On completion (GS 47), frog metamorphs were sampled and euthanized in 0.1% MS-222 and time to metamorphosis was calculated individually. Body length was evaluated at the end of metamorphosis using the software AxioVision® (Carl Zeiss; Oberkochen, Germany) using a digital photograph (Finepix F500EXR, Fujifilm) of the metamorph. Associated wet body mass was recorded by weighing the dabbed dry metamorph to the nearest 100 milligram (Mettler PM6000, Colombus, USA). Body length and mass were used to compute body condition of individuals, using the scaled mass index ($\widehat{M}_{\mathrm{i}}$) (Peig and Green, 2009).

2.4.2 Single exposure experiment

To account for the impact of repeated Bti applications on biochemical responses of *R. temporaria* tadpoles in early developmental stages, a single exposure experiment was performed. In contrast to the multiple exposure experiment, tadpoles were exposed to Bti (Ice, Liquid, Sand) the first time between GS 24 and 28. 48 h after the application tadpoles assigned to GS 25 (n=10) were euthanized using 0.1% MS-222, shock-frozen in liquid nitrogen and individually stored at -80°C for further biomarker analyses. To reduce the number of animals due to animal protection regulations, the experiment consisted of a control and all formulations in one field rate (2x FR). If not stated otherwise the experiment was conducted under the same conditions mentioned in 2.1 and 2.4.1.

2.5 Biomarker assays

Since Lajmanovich and co-workers (2015) found signs of oxidative stress in larval amphibians after Bti exposure, we investigated the enzymatic biomarkers GST and GR. GST is a phase II detoxifying enzyme while GR serves as an antioxidant enzyme (Steinberg, 2012). Both are widely used to assess pesticide effects on amphibians (Venturino and D'Angelo, 2005). Additionally, AChE was examined since it plays an important role in the function of nerve impulse transmission which makes it an important and well-studied biomarker for the detection of neurotoxic properties in aquatic organisms (Venturino and D'Angelo, 2005). The specific activities of the selected enzymes were determined spectrophotometrically using a multi plate reader (Synergy HT-I, BioTek, Winooski, USA) at 25°C in duplicates. All biomarker assays were conducted according to the protocol described in Mingo et al., (2017) modified for tissue using a 1:10 dilution of tadpole homogenate. Protein concentrations, needed to calculate enzyme specific activities, were determined according to the Bradford method, using bovine serum albumin (BSA) as a standard (Bradford, 1976). GST activity was measured following Habig et al. (1974). GR activity was assayed according to Carlberg and Mannervik (1985). AChE activity was measured following the Ellman method (Ellman et al., 1961).

2.6 Calculations and statistics

2.6.1 Calculations

The scaled mass index ($\widehat{M}_i$) as described by Peig and Green (2009) was chosen as a body condition index (CI) to assess test animals' fitness based on length and mass data. Compared to other conventional body condition indices, it proved to successfully account for the detection of body size changes (Peig and Green, 2010). It is calculated according to the following equation:

$$\widehat{M}_i = M_i \lfloor L_0 / L_i \rfloor^{b_{SMA}}$$

To compute this index, a standardized major axis (SMA) regression was performed on ln-transformed data of all body mass versus length measurements. The index adjusts the body composition of each individual to the arithmetic mean of all individual length measurements L_0. When two individuals reached metamorphosis, the mean values of body mass, length, time to metamorphosis and $\widehat{M}_i$ were calculated for each replicate.

2.6.2 Statistical analyses

To test for statistically significant differences in enzymatic responses, body condition parameters, time to metamorphosis and mortality rates in the Bti treatments compared to the respective control, analysis of variances (ANOVA) was used whenever the assumptions of normality and homoscedasticity were met. Normality of data was examined using the Shapiro–Wilk test, as well as visual inspection. Homoscedasticity was tested with the Levene test. ANOVA was followed by Dunnett's post-hoc test. If assumptions of normality and homoscedasticity were not met or the number of observations was unequal, a Kruskal–Wallis test was used followed by Dunn's test for post-hoc comparisons of control and treatments (Zar, 2010).

To test whether the exposure to Bti influenced the activity of the enzymatic biomarkers, a generalized linear model (GLM) was implemented. Time (from experiment start), FR, formulation and the interaction between formulation and time and between dose and time were implemented in the model as explanatory variables. Time after experiment start was included as a continuous variable to account for proceeding tadpole development under the increasing number of applications. The best model was chosen using F-test-based backward model selection. For GST, a gamma probability distribution and an identity link function was used. Since GR and AChE activities showed a high number of zero-values, a GLM following a tweedie distribution (var.power=1.7, link.power=0) was conducted. The tweedie distribution was chosen since it reflected the data properly, adjusting a point mass at zero before following a regular exponential curve (Dunn and Smyth, 2005). All calculations and statistical analyses were performed in R (R developmental Core Team, Vienna, R version 3.3.2).

3. Results

3.1 Multiple exposure experiment

3.1.1 Mortality, body condition parameters and time to metamorphosis

Common frog survival was neither significantly affected by the different Bti substances nor the application rate (chi-square=3.35, p=0.95), although mortalities of up to10% occurred with the nominal and the doubled field rate treatments (Table 2). The number of days tadpoles needed to finish metamorphosis ranged from the highest value of 59.75 (± 4.12) in the control to the shortest time of 51.90 (± 2.90; ±3.09) in Ice and Sand applications (1x FR) where frogs left the water around 8 days prior to the control, but without statistically significant difference (chi-square=11.7, p=0.23). Body length ranged from 13.80 mm (±1.27) in the control to 15.10 mm (± 1.25) in Ice (1xFR) without statistically significance differences (chi-square=9.08, p=0.85). Tadpoles showed no significant difference in weight (chi-square=7.18, p=0.62), although the lowest weight of 0.17 g (± 0.02) in the control and Ice (2xFR) while the highest weight (0.21 g ± 0.04) was recorded in Ice (1xFR) and Liquid (2xFR). $\widehat{M}$ was comparable throughout all treatments and ranged between 0.18 and 0.20 (F=0.52, p=0.85).

Table 2: Mortality, time to metamorphosis (TTM) and physiological parameters of *R. temporaria* metamorphs at Gosner stage 47 when metamorphosis was completed in different treatments (mean values ± 95% Confidence Interval, $\widehat{M}$ - scaled mass index, FR – field rate, n=10).

Treatment (Application rate)	Formulation	Mortality [%]	TTM [d]	Body length [mm]	Body mass [g]	$\widehat{M}$
Control	-	0	59.75 ± 4.12	13.80 ± 1.27	0.17 ± 0.02	0.19 ± 0.02
1x FR	Ice	10 ± 13.07	51.90 ± 2.90	15.10 ± 1.25	0.21 ± 0.04	0.18 ± 0.02
	Liquid	10 ± 19.60	56.94 ± 5.96	13.94 ± 1.36	0.19 ± 0.05	0.20 ± 0.02
	Sand	5 ± 9.80	51.90 ± 3.09	14.34 ± 1.65	0.19 ± 0.02	0.19 ± 0.02
2x FR	Ice	0	53.44 ± 3.43	14.02 ± 1.10	0.17 ± 0.02	0.20 ± 0.03
	Liquid	10 ± 13.07	57.05 ± 5.42	14.94 ± 1.30	0.21 ± 0.04	0.20 ± 0.02
	Sand	5 ± 9.80	54.80 ± 3.70	14.29 ± 1.24	0.17 ± 0.03	0.18 ± 0.03
10x FR	Ice	5 ± 9.80	54.89 ± 3.59	14.07 ± 0.77	0.19 ± 0.02	0.20 ± 0.01
	Liquid	0	52.70 ± 2.17	14.73 ± 1.08	0.18 ± 0.02	0.18 ± 0.02
	Sand	5 ± 9.80	54.30 ± 2.89	14.94 ± 0.63	0.20 ± 0.03	0.19 ± 0.03

3.1.2 Enzymatic activities

3.1.2.1 GST activity

Generally, mean GST activity levels in tadpoles decreased significantly over the course of the experiment and proceeding tadpole development (t=-9.98, p<0.001) (Fig. 2). However, GST activity levels of all treatments increased statistically significant compared to the respective control activities after Bti applications in all three formulations (Ice: t=6.26, p<0.001; Liquid: t=4.70, p<0.001; Sand: t=6.26, p<0.001). The mean increase in activity was about 37% after Application 1, 150% after Application 2 and 550% after Application 3 irrespective of the applied formulation. The application of higher field rates tended to lead to lower GST activities (t=-1.90, p=0.06) primarily apparent with Liquid and Sand. Proceeding time of the experiment interacted significantly negative with the application of the formulations Ice (t=-

3.11, p<0.01) and Sand (t=-2.03, p<0.05), leading to higher treatment effects at the early phases of the experiment.

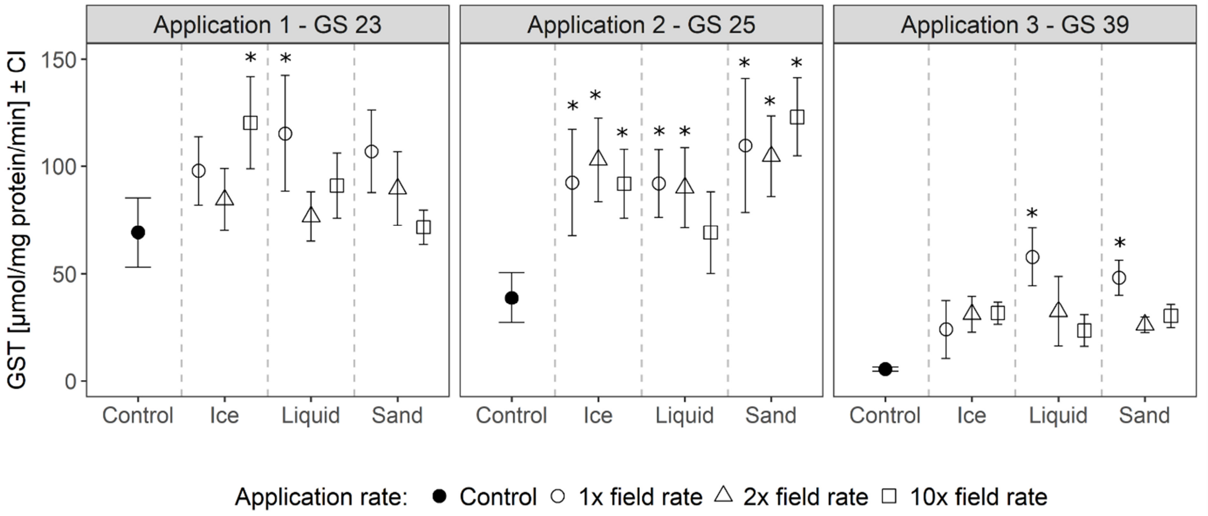

Figure 2: Mean GST activity rates (± 95% CI) of *R. temporaria* larvae in control and Bti treatments (Ice, Liquid, Sand) at different application rates (1x, 2x, 10x field rate) for three Gosner stages (GS) 23, 25, 39 (n=10, except n=5 in 2x and 10x FR at Gosner 39). Asterisks indicate statistically significant differences to the respective control p<0.05.

3.1.2.2 GR activity

GR showed decreasing activity rates with proceeding experimental time (t=-11.87, p<0.001), but less distinct than in GST responses (Fig. 3). Mean GR actvities increased after the Bti applications compared to control levels, showing the highest increase of 140% after the second application at GS 25 (Application 1: 5%, Application 3: 24%). While some treatments showed differences after the first two applications, there was no statistically significant difference of any treatment combination after the third application (Fig. 3). Increases were not driven by the different Bti formulations (Ice, Liquid, Sand: p>0.05) or any interactions with time but rather by field rate. The application of higher field rates significantly increased GR activities (t=4.13, p<0.001) which is in contrast to GST results.

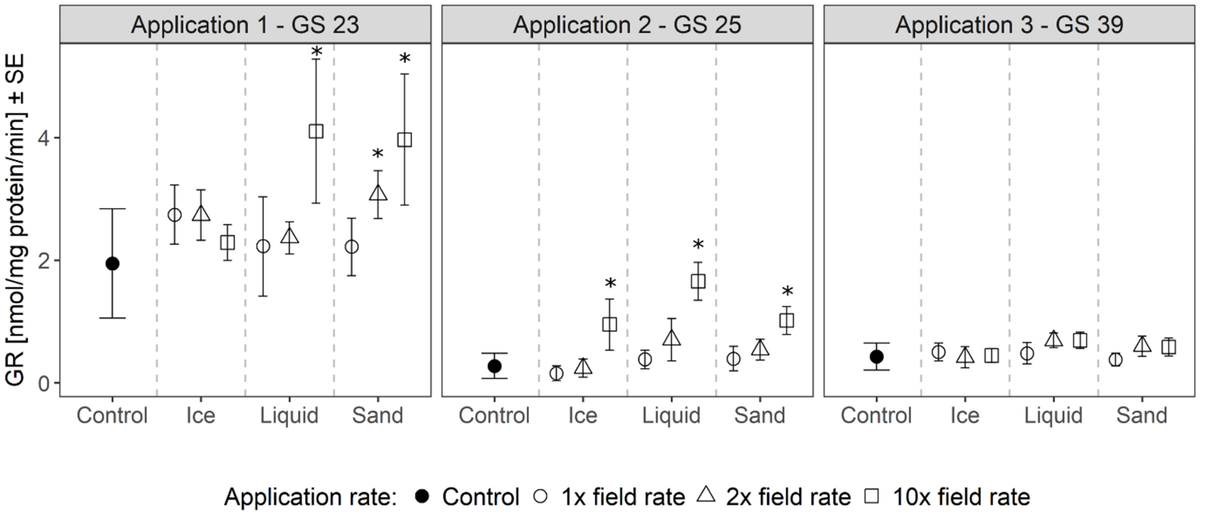

Figure 3: Mean GR activity rates (± 95% CI) of *R. temporaria* larvae in control and Bti treatments (Ice, Liquid, Sand) at different application rates (1x, 2x, 10x field rate) for three Gosner stages (GS) 23, 25, 39 (n=10, except n=5 in 2x and 10x FR at Gosner 39). Asterisks indicate statistically significant differences to the respective control p<0.05.

3.1.2.3 AChE activity

The activity pattern of AChE was similiar to the GST and GR responses and showed decreasing actvity rates with proceeding experimental treatment and tadpole development (Time: t=-30.21, p<0.001) (Fig. 4). After the first two applications, AChE levels of all treatments increased by an average of 38% (Application 1) and 137% (Application 2) while the third Bti application showed no increases compared to control levels. AChE increases occurred independently from the applied formulation (Ice: t=4.20, p<0.001; Liquid: t=4.02, p<0.001; Sand: t=3.10, p<0.01). As with GR activites, field rate generated a significant positive input to the AChE responses (t=0.02, p<0.01). Detailed results on the model output of all biomarker GLMs can be found in the Supplementary material B.

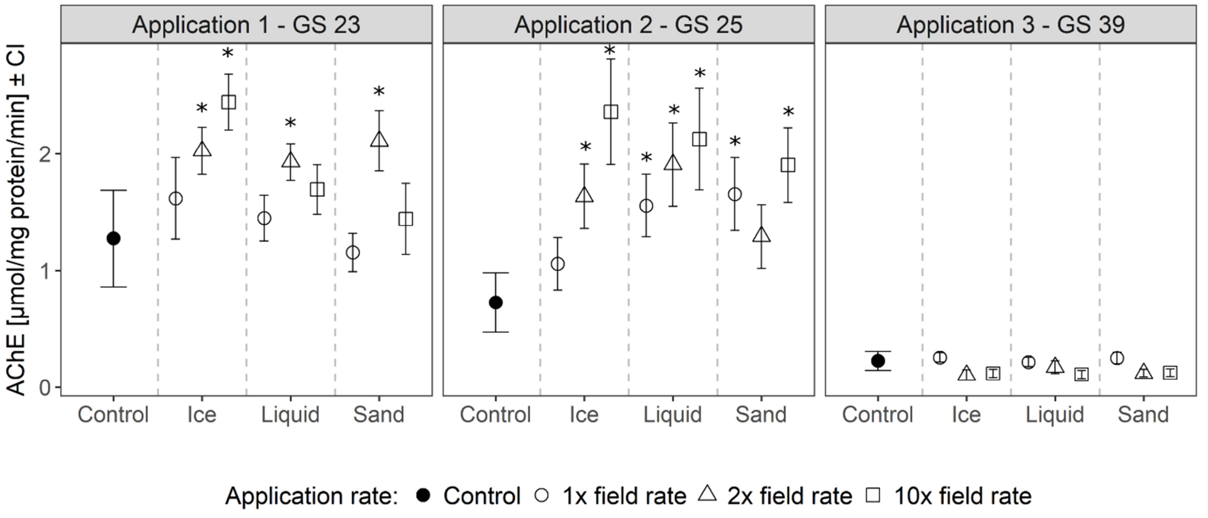

Figure 4: Mean AChE activity rates (± 95% CI) of *R. temporaria* larvae in control and Bti treatments (Ice, Liquid, Sand) at different application rates (1x, 2x, 10x field rate) for three Gosner stages (GS) 23, 25, 39 (n=10, except n=5 in 2x and 10x FR at Gosner 39). Asterisks indicate statistically significant differences to the respective control p<0.05.

3.2 Single exposure experiment

After the first Bti application at GS 25, mean GST activity levels increased of about 48% compared to control levels, showing statistical significance in Liquid ($p<0.05$) and Sand ($p<0.01$). GR activity increased significantly after the exposure to all formulations (Ice: $t=2.61$, $p<0.05$; Liquid: $t=2.5$, $p<0.05$; Sand: $t=4.65$, $p<0.001$), resulting in a mean increase of 88%. AChE showed similar activity levels to the control, except for a statistically signifcant decrease in Ice ($p<0.05$).

4. Discussion

In our experimental setup, common frog larvae experienced exposure conditions similar to realistic mosquito control in the Upper Rhine Valley. Regardless of the formulation, delivery form or application rate, tadpole survival rates and time to metamorphosis tended to be reduced after repeated Bti exposures. All exposed individuals, once again irrespective of application rate or formulation, revealed statistically significant deviations in the level of antioxidant enzyme activity when compared to control individuals. Our study indicates that field-relevant Bti applications induce metabolic processes of detoxification, antioxidant defenses and alter neuronal activity in tadpoles.

Contrary to our expectations, neither of the applied Bti formulations (VectoBac®12AS and VectoBac®WG), in any of the different delivery forms (Liquid, Ice, Sand) induced acute mortality to *R. temporaria* tadpoles. Nevertheless, we found a slight, but not significant, increase in the cumulative mortality after three consecutive Bti applications (Table 2). However, high survival rates made it possible for this study to examine adverse effects on a sublethal level. While our study is in line with older studies that show no Bti induced mortality in larval frogs (inter alia *R. temporaria*), newts, salamanders or toads (Becker and Margalit, 1993; Boisvert and Boisvert, 2000; WHO, 1999), it contradicted the findings of a recent study on the south American common frog *Leptodactylus latrans*. Lajmanovich et al. (2015) found toxic effects of the liquid Bti formulation Introban®, causing 100% mortality at a concentration of 22.45 mg/L which corresponds to 48,000 ITU/L. ITU is referring to the quantity of toxicity driving endotoxins, responsible for toxic effects in targeted insects (Skovmand and Becker, 2000). In terms of ITU, the concentration of the liquid VectoBac®12AS in our study was even higher (64,940 ITU/L), but did not significantly affect survival rates. Lethal effects towards amphibians that are based on the same very specific toxic mode of action of Bti towards insects are rather unlikely, due to the absence of suitable receptor sites in the neutral intestine of amphibians (Broderick et al., 2006; McDiarmid and Altig, 1999). Besides endotoxins, formulations also contain additives, which do not need a public declaration being necessary but constitute a major part of the formulation, varying from 62.6% (VectoBac®WG) to 98.8% (Introban®). These additives may be as responsible for the divergent results on mortality as the potential difference in the sensitivity of the tested amphibian species. In fact, many studies concerning the risks of pesticide applications for human health and wildlife have already highlighted the importance of additives and surfactants as drivers for toxicity (Cox and Surgan, 2006; Puglis and Boone, 2011; Wagner et al., 2013).

Surprisingly, we found a trend towards a faster larval development in the Bti treated tadpoles compared to control animals (Table 2). This trend was consistent throughout all Bti treatments. Amphibian larvae are able to change their behaviour, morphology or physiology in order to adapt to different environmental conditions, collectively termed as phenotypic plasticity (Newman, 1992). In this case, tadpoles could have used Bti proteins as an additional food source, which would enable them to capitalize from better aquatic growth opportunities, gain body mass and allow for a faster growing. However, this could not be supported by the data on body condition at the time of metamorphosis, since body mass, as well as body condition, did not differ between control and treated individuals (Table 2). Alternatively, amphibian larvae are also known to escape unfavorable conditions in their larval environment as soon after they reach a certain threshold body mass (Morey and Reznick, 2000). Such a reaction was already detected under the influence of pesticides (Cauble and Wagner, 2005). In addition to that, all the examined biochemical markers showed significant changes in activity levels after contact to Bti throughout the course of the experiment (see 3.2). These alterations indicate that tadpoles possibly underwent enhanced stress conditions during their stay in Bti treated water, determined by increases in the detoxification process (GST), the antioxidant defence (GR) and in AChE levels. Moreover, higher activities of the antioxidant enzymes GPx and GR have already been found in amphibian larvae experiencing development acceleration (Burraco et al., 2017).

In fact, Bti induced higher activity of GST and GR after each application in our study (Fig. 2, 3). As detoxification enzyme, GST can be involved in the detoxification of Bti which was already suggested in association with resistance against Bti in mosquitoes (Boyer et al., 2007). However, given the size of the toxin and the extracellular location (the mode of action requires binding to a receptor in the midgut epithelium) it is unlikely that detoxification occurs before the interaction with the receptors. To our knowledge there is no biochemical evidence how Bti toxins could be detoxified by GST which is why Boyer et al. (2012) suggested that the increase in GST could also be a response to stress caused by either Cry toxins or associated additives.

GR is one of the key antioxidant enzymes that protects cells in stress conditions by re-establishing or maintaining redox homeostasis after an excessive increase of reactive oxygen species (ROS). Therefore, high antioxidant activities could be seen as an adaptive response in order to avoid oxidative stress. However, if ROS is not sufficiently balanced by an upregulation of the antioxidant defences, it may lead to oxidative stress (Monaghan et al., 2009). There are two conceivable ways for ROS to actively be created: first off, within phagocytic cells as immune response to fight the bacteria or other harmful particles contained in the Bti formulation (Steinberg, 2012). Secondly, ROS may also be induced when ingested Bti is detoxified in the biotransformation system. In the phase I metabolism, xenobiotic compounds get functionalized by cytochrome P450 enzymes (Steinberg, 2012). As a result, ROS can be generated although this step is necessary for the provision of reactive sites needed in the conjugating reaction (phase II) involving GST (Steinberg, 2012).

Increasing GST activities have also been found in tadpoles exposed to sublethal concentrations of another Bti formulation (Introban®) (Lajmanovich et al., 2015). A notable difference is, however, that the increases in GST activities after VectoBac® treatments are dose-independent and led to rather constant or even slightly decreasing activities. A potential explanation might be that the detoxification potential of GST is limited and the threshold is already reached after the exposure to nominal field rates. Subsequently this leads to free ROS that could not be degraded by GST anymore in higher field rates (Steinberg, 2012). An oversaturation of the complete glutathione related uptake pathway at the nominal field rate could also be a feasible alternative, when GR responses would show a similar pattern to GST. However, considering that the activity pattern of GR displays an activity increase (indicating the emergence of ROS) with increasing application rates, the first assumption seems more plausible. Presuming the limitation of the GST detoxification capacity, increased field rates may nevertheless lead to higher stress levels which needs to be clarified with more biomarker assays within the phase II metabolism or ROS measurements. If the increased antioxidant responses already indicate the presence of oxidative stress, cannot be finally stated by means of the analysed biomarkers in this study. Building on this, biomarker of oxidative damage in the targeted key molecules, notably DNA, proteins or lipids would need to be evaluated further (Monaghan et al., 2009).

Moreover, common frog tadpoles responded to the first two Bti applications with significant increases in AChE activity, which were up to 137% higher than control levels. As a biomarker for neurotoxic effects, it is widely accepted that a change of more than 25% in AChE activity indicates harmful effects after pesticide exposures (Beyers and Sikoski, 1994; Stansley, 1993; Sturm et al., 2007). However, deleterious neurotoxic effects are known from pesticides like organophosphates, carbamates or organochlorines and are linked to AChE inhibitions, rather than excitation (Venturino and D'Angelo, 2005). Regarding amphibians, AChE increases have also been observed in *Rhinella arenarum* and *Rana clamitans* tadpoles being exposed to organophosphorus pesticides (Rosenbaum et al., 2012; Sparling et al., 1997). As an explanation for AChE increases, Sparling and co-workers (1997) assumed that suffering under prolonged exposure stress could have stimulated the nervous system in tadpoles leading to the production of more ACh and consequently AChE. Besides, increased AChE levels have been found in bees after sublethal concentrations (Badiou et al., 2008). Thus, AChE increases may also be a response in order to avoid neurotoxicity to a certain degree. However, mechanisms behind AChE increases after Bti exposures have not been described so far, but should be studied further since our results indicate some unknown alterations in the neural transmission.

Subcellular responses were induced independently of formulation and delivery form, suggesting that either certain proteins, chitinases, spore-associated factors (Benz and Perron, 1967; Sampson and Gooday, 1998), a common additive or some Bti specific virulence factors such as beta-exotoxins or vegetative insecticidal may trigger biochemical alterations. However, as the surfactants of the formulations remain unknown, definitive assertions regarding the mode of action cannot be made. Furthermore, all enzymatic activities decreased with proceeding time of the experiment. Since the decrease can be seen in the control as well, changes in activity levels are probably associated with metabolic changes during the larval development (Ferrari et al., 2008). The extent of the Bti effect after individual applications varied, showing lowest activity increases after the first application followed by increases about 140% when Bti is applied a second time at a later developmental stage. Additionally, treatment effects on GST were higher in the early stages of the experiment according to the significant interaction term (3.1.2.1). Due to the linkage of application frequency and larval development in the experimental design, it can hardly be distinguished if either GS 25 is a very sensitive larval stage or short-term consecutive applications intensify observed effects. In fact, the latter can be supported by the enzymatic activities gained from the single exposure experiment where Bti was applied at GS 25 for the first and only time (3.2). While the increase in GST (37%) after an application at GS 23 is comparable to the increase (48%) at GS 25, a second consecutive application at the same developmental stage enhanced GST activities twice as much (Fig. C.1a). At the same time, GR activities increased as well, about 15% (Fig. C.1b). Thus, our results suggest that consecutive Bti applications in a short period of time may increase the risk for the induction of detoxification and antioxidant responses. At the time of the third exposure (32 days following the second) a recovery of the cellular responses seems most plausible (Mingo et al., 2016) and is supported by GR and AChE activities. However, latent effects from former exposures cannot be completely excluded since GST activities showed further increases.

Our results are of particular importance for mosquito control strategies in seasonal wetlands, because Bti can be applied up to 12 times a year (Becker, 1997) in intervals less than one week (KABS e.V., unpublished) depending on the incidence of flooding events that induce massive mosquito hatchings. Therefore, an application frequency of three times, chosen for this study, is a rather conservative approach in assessing Bti induced effects. While early Bti applications against snowmelt mosquitoes in marshy woodlands are implemented in March or early April, treatments against floodwater mosquitoes in temporary flooded ponds along streams are applied during summer months. Hence, various native amphibian species can come into contact with Bti, ranging from early spawning anurans that reach their spawning habitats in early spring (mid of March) such as *R. temporaria* or *R. dalmatina*, to the *Phelophylax* frogs which spawn later during May and June (Günther, 1996). Anuran tadpoles often spend their larval time in very shallow waters where the assumed water phase of 7.5 cm in height is indeed low but still realistic. For example, standardized calculations on Bti concentrations in ponds are mostly based on an assumed mean water height of 10 cm (Schnetter et al., 1981). Besides, application rates in mosquito control programs are calculated depending on the surface area of waterbodies which can vary greatly in depth or structure. Additionally, according to our results, effects on the subcellular level do not show strong dose-effect relationships.

Generally, investing in cellular responses to xenobiotics is an energy demanding process for animals (Steinberg, 2012). Tadpoles will take trade-offs due to increased costs for maintenance, which, if not resulting in direct mortality, may lead to the impairment of other life-history components such as behaviour, reproduction or life-span that, again, affect their fitness (Lushchak, 2011; Monaghan et al., 2009). Furthermore, experiences in early larval development result in latent effects that may be first exhibited in juveniles or adults (Pechenik, 2006). Such effects yet cannot be assessed by this study but are likely to determine amphibian health in later life. Amphibians inhabiting any kind of wetlands largely contribute to habitat interconnectivity, thus, potential effects on life-history or reproduction would adversely influence the transfer of an appreciable portion of energy and biomass across ecosystem boundaries (Gibbons et al., 2006).

5. Conclusion

In the light of global climate change, proceeding globalization and the ongoing spreading of tropical mosquito species in Europe, in future, mosquito control will gain in importance with regard to human health. Consequently, the application of products based on Bti will probably rise alike worldwide due to its propagated environmental compatibility in various aquatic habitats. In view of the above, the present work indicates that the decision for such an expanded use should not be taken lightly. This is due to the induced subcellular biochemical alterations in young amphibian larvae after consecutive exposures with Bti. Subsequently, this may adversely affect amphibian health. The mode of action behind these alterations is probably different to the toxic mechanisms involved in insects. Hence integrative approaches that combine several different enzymatic biomarkers and endpoints related to the reproductive potential, need to be incorporated in further research in order to fully understand the extent of effects of Bti on non-target amphibians. Unfortunately, the current environmental risk assessment for Bti strain AM65-52 bases the risk for all non-target aquatic organisms, like basically for all insecticides, on toxicity data of fish and daphnids (European Comission, 2011). In the context of worldwide amphibian declines (Stuart et al., 2004), the implementation of a threshold on application numbers as well as a minimum interval between individual Bti treatments may help to reduce the potential risk for adverse effects on amphibians. Such precautions should be especially considered for wetlands located in designated nature conservation areas further apart from human residential areas. These areas, by definition, focus on the protection of nature and environmental health over human convenience, sometimes even in a legally binding matter. Adopting existing management accordingly would be an important first step towards environmentally safe mosquito control programs.

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Ethical approval

All experimental procedures in our study were evaluated and approved by the Institutional Animal Care and Use Committee at the University Koblenz-Landau and the federal investigation office (Landesuntersuchungsamt – LUA Rhineland-Palatinate, Germany, NTP-ID: 00008349-1-2).

Declaration of interest

The authors declare that they have no competing interests.

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Appendix VI: Scientific publication VI

Mosquito control based on *Bacillus thuringiensis israelensis* (Bti) interrupts artificial wetland food chains

Stefanie Allgeier, Anna Friedrich, Carsten A. Brühl

Authors' contribution:

Conceptualization: SA, AF, CAB; Investigation, Methodology: SA, AF; Formal analysis: SA; Writing – Original draft preparation: SA; Supervision: CAB

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Highlights:

- Chironomidae are the non-target aquatic invertebrates most affected by Bti.
- Development of newt larvae was indirectly affected in Bti-treated food chains.
- The dragonfly *Aeshna cyanea* decreased newt survival by 27% when Bti was applied.
- Larval chironomids are a key component in the diet of aquatic predators.

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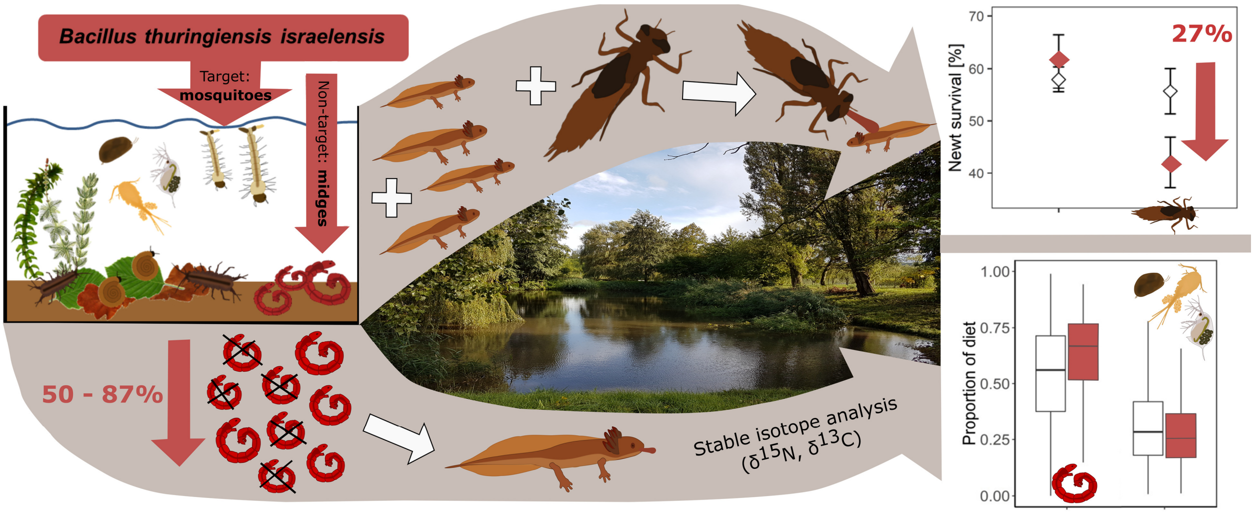
Abstract

The biocide *Bacillus thuringiensis israelensis* (Bti) has become the most commonly used larvicide to control mosquitoes in seasonal wetlands. Although Bti is considered non-toxic to most aquatic organisms, the non-biting chironomids show high susceptibilities towards Bti. As chironomids are a key element in wetland food webs, major declines in their abundance could lead to indirect effects that may be passed through aquatic and terrestrial food chains. We conducted two mesocosm experiments to address this hypothesis by assessing direct and indirect effects of Bti-modified availability of macroinvertebrate and zooplankton food resources on the predatory larvae of palmate and smooth newts (Urodelans*: Lissotriton helveticus, Lissotriton vulgaris*). We examined newt survival rates and dietary composition by means of stable isotope (δ^{15}N and δ^{13}C) analysis in the presence of Bti treatment and a predator (Odonata: *Aeshna cyanea*). We assessed body size at and time to metamorphosis of palmate newts while developing in Bti treated mesocosms. Chironomid larvae were the most severely affected aquatic invertebrates in all Bti treated food chains and experienced abundance reductions by 50 to 87%. Moreover, stable isotope analysis revealed that chironomids were preferred over other invertebrates and comprised the major part in newts' diet (56%) regardless of their availability. The dragonfly *A. cyanea* decreased survival of newt larvae by 27% in Bti treated mesocosms showing affected chironomid abundances. Increasing intraguild predation is most likely favored by the Bti-induced reduction of alternative prey such as chironomid larvae. The decreased food availability after Bti treatment led to slightly smaller *L. helveticus* metamorphs while their developmental time was not affected. Our findings highlight the crucial role of chironomids in the food webs of freshwater ecosystems. We are also emphasizing the importance of reconsidering human-induced indirect effects of mosquito control on valuable wetland ecosystems particularly in the context of worldwide amphibian and insect declines.

Keywords: *Bacillus thuringiensis israelensis*, amphibians, Chironomidae, intraguild predation, stable isotope analysis, food chain

Graphical abstract



1. Introduction

The biocidal usage of the bacterium *Bacillus thuringiensis israelensis* (Bti) is considered one of the most environmental friendly methods to control mosquito populations emerging from stagnant water (Lawler, 2017). The treated water bodies range from temporary flooded wetlands to small ditches that are inhabited by various freshwater organisms (Becker et al., 2010). In principle, Bti is believed to be relatively safe for aquatic non-target organisms such as freshwater molluscs, crustacean, vertebrates and most insects due to its' specific toxic mode of action (Boisvert and Boisvert, 2000; Lacey and Merritt, 2003; Lagadic et al., 2016, 2014). The larvicidal activity of Bti is based on δ-endotoxins produced during sporulation (Ben-Dov, 2014). After ingestion, they bind to specific receptor sites in the midgut epithelium of the targeted species, mosquitoes (Culicidae) and black flies (Simuliidae), which leads to perforation and subsequently to a rapid death of their larvae (Becker and Margalit, 1993; Ben-Dov, 2014).

Next to mosquitoes and black flies, non-biting midges of the family Chironomidae also show a high susceptibility towards Bti (Kästel et al., 2017; Stevens et al., 2005). Therefore, Bti is even applied against chironomids to reduce nuisance associated with their ability to form huge swarms upon emergence (Ali et al., 2008; Vaughan et al., 2008). However, chironomids are usually non-target organisms with regard to Bti applications in wetland mosquito control. Nonetheless, several field studies showed that Bti reduced abundances of chironomid larvae and adults by 50% and more in wetlands that were subject to regular mosquito control activities (Allgeier et al., 2019; Hershey et al., 1998; Jakob and Poulin, 2016) while other non-target invertebrates were not affected (Duchet et al., 2008; Lagadic et al., 2016, 2014). In contrast, other studies did not find negative effects of Bti on chironomids (Duchet et al., 2015; Lagadic et al., 2016; Lundström et al., 2010b; Wolfram et al., 2018) which is why the susceptibility of chironomids in the field is still a matter of debate in the current literature.

Nevertheless, a Bti-induced reduction in chironomid availability is of great concern since chironomids form a major component of the macroinvertebrates in most ecosystems as they are characterized by high abundances and species richness (Armitage et al., 1995). Besides their ubiquitous occurrence, their high protein content and digestibility makes them a quality food resource for aquatic and terrestrial organisms such as predatory insects, fish, amphibians, bats and birds (Armitage et al., 1995; Arnold et al., 2000; De La Noüe and Choubert, 1985; Jakob and Poulin, 2016). Some field studies on routine Bti treatments linked reduced availability of chironomid adults to lower breeding success in birds (Poulin et al. 2010; Poulin 2012) or declining numbers and richness of adult dragonflies (Jakob and Poulin, 2016). In contrast, other studies did not find any indirect effects on birds that are related to Bti applications (Hanowski et al., 1997; Niemi et al., 1999; Timmermann and Becker, 2017).

Compared to insectivorous birds which are relatively mobile terrestrial predators, aquatic predators cannot escape from Bti treated ponds where they are exposed to Bti and have to subsist on available prey. The predatory performance of backswimmers on the mosquito *Aedes aegypti* was enhanced after Bti exposure (Gutiérrez et al., 2017) whereas Bti-based control of floodwater mosquitoes increased the abundance of medium-sized diving beetles (Vinnersten et al., 2009). However, there have been no studies on aquatic predators investigating indirect effects of Bti-induced changes in wetland food resources aside from mosquitoes.

Seasonal ponds and flood areas of small creek offer optimal breeding grounds for mosquito larvae but also amphibians by providing an aquatic environment with standing water free of predatory fish (Batzer and Wissinger, 1996; Rubbo et al., 2011). Larval development of most European amphibian species coincides with recurrent mosquito control activities in spring and summer months (Allgeier et al., 2019; Becker et al., 2010; Günther, 1996). In these ecosystems, carnivorous newts (Urodelans) are among the top-predators and can have a major impact on freshwater invertebrate communities as they are opportunistic feeders that select their prey on availability and size (Mettouris and Giokas, 2017). The quality and quantity of food accessible during their larval stage has implications for time to emergence and body size at metamorphosis. Decreased food levels lead to smaller anuran metamorphs (Leips and Travis, 1994) whereas the length of the larval period largely depends on growth rates (Newman, 1992; Tejedo and Reques, 1994). Body size at metamorphosis is closely associated with individual fitness further affecting fecundity, reproductive success or survival as adults (Leips and Travis, 1994; Semlitsch et al., 1988).

Although Bti is supposed to have no direct lethal effect on amphibians, field application rates can evoke stress in amphibian larvae that is manifested in alterations of oxidative stress enzymes and changes in swimming behavior (Allgeier et al., 2018; Junges et al., 2017; Lajmanovich et al., 2015). Other than human-induced contaminants, amphibian larvae face multiple biotic and abiotic stressors in wetland ecosystems such as pond drying, varying temperatures, food limitation and the presence of natural predators (Wilbur, 1997). The combination of several sublethal stressors can have adverse interactive effects on amphibians (Boone et al., 2007; Rohr et al., 2004). For example, pesticides can become more lethal to amphibians in the presence of stress induced by predator cues (Relyea, 2003; Relyea and Mills, 2001). Conversely, predator-prey interactions may be affected by pesticide-induced changes in antipredator traits (Janssens and Stoks, 2012; Relyea and Edwards, 2010).

Amphibians are engaged in predator-prey interactions with dragonfly nymphs that prey on anurans and newts (Blois, 1985; Van Buskirk and Schmidt, 2000). Furthermore, predatory newts and dragonfly nymphs are also competitors for shared food resources. This mixture between competition and predation of species with comparable trophic niches is defined as intraguild predation (Holt and Polis, 1997). However, the coexistence of different species in the same trophic guild can be facilitated by high abundances of alternative prey, like chironomids, being present in wetland ecosystems (Preston et al., 2017).

This study addressed direct and indirect effects of Bti-based mosquito control on experimental mesocosm food webs with focus on larval newts (genus: *Lissotriton*). Due to the essential role of chironomids in wetland food webs (Leeper and Taylor, 1998; Williams, 2006) and their controversially discussed vulnerability in higher tier studies, we focused this research on chironomids. Our aim was to examine how Bti treatments affect resource use and development of newt larvae and the trophic dynamics in a more complex trophic food web including a predator, the dragonfly nymph *Aeshna cyanea.* We used stable carbon and nitrogen isotopes (δ^{15}N and δ^{13}C) analysis to examine dietary composition and isotopic niche widths of the predators. The latter provides quantitative information on the isotopic niche space occupied by newts and dragonflies and thus insights to their adapted ecological niches and trophic diversity (Newsome et al., 2007). First, we hypothesized that (i) the dietary composition of newts feeding in Bti-treated aquatic communities would change towards the consumption of less chironomid larvae as a result of their reduced availability. On this basis, we assumed that (ii) a reduced availability of nutritional chironomids would affect the development of individual newt larvae leading to smaller metamorphs in terms of body condition. Moreover, we expected that the Bti-induced modification of basic food resources (iii) increased intraguild predation between dragonfly nymphs and newts due to reduced availability of alternative prey.

2. Materials and methods

2.1 Newt sampling and breeding

To obtain larval newts, we collected fertilized *Lissotriton helveticus* and *Lissotriton vulgaris* females in a pristine pond in the Bienwald forest, Rhineland-Palatinate, Germany (49°01’ N, 8°10’ E) and brought them to the laboratory for oviposition. Two females at a time were placed in 30 L glass tanks (50 × 30 × 20 cm) containing filtered tap water (0.2 µm Supor, Pall Corporation, Port Washington) and blades of couch grass (*Elymus sp.)* for egg deposition. Newt females were fed *ad libitum* with larvae of mosquitoes and chironomids obtained from forest ponds. Grass blades were checked for eggs every other day and replaced if necessary, along with the renewal of water. Eggs were placed into aerated plastic aquaria (7 × 16 × 22 cm) containing pond water. After hatching, larvae were fed with small *Artemia* and *Daphnia* from in-house cultures. Female newts were released back in the pond of origin after 21 days if enough eggs had been obtained for the experiments. All experimental procedures were evaluated and approved by the Institutional Animal Care and Use Committee at the University Koblenz-Landau and performed in accordance with Directive 2010/63/EU.

2.2 Study system

The present study comprised two mesocosm experiments conducted at the Landau Stream Mesocosm Facility at the University of Koblenz-Landau (Germany). The experiments were implemented in two subsequent years and followed the same study system, set-up, inoculation with invertebrates and sampling procedure as explained in more detail in Allgeier et al. (2019).

We established 48 (Experiment 1) and 16 (Experiment 2) aquatic model ecosystems in artificial ponds (70 × 40 × 30 cm, 90 L, polyethylene mortar buckets, JOPA, Ahlen, Germany). Mesocosms were placed into the water flow-through stream channels and covered by a shading net (30 % reduction) (Fig. S.1). All mesocosms were filled with 40 L tap water, inoculated with 30 L pond water and supplied with quartz sand, a black alder/oak leaf mixture typically found in swamp forests and two aquatic plants (*Elodea canadensis, Myrophyllum spicatum)*. To establish suitable prey for predators, we obtained aquatic invertebrates from local wetlands (*Asellus aquaticus*, snails, zooplankton, chironomids, mosquitoes) and in-house cultures (*Culex pipiens*, *Chironomus riparius*, *Daphnia magna*). Aquatic invertebrates were added to each mesocosm continuously in the same abundance or volume starting six weeks before the experimental phases in order to obtain standardized invertebrate communities. In total, 14 snails (Physidae, Planorbidae, Lymnidae) and 24 *Asellus aquaticus* were initially added to each mesocosm. A zooplankton mixture (Cladocera, Copepoda, Ostracoda) obtained from local wetlands (60 µm net) was homogenized with *Daphnia magna* and allocated into 48 (Experiment 1) and 16 (Experiment 2) equal volumes prior to their addition to the mesocosms. Chironomid larvae (pet-shop, 90 mL; in-house culture) and eggs (in-house culture; local wetlands) were portioned into aliquots of the same volume and added to the mesocosms once a week to ensure a diverse age structure and species mixture. Mosquitoes were treated equally to chironomids and added three and one week prior to the experiment start.

Each mesocosm was covered with an emergence trap (70 × 40 × 85 cm) to prevent colonization by external organisms and to catch emerging insects in collecting bottles positioned on top of the traps. Collecting bottles were filled with glycol and a detergent and activated once a week for a period of three consecutive days. Emerged insects were preserved in 70% ethanol and determined to order level and the order Diptera to family level using a Leica M80 binocular with a 10× magnification. Insect emergence within the entire sampling period expressed the number of successfully emerged insects that were not used as prey during their larval stage. Additionally, sampling emergence enables the detection of effects on 1st or 2nd instar chironomids which are difficult to count in natural samples due to their small sizes.

Aquatic invertebrates were sampled once prior to the Bti application and otherwise once a week during the course of the experiments using a tube sampler (Fig. S2, method described in (Allgeier et al., 2019). After gently mixing the mesocosm water, the tube (Ø 12 cm) was lowered quickly through the water column into the sand of each mesocosm and closed with a Perspex lid. The invertebrates contained in the water/sediment sample of the tube (volume:

1.5 L) were immediately identified and counted on site to class (Ostracoda, Hirudinea), order (Copepoda, Cladocera), genus (*Hydra, Tubifex*), family (Physidae, Planorbidae, Chironomidae, Culicidae, Chaoboridae) or species (*Asellus aquaticus*). Samples were poured back into the respective mesocosm after determination.

2.3 Experiment 1: Community experiment

2.3.1 Experimental design

The first experiment examined the effects of a regular Bti application and predation on the dietary intake and survival of a natural newt larvae assemblage comprising *L. helveticus* and *L. vulgaris*. Both species are common in Central Europe and among the smallest newts in seasonal wetlands. They breed in early spring and their aquatic larval period coincides spatially and temporally with the application of Bti for mosquito control (Allgeier et al., 2019; Günther, 1996). Both species further share a similar feeding behavior and have a high food niche overlap which is why they are considered as a single group within food analyses (Griffiths, 1986). We used a randomized 3 × 2 factorial design manipulating two factors in the aquatic communities to assess the impacts on the food chain: three levels of predators [*no predators (predation control (PC))/ newt larvae (N)/ newts and dragonfly larvae (ND)*] and two levels of Bti treatment [*control/ Bti treated*]. *Aeshna cyanea* (Odonata: Anisoptera) nymphs were inserted as a generalist predator that is efficient in consuming a wide range of prey in the wild including zooplankton, amphipods and amphibian larvae (Blois, 1985; Van Buskirk and Schmidt, 2000). All treatment combinations were replicated eight times and randomly assigned to 48 previously established pond mesocosms. Results on invertebrates in PC were recently published in Allgeier et al. (2019) and are used again here for the assessment of the impact of predators on the aquatic communities in N and ND.

The community experiment lasted for nine weeks from May 8 to July 12, 2015. On May 10, we applied 34.8 mg VectoBac®WG granules (3000 International Toxic Units (ITU)/mg, Valent BioSciences Corporation, Illinois, USA) directly onto the water surface (0.29 m²) of the treated mesocosms. The application rate equaled a high field rate (2.88×10^9 ITU/ha) which is regularly applied in the German mosquito control program at water levels >10 cm and when mosquito larvae are larger than 2nd larval stage (Allgeier et al., 2019; Becker, 2003). We performed six samplings of aquatic invertebrates at -2, 2, 9, 18, 32, and 47 days after application (DAA) and emergence at DAA 0, 5, 12, 18, 32, 45. Dissolved oxygen, pH and water temperature were measured mid-morning of each sampling day in a randomized design using a WTW Multi 340i (WTW GmbH, Weilheim, Germany) (Appendix).

Late-instar *A. cyanea* nymphs were collected by dip-netting from a Bti-untreated pond near Landau (49°15’N, 7°57’E). One nymph (mass: 568.75 mg ± 47.6, length: 36.69 mm ± 1.4) was randomly added to each ND mesocosm on May 6. Initially, six newt larvae (length: 10 mm ± 1) were inserted in N and ND mesocosms on DAA 3. We added another five newt larvae (length: 15 mm ± 1) into each mesocosm three weeks later because of high mortality rates at the beginning of the experiment. As a reaction to progressing hatching rates of dragonfly nymphs we measured body length (ruler) and mass (Mettler PM6000, Columbus, USA) on site at DAA33 (38 days after addition). Newt assessment was performed twice per week as soon as the first newt larvae showed signs of metamorphosis such as the resorption of the external gills and the tail. Shortly before metamorphosis, individual newts were transferred into sealed aquaria to finish metamorphosis without escaping the mesocosm tanks. We recorded newt survival as the proportion of newts that finished metamorphosis in each mesocosm and kept 54 individuals for stable isotope analyses.

2.3.2 Stable isotope analysis

Sampling of aquatic food web components took place between July 9 and July 12 after the last newts left the mesocosms because tanks needed to be emptied entirely to reach a sufficient sample amount of resources (Cladocera, Copepoda, Ostracoda, Chironomidae, *Asselus aquaticus*, Planorbidae, Physidae, Lymnaeidae). For the evaluation of newts’ dietary composition we selected newt individuals of N-control and N-Bti that finished metamorphosis between July 8 and July 20 (n = 19). Moreover, we analyzed the trophic niche spaces occupied by dragonflies and newts that finished metamorphosis between June 29 and July 5 when metamorphosis rates peaked in all treatment combinations (n(dragonfly) = 13, n(newts) = 35).

Newt metamorphs and dragonflies were euthanized by immersion in 0.1% MS-222 and individually stored at -20°C until processing for stable isotope analyses. MS-222 is commonly used for euthanizing amphibians and no effects on stable isotope measurements have been reported (Caut et al., 2013). We dissected tail muscle samples from newt metamorphs and thoracic muscle tissue from dragonflies for bulk stable isotope analyses. All samples were kept separate in 1.5 mL safe-lock tubes (Eppendorf, Hamburg, Germany), oven-dried at 60°C for at least 48 h and grounded to a fine and homogenous powder using stirring spatulas (120 mm, 30 × 3 mm, Ø 3 mm, PS, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). 0.4 to 1 mg of ground sample was weighed into tin capsules (8 × 5 mm, ThermoFisher Scientific) and stored at 60°C until analysis of carbon and nitrogen isotopic values ($\delta^{13}C$ and $\delta^{15}N$). Bulk stable isotope analysis was performed at the Landau Stable Isotope Facility using a flow ratio mass spectrometer (Delta V Advantage) interfaced to a high-performance Flash 2000 HT elemental analyzer via a ConFlo IV (Thermo Finnigan, Bremen, Germany). Stable isotope ratios of carbon and nitrogen are expressed in the standard δ-notation, which is defined as the difference between the stable isotope value of the sample and the international reference standards relative to Vienna Pee Dee Belemnite and atmospheric N2 in per mille (‰). The accuracy of repeated analysis of the international standard Casein was ≤ 0.057‰ and 0.097‰ for carbon and nitrogen, respectively.

2.4 Experiment 2: Newt development experiment

The second experiment examined the individual development of *L. helveticus* larvae under regular Bti treatments by assessing time to metamorphosis (TTM) and body size at metamorphosis. Therefore, we established eight controls and eight Bti treatments following the general study system and set-up. The experimental phase lasted for nine weeks between May 25 and July 22, 2016. Mesocosms were treated with Bti twice (May 26 and June 8, 2016) using ice pellets (IcyPearls) from the stock material of the German Mosquito Control Association. IcyPearls were manufactured with a VectoBac®WG suspension that is converted to 4 mm grain sized granules with the help of liquid nitrogen (Becker, 2003). Again, we applied the high field rate (2.88×10^9 ITU/ha) which equals the application of 870 mg IcyPearls on the water surface of each mesocosm. At DAA11, two days prior to the second Bti application, we added additional mosquito larvae to record the efficiency of the second Bti treatment. We sampled aquatic invertebrates at DAA -1, 6, 13, 21, 26, 35 and 40 and emergence at DAA 6, 13, 19, 26, 33 and 42. Temperature and pH were measured three times while dissolved oxygen and conductivity were measured two times during the sampling period (Appendix).

One larval *L. helveticus* individual (11.44 ± 1 mm) was added to each mesocosm the day after the initial Bti application. To prevent newt metamorphs from escaping the mesocosms, we transferred the nearly finished metamorphs into closed plastic aquaria for a maximum of four days until they finally finished metamorphosis. Time to metamorphosis displays the time between experiment initiation and date of complete metamorphosis. Body length and snout to vent length (SVL) were evaluated at the beginning of the experiment and at the end of metamorphosis using the software AxioVision® (Carl Zeiss; Oberkochen, Germany) on a digital photograph of the individuals. Associated body mass was recorded by weighing the dabbed dry metamorphs (Mettler Toledo XA105 DualRange; Giessen, Germany). Metamorphs were released next to their pond of origin after final body size was assessed.

2.5 Data analysis

All analyses were performed using the statistical software R, version 3.5.1 (R Core Team, 2014). Significant differences were denoted by asterisk in graphical representations of the data.

2.5.1 Food web composition

Differences in taxa abundances due to a certain treatment referred to cumulative reductions over the entire sampling period. Taxa contributing >0.1% to the overall invertebrate abundances were included in multivariate analyses on invertebrate communities. We performed a permutational multivariate analysis of variance (PERMANOVA) to reveal the influence of time, Bti, predator and their interactions on invertebrate communities in 999 permutations. Pairwise comparisons (post-hoc) were performed using Wilks test statistic, 999 permutations and Bonferroni corrections (function: pairwise.perm.manova). Furthermore, Bti effects on the invertebrate composition in the newt development experiment were analyzed using the principal response curve (PRC) method (Van den Brink and Braak, 1999). We ln(2x+1) transformed the abundance data beforehand to avoid false discrepancies between zero abundances and low abundances (Van den Brink et al., 2000). Differences at single sampling dates were assessed by redundancy analysis (RDA) followed by Monte Carlo permutation tests.

A generalized linear mixed-effect model (GLMM) with negative binomial errors was implemented to assess whether the treatments influenced taxa abundances. We specified taxa abundance as response variables and included DAA and Bti, predator and Bti*predator (community experiment) or DAA and Bti (development experiment) as predictor variables. Mesocosms were included as random effect to account for repeated measures. The interaction term was removed when non-significant. Differences at each sampling day were analyzed using Student’s t-test with Benjamini-Hochberg correction (Benjamini and Hochberg, 1995). We applied Kruskal-Wallis test when data were not normally distributed (tested with Shapiro-Wilk test) and Welch’s t-test with unequal variances (tested with Levene’s test). Differences in abiotic parameters were tested with a generalized linear effect model using a poisson distribution and displayed in supplementary material A. We used the R packages "vegan" (Oksanen et al., 2018) and "glmmADMB" (Skaug et al., 2013) for the analyses.

2.5.2 Predator performances

We estimated chironomid prey intake by predators as difference between the amount of emerged chironomids in the predator-free controls and both predator scenarios during the entire sampling period. To assess individuals fitness we calculated a body condition index (BCI) for newts and dragonfly nymphs according to the scaled mass index described by Peig and Green (2009). For the index, ln-transformed data of individual mass and length measurements were used to perform a standardized major axis (SMA) regression. Furthermore, the slope was used to adjust the body composition of each individual to the mean length of all newt or dragonfly individuals. Newt survival was analyzed with a two-way analysis of variance (ANOVA with Type III SS without sequentially fitting of terms) of fitted linear mixed effect models with treatment, predator and their interaction as fixed and mesocosm as random effect. Residuals were tested for normality using a Shapiro-Wilk's test. We calculated the mean increases in mass, length and the respective BCI of dragonfly nymphs and analyzed differences in body parameters using a Student's t-test. Whether Bti treatment had an effect on individual newt development (mass, SVL, length, length increase, BCI) was assessed by an ANCOVA in which TTM was included as covariate to account for possible time-dependent effects. Influences on TTM were analyzed using a two-way ANOVA. The analysis of mass related parameters is based on n(Bti) = 7 and n(control) = 8 newt individuals since one body mass value was noted incorrectly.

2.5.3 Stable isotope analysis

Differences of raw $\delta^{13}C$ and $\delta^{15}N$ values of analyzed organisms in control and Bti treatment were evaluated using Student's t-test. Effects of treatment or predator on $\delta^{13}C$ and $\delta^{15}N$ values of newts at the metamorphosis peak were analyzed with a two-way ANOVA. We used "MixSIAR", a Bayesian framework for conducting stable isotope analysis in R (Stock et al., 2018), to estimate newts' relative consumption of prey. We applied trophic discrimination factors (TEFs) specified for the analysis of muscle tissue without any treatment for lipid removal as recommended by McCutchan et al. (2003) (1.1 ± 0.35 for $\delta^{13}C$; 2.8 ± 0.4 for $\delta^{15}N$). To reduce the number of sources in the model, we summarized isotopic ratios of Cladocera, Copepods and Ostracods as "zooplankton" and Planorbidae, Physidae and Lymnidae as "snails" a posteriori resulting in four systematically distinguished prey types: chironomids, zooplankton, snails and *Asellus aquaticus*. We used an informative prior for the mixing model based on abundance data to account for the Bti effect on prey abundances. Cumulative abundance data for control and Bti treatment were scaled so that the sum of α for each prey type is 4 which is the number of sources (Table S1). The mixing model was fitted on three Markov chain Monte Carlo (MCMC) chains and convergence was assessed with the Gelman-Rubin diagnostic (Gelman et al., 2013).

We explored their isotopic niche widths to analyze dietary similarity and potential niche shifts between predators in control and Bti mesocosms. Although the isotopic niche of a consumer is derived from abiotic and biotic processes within a biological system, it highly correlates with the trophic niche when it is primarily driven by consumer-resource interactions (Bearhop et al., 2004). For its calculation, we used Bayesian methods for populations with unequal and small sample sizes included in the R package 'SIBER' (Jackson et al., 2011). The analysis generates a standard ellipse area (SEA) of the isotopic niches which is a measure of the mean core population isotopic niche not affected by sample size. Moreover, we calculated a corrected SEA_C value (minimizes bias due to small sample sizes) and a Bayesian SEA_B value to compare niche width statistically (based on 10000 posterior draws). Differences in SEA_C were estimated via Bayesian inference according to Jackson et al. (2011). Dietary niche overlap is given as a proportion of the non-overlapping area of two isotopic niches.

3. Results

3.1 Experiment 1: Community experiment

3.1.1 Food web composition

Invertebrate community structure differed significantly as a result of proceeding time, the Bti treatment and the presence of predators (PERMANOVA, Table 1). Both predator treatments influenced the invertebrate community composition when compared to PC (pairwise PERMANOVA, N: $P = 0.003$, ND: $P = 0.03$). However, there were no differences in the invertebrate composition between N and ND (pairwise PERMANOVA).

Table 1: Results of PERMANOVA of invertebrate community compositions in relation to sampling time Bti treatment and predator groups. *P* values in bold indicate statistical significance.

Factors	**F**	**r^2**	***P***
Main effects			
Time	91.87	0.261	**0.001**
Bti	18.95	0.054	**0.001**
Predators	2.77	0.015	**0.008**
Interactions			
Bti × Time	2.06	0.006	0.081
Predators × Time	0.63	0.004	0.764
Bti × Predators	1.36	0.008	0.200

Total invertebrate density decreased as a result of Bti treatment (GLMM: $z = -5.73$, $P < 0.001$) whereas differences were larger in the presence of predators (GLMM, N: $z = -2.88$, $P = 0.004$; ND: $z = -1.69$, $P = 0.09$; Fig. B.1). Available invertebrate prey occurred in varying abundances (Fig. 1). Chironomid larvae and small-bodied zooplankton (Cladocera, Ostracoda, Copepoda) were highly abundant in all treatments. Chironomid larvae were evenly spread over the predator treatments, however, Bti reduced their abundances by 53 to 64% (GLMM: $z = -10.47$, $P < 0.001$). Within the zooplankton, relatively less Cladocera and Copepoda occurred with Bti (GLMM: Cladocera: $z = -1.89$, $P = 0.059$, Copepoda: $z = -3.38$, $P < 0.001$) while the presence of newts reduced the abundances of Cladocera and Ostracoda in N (GLMM: Cladocera: $z = -3.09$, $P = 0.002$; Ostracoda: $z = -2.54$, $P = 0.01$; Fig. 1). Other less abundant organisms like snails, annelids and other dipteran larvae appeared too infrequently to evaluate a pattern (mean abundance <4). The most abundant organisms were included in the subsequent analysis of stable isotopes except Hydra which was no longer found in the final sampling.

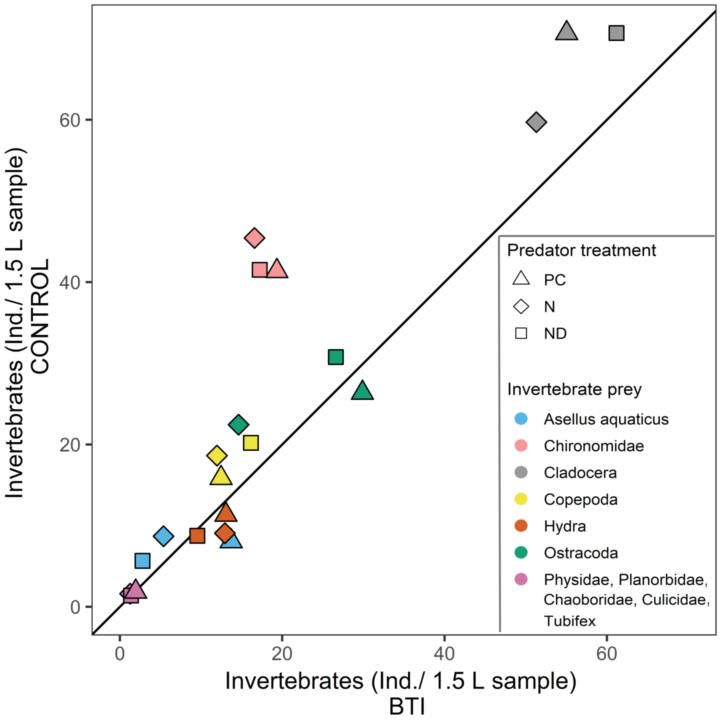


Figure 1: Mean abundances of aquatic invertebrate prey (Individuals in 1.5L sample) over all sampling days (N = 5) in Bti relative to control treatments. Abundances are displayed in predator treatments (predation control: PC, newt larvae: N, newts + dragonfly larvae: ND). Taxa with mean abundances <4 are summarized in one group (purple). Taxa above the solid line indicate that taxa are more abundant under control conditions while taxa under the line are more abundant in Bti treatments.

3.1.2 Predator performance

The presence of the different predators influenced the overall number of emerging chironomids (GLMM: N: *z* = -1.92, *P* = 0.055; ND: *z* = -2.81, *P* = 0.005; Fig. 2) that comprised the majority (>90%) of emerging insects. Predators reduced the number of chironomid adults by 22% in N-control (mean: 34.90 ± 6.4) and 41% in ND-control (mean: 26.38 ± 3.0) when compared to PC-control (mean: 44.88 ± 6.1) (Fig. 2; Table S2). The presence of predators in Bti treated mesocosms equally reduced chironomid emergence by 34% in N-Bti (mean: 15.60 ± 2.3) and ND-Bti (mean: 15.4) (Fig. 2, Table S2). In Bti treated mesoscoms, newts consumed 22% less chironomids than in the control while 57% less chironomids were consumed by newts and the dragonfly nymph. Chironomid adults exhibited Bti-induced abundance reductions by 43 to 56% (GLMM: *z* = -5.05, *P* < 0.001) comparably to the reductions in their larval state. Mosquitoes emergence (<2% of all emerged insects) were reduced by 45% due to Bti (GLMM: *z* = -2.46, *P* = 0.014) but were not influenced by predators.

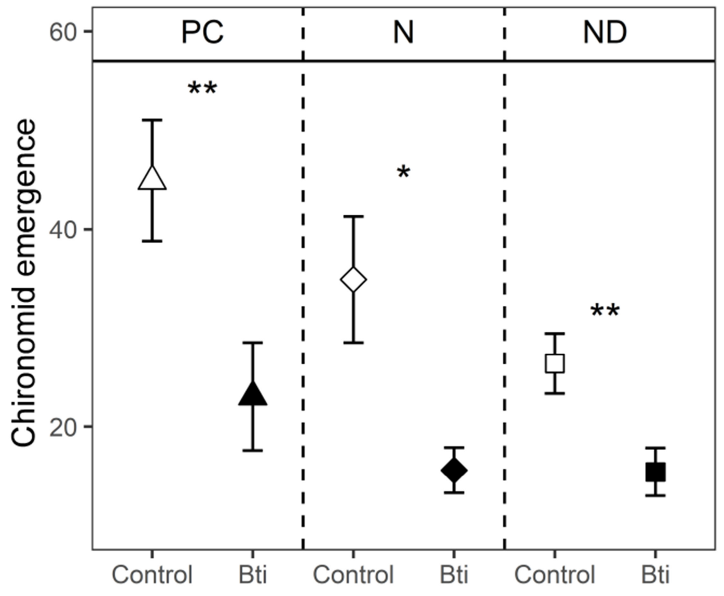


Figure 2: Mean chironomid emergence rates (±SE) over the entire sampling period (7 weeks) in the different predator groups (predation control: PC, newt larvae: N, newts + dragonfly larvae: ND) and control (white) and Bti (black) treatment (n = 8 mesocosms for each treatment).

Proportional newt survival was affected by *A. cyanea* ($X^2 = 10.09$, $P = 0.001$) but not by the Bti treatment (Fig. 3). However, Bti caused a significant decrease in survival rates by 27% when applied in mesocosms with dragonfly predator ($X^2 = 3.93$, $P = 0.047$; Fig. 3).

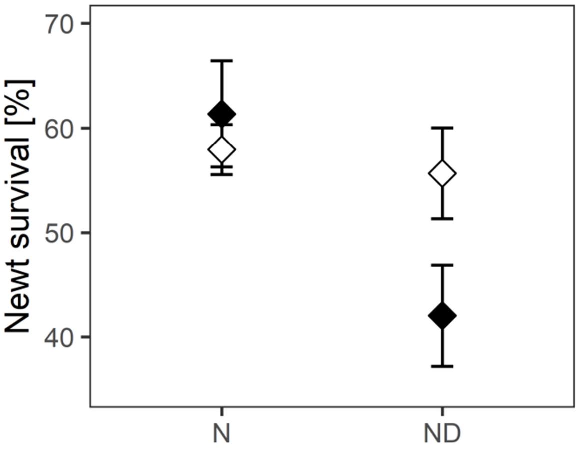


Figure 3: Mean proportion (±SE) of surviving newts (*L. helveticus, L. vulgaris*) in the absence (N) and presence (ND) of one *A. cyanea* nymph under control (white) and Bti treatment (black) (initially introduced newt larvae: n = 11).

Dragonfly body length, mass and the respective increases did not significantly differ between Bti and control at DAA33 (Fig. 4). However, whereas dragonflies had an equal initial BCI in both treatments at the beginning of the experiment (mean: 599.12 ± 36.2; *t* = 1.448, *P* = 0.17), nymphs in the Bti treatment showed a significantly lower BCI after 38 days (Fig. 4C).

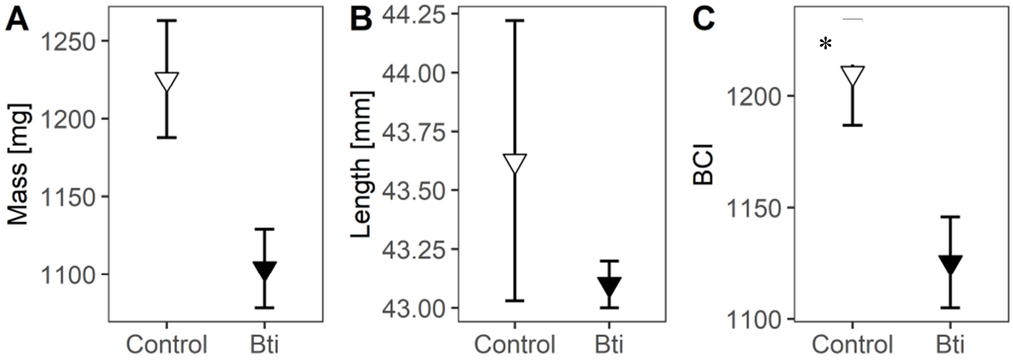


Figure 4: Mean (±SE) (A) body mass, (B) length and (C) body condition index (BCI) of *A. cyanea* nymphs in control (n=8, white) and Bti treatment (n=5, black) at DAA33.

3.1.3 Stable isotope analysis

Newts' δ^{15}N values were enriched relative to their prey (Table 2). No significant shifts in stable isotope values of either predator or prey occurred between control and Bti, except for slightly higher δ^{13}C in chironomids in the Bti treatment (Table 2, dual isotope spaces: Fig. S3). Isotopic values of newts at the metamorphosis peak did not differ significantly as a result of Bti, predator presence or their interaction (two-way ANOVA, $P > 0.2$). The dragonfly nymph showed highest δ^{13}C values of all taxa and 1.4‰ lower δ^{15}N values than newts (Table 2) which is why we could not perform a stable isotope mixing model to assess dragonflies' dietary composition. Thus, the mixing model analyses were focused on the dietary composition of newts.

Table 2: Mean ± SD δ^{13}C and δ^{15}N values from bulk stable isotope analysis of predators and prey in control and Bti treated mesocosms (uncorrected for isotopic discrimination) used in the analyses of dietary composition of *Lissotriton* newts (N) and for isotopic niche widths of newts and *A. cyanea* at the metamorphosis peak (N/ ND). n= number of individuals (*Lissotriton*, *A. cyanea*) or prey samples analysed. Values written in bold indicate statistically significant difference from control.

	Control			**Bti**		
	n	δ^{13}C ± SD (‰)	δ^{15}N ± SD (‰)	n	δ^{13}C ± SD (‰)	δ^{15}N ± SD (‰)
Dietary composition (N)						
Lissotriton	10	-24.21 ± 0.38	5.73 ± 0.47	9	-24.32 ± 0.36	5.73 ± 0.26
Chironomids	9	-25.17 ± 0.51	3.01 ± 0.9	9	**-25.84 ± 0.46**	2.71 ± 0.64
Asselus aquaticus	5	-24.00 ± 0.57	2.04 ± 0.73	5	-24.32 ± 0.38	1.69 ± 0.27
Snails	15	-26.03 ± 1.92	1.94 ± 0.75	13	-26.71 ± 2.15	2.18 ± 0.79
Zooplankton	15	-23.91 ± 3.29	3.37 ± 1.34	15	-23.14 ± 3.68	3.04 ± 1.38
Isotopic niche width (N / N						
Lissotriton (N)	8	-24.78 ± 0.49	5.76 ± 0.27	10	-24.19 ± 0.83	6.00 ± 0.38
Lissotriton (ND)	10	-24.39 ± 0.90	5.86 ± 0.43	7	-24.32 ± 0.71	5.97 ± 0.54
A. cyanea (ND)	7	-29.11 ± 0.43	4.39 ± 0.26	6	-28.96 ± 1.29	4.49 ± 0.43

The diet of newts was dominated by a combination of mostly chironomids (>56%) and zooplankton (>25%, Fig. 5) according to the mixing model estimates including the abundance priors. Moreover, chironomids comprised a similar proportion of newts' diet in control and Bti treatment despite their different abundances.

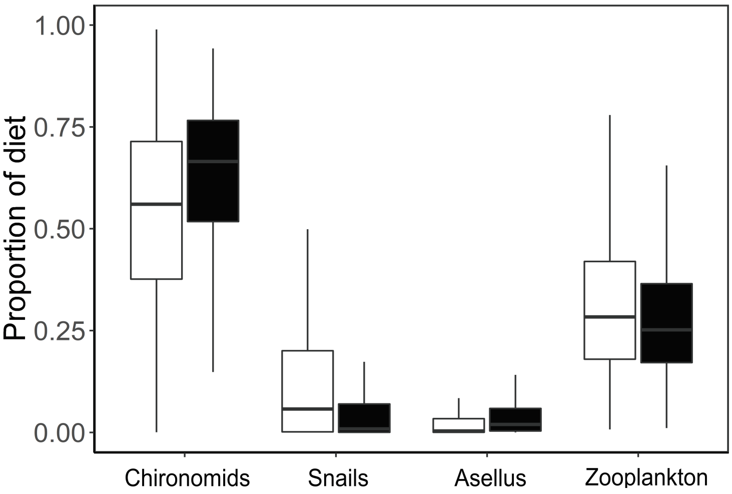


Figure 5: Proportional contributions (lines = median, box = 50% CI, error bars = 95% CI) of each prey item to the diet of *Lissotriton* newts calculated with the abundance data (abundance prior) in control (white) and Bti (black) mesocosms.

Isotopic niche widths reflect population food resource diversity and varied in-between treatments indicated by increasing SEA_B and SEA_C values (Fig. 6). Newts without any treatment (N-control) had the smallest isotopic niche width (SEA_C, Fig. 6A). Treatment with Bti resulted in 1.3 (N) and 1.7 times (ND) enhanced niche widths (SEA_C) of newts compared to their respective controls. Accordingly, the presence of *A. cyanea* increased newt niche widths by 2.3 times compared between controls and 1.7 times when comparing Bti treatments (Fig. 6A). Lowest niche overlap (33%) was found between newts in N-control and ND-Bti whereas highest niche overlap (74%) occurred in newts of N-Bti and ND-control (Fig. S4). Isotopic niche widths of *A. cyanea* individuals were five times wider when feeding in Bti treated mesocosms than in Bti-free controls (Fig. 6B). Moreover, dragonflies in Bti and control conditions showed just a small niche overlap of 20% (Fig. S5).

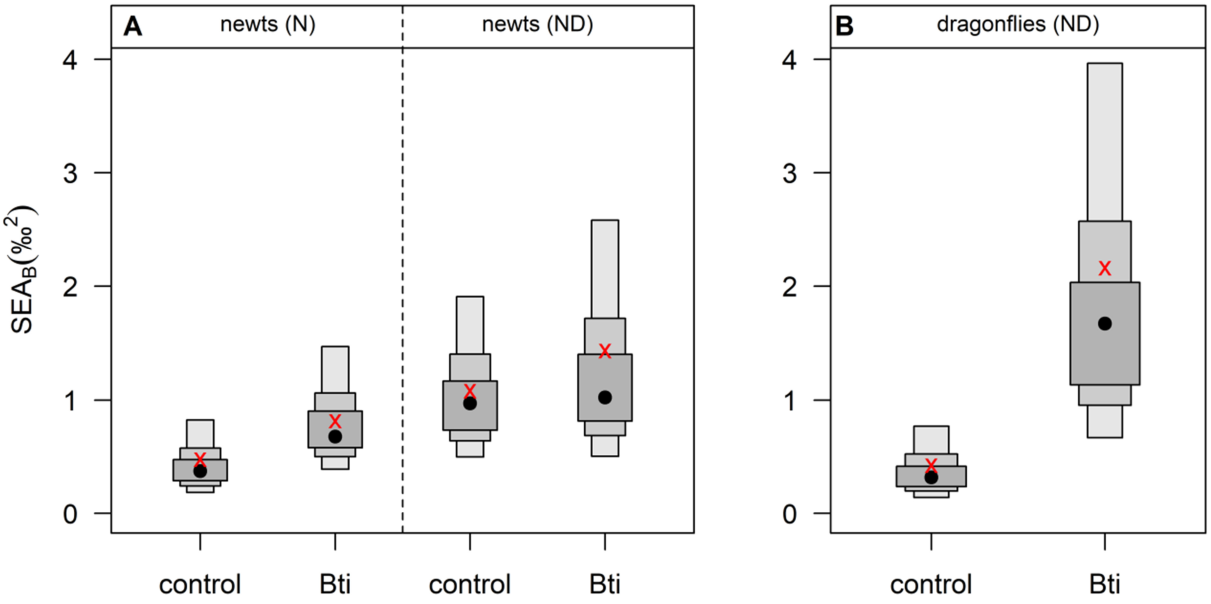


Figure 6: Density plots of standard ellipse area (SEA) displaying the isotopic niche width for (A) newts and (B) *A. cyanea* in predator (N/ ND) and Bti treatments (control/Bti). Boxed areas represent SEA_B value (black dot) with Bayesian 50, 70 and 95% credible intervals (grey bars). The red cross indicates the sample size corrected SEA_C value.

3.2 Experiment 2: Newt development experiment

3.2.1 Food web composition

The aquatic invertebrate communities were dominated by zooplankton (Cladocera: 71% of individuals, Ostracoda: 5%, Copepoda: 5%) and midge larvae (Chironomidae: 9%, Culicidae: 4%). Mean invertebrate densities were higher than in the community experiment (control: 230.25 ± 10; Bti: 206.61 ± 9.9) but also reduced by the Bti treatment (GLMM: $z = -1.96$, $P =$

0.05). Control and Bti treated mesocosms differed in their invertebrate community (Bti: F = 25.00, *P* = 0.001) according to the PRC analysis (Fig. S6). Differences were statistically significant at all sampling dates except the initial sampling prior to the first Bti treatment (DAA -1). Monte Carlo permutation tests indicated that 76% of the total variance was captured by the first PRC axis. Overall, 41% of the variance could be attributed to time whereas Bti treatments explained 16%. Analysis of taxa weights (b_k) showed that chironomids were the most affected taxa ($b_k = 3.28$) followed by mosquitoes ($b_k = 1.24$). Mosquito larvae were reduced by at least 99% within the first six to eight days after both Bti applications (DAA6: $X^2 = 12.39$, $P < 0.001$; DAA21: $X^2 = 8.45$, $P = 0.003$) (Fig. S7). Moreover, Bti affected chironomid larvae from six days after the initial Bti application and cumulatively reduced their abundances by 87% over the entire sampling period (GLMM: $z = -12.10$, $P < 0.001$) (Fig. S7). While Cladocera decreased over time (GLMM: $z = -6.36$, $P < 0.001$) other aquatic invertebrates showed increasing abundances. However, none of them was affected by the Bti treatment.

3.2.2 Newt development

Body sizes of newt metamorphs ranged from 167.62 to 203.81 mg (33.08 – 38.25 mm) in the Bti treatment and from 185.27 to 219.10 mg (34.66 – 37.94 mm) in the controls. Newt metamorphs appeared to be slightly smaller in Bti treated mesocosms, while TTM showed no differences between control and Bti treatment (Fig. 7, Table S4). The smaller sizes were reflected in a trend towards a 7% lower body weight of *L. helveticus* newts at metamorphosis (F = 4.07, *P* = 0.067).

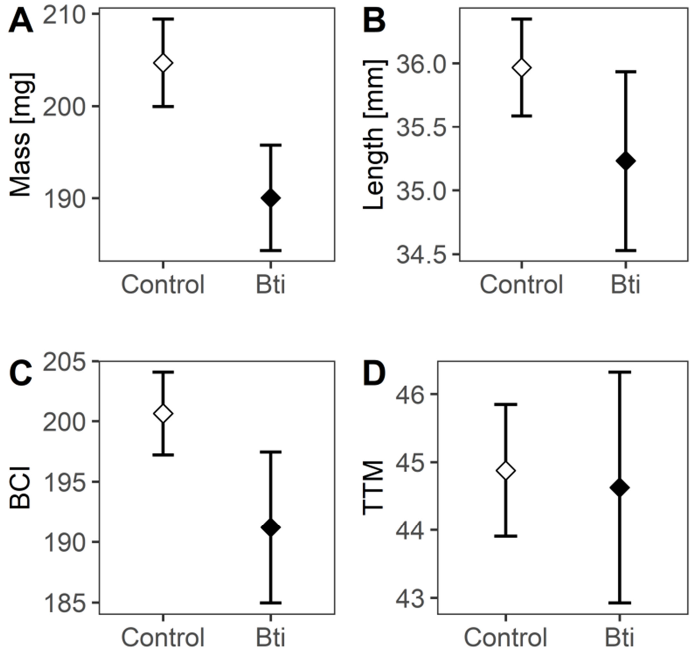


Figure 7: Mean (±SE) (A) body mass, (B) length, (C) body condition index (BCI) and (D) time to metamorphosis (TTM) of *L. helveticus* metamorphs in control (n=8, white) and Bti treatment (n=7, black).

4. Discussion

This is the first study that showed adverse effects of Bti-based mosquito control in environmentally relevant application rates on aquatic predators like newt larvae. Our experiments revealed that Bti increased vulnerability of newt larvae to intraguild predation by a dragonfly nymph, while both predators co-existed in Bti-free environments. As chironomids are the most severely affected non-target food resource in the Bti treated food chains (Allgeier et al., 2019), this study highlights the crucial role of chironomids as food resource in confined aquatic systems.

4.1 Bti effects on food web composition

Bti treatment changed the invertebrate community structure and affected the density of available food resources by significantly reducing abundances of mosquitoes but also non-target chironomids (Fig. 1). Availability of chironomids was reduced by more than half (53 to 87%) which was confirmed repeatedly in both our mesocosm experiments. In accordance with previous studies, other aquatic invertebrates were not directly affected due to the Diptera-specificity of Bti (Boisvert and Boisvert, 2000; Lacey and Merritt, 2003). Chironomids usually constitute a major proportion of invertebrate biomass and secondary production in temporary ponds and other wetlands in terms of abundances (Allgeier et al., 2019; Leeper and

Taylor, 1998; Lundström et al., 2010b; Williams, 2006) and species diversity (Lundström et al., 2010a; Theissinger et al., 2018; Wolfram et al., 2018). On this account, chironomids as the only adversely affected non-target invertebrates are central for our further considerations, especially since previous results on the existence and extent of negative effects on chironomids have been inconsistent (Allgeier et al., 2019; Lagadic et al., 2014; Lundström et al., 2010b).

Chironomids showed similar effect sizes (55 to 75% reduction) in another pond mesocosm study where the formulation VectoBac®G was used in application rates up to 9×10^9 ITU/ha (Liber et al., 1998). Moreover, the effect on chironomids in our mesocosm was verified by several field studies that analyzed chironomid communities and found reduced abundances (Allgeier et al., 2019; Hershey et al., 1998; Jakob and Poulin, 2016; Theissinger et al., 2018). Nevertheless, there are other semi-field and field approaches in which chironomid abundances or species diversities were unaffected (Charbonneau et al., 1994; Lagadic et al., 2014; Lundström et al., 2010b, 2010a; Pont et al., 1999; Wolfram et al., 2018).

There may be several reasons for the discrepancy of the results in field studies. First, the detection of Bti effects on chironomid communities in the field may be facilitated in sampling periods longer than four days after the Bti application were effects on chironomids could recently not be detected (Wolfram et al. 2018). Furthermore, not all of the above mentioned field studies on non-target effects explicitly assessed abundances of mosquitoes (Allgeier et al., 2019). However, significant effects on the target organism should be used as a proxy for Bti treatment efficiency that allows estimating non-target effect sizes on other organisms. Besides, several environmental factors (e.g. biotic and abiotic wetland characteristics, desiccation and flooding events, species community structure, timing, rate and formulation of Bti applications) might modify or superimpose Bti-induced effects in natural wetlands. Even if these factors generally influence chironomid abundances, a Bti-induced reduction of this magnitude would additionally limit prey available for several aquatic predators such as newts, insects or fish.

4.2 Diet of larval newts

According to the stable isotope mixing model, chironomids constituted the major part of newts' diet when compared to more abundant zooplankton or Cladocera in particular (Fig. 5). A diet comprised of chironomids and zooplankton is in accordance with stomach content observations of *L. helveticus* larvae and the syntopic species *Ichthyosaura alpestris* and *Triturus cristatus* (Braz and Joly, 1994). However, stomach contents in Romanian *L. vulgaris* contained mainly Cladocera in quasi-permanent ponds (Covaciu-Marcov et al., 2010) and Asselidae in the Carpathian Mountains (Roşca et al., 2013). Mosquitoes were considered less important in the diet of crested and smooth newt populations (Bogdan et al., 2013) due to their peak occurrences after flooding events and the fast larval development (< three weeks) at temperatures above 15°C (Becker et al., 2010). Chironomids are independent of flooding events and show continued availabilities with developmental times ranging from several months to more than one year (Armitage et al., 1995). Hence, chironomids depict an important and reliable insect food resource for predators in wetlands.

In contrast to our assumption, newt larvae seemed to prey on chironomids preferentially instead of feeding opportunistically as a result of higher availability of other prey. This is probably due to their high conspicuousness to optical predators associated with the benthic zone (Dolmen and Koksvik, 1983). *Lissotriton* newts effectively suppressed chironomid emergence rates but consumed 22% less chironomids in Bti-treated mesocosms. Thus, instead of switching to alternative prey in the Bti treatment, newts might have eaten less in terms of actual biomass. This is surprising as at least *L. vulgaris* adults are considered generalists with high trophic prey diversities (Covaciu-Marcov et al., 2010; Griffiths, 1986; Roşca et al., 2013). Larvae of the genus *Lissotriton* change their feeing behavior during ontogeny ranging from small crustaceans to larger insects (predominantly chironomids) (Kuzmin, 1991) which can both be accounted for with the analysis of stable isotopes.

Newt larvae neither exposed to Bti nor predator had the most specialized diet while these factors caused increasing niche widths (Fig. 6). A trophic niche expansion can indicate a higher diversity of used resources for instance within the group of zooplankton that have different feeding strategies ranging from predatory copepods to filter-feeding daphnids. Wider niches might also reflect suboptimal environmental conditions due to increased stress triggered by the presence of a contaminant (Bayona et al. 2014), food limitation or inadequate food quality (Karlson et al., 2018).

4.3 Development of larval newts

The development of *L. helveticus* individuals indicated a trend towards slightly smaller metamorphs in Bti treatments, albeit the difference was not significant at a 0.05 significance level (Fig. 7). Amphibian body size at metamorphosis is determined by food intake experienced during the later developmental stages (Alford and Harris, 1988; Leips and Travis, 1994) which is why reduced chironomid prey can be reflected in smaller newts. However, sublethal Bti concentrations induced early signs of detoxification and oxidative stress in tadpoles causing additional stress for amphibians (Allgeier et al., 2018; Lajmanovich et al., 2015). Thus, reduced growth can be a result of the prey limitation but also of a higher energetic investment in detoxification mechanisms (Wright and Wright, 1996).

Freshly metamorphosed *L. helveticus* were recorded to reach mean body weights of 100 mg (Schlüpmann et al., 1996). As this is less than observed in our mesocosm (190 mg - 205 mg) we cannot exclude that a potential food oversupply in the development experiment led to an underestimation of effects. However, natural wetland conditions, such as higher habitat complexity, lower invertebrate densities and the presence of competitors, might increase the adverse effects on newt development in the field. Reduced sizes during the aquatic phase show several disadvantages such as lower survival probabilities during their juvenile period and higher vulnerability towards predation by fish, beetles or dragonflies (Formanowicz, 1986; Smith, 1987).

4.4 Trophic interactions in Bti-treated food webs

Depending on wetland community structures, dragonflies and newts are engaged in intraguild predation that can be intensified when dragonflies as intraguild predators utilize resources of newts as the intraguild prey (Holt and Polis, 1997; Yurewicz, 2004). In our experiment, the dragonfly *A. cyanea* was 27% more lethal to newt larvae when they were exposed to Bti. Interestingly, *A. cyanea* had a minor effect on newt survival in control mesocosms (Fig. 3) even though dragonflies are one of the most efficient predators in seasonal wetlands (Van Buskirk and Schmidt, 2000; Wilbur and Fauth, 1990). Amphibian tadpoles recognize predators mainly through chemical cues (Relyea and Mills 2001). To escape predation risk they develop physiological modifications designed to move faster (deep tails, small bodies) or adapt a antipredator behavior by decreasing their activity (Relyea, 2001; Van Buskirk and Schmidt, 2000). Larval *L. helveticus* showed increasing refuge use (resulting in lower activity rates) to reduce detection and encounters by predatory brown trouts (Orizaola and Braña, 2003).

While the exposure to predator cues resulted in adaptive behavior or physiology (Reeves et al., 2011; Van Buskirk and Schmidt, 2000) the presence of actual predators reduced amphibian survival significantly (Relyea, 2018; Van Buskirk and Schmidt, 2000). Nymphs of *Anax junius* reduced tadpoles by 30 and 92% irrespectively of their potential antipredator behavior (Relyea 2018). The increased amphibian mortality when dragonflies interfere with Bti is in line with a mesocosm study by Pauley et al. (2015) where Gray treefrog (*Hyla versicolor*) tadpoles experienced even higher mortalities of more than 80%. However, dragonflies reduced their survival by around half even when not exposed to any insecticide (Pauley et al. 2015). The mechanism behind this synergistic effect was not explicitly studied but did not involve intraguild predation as herbivores treefrogs are no competitors to dragonflies.

High chironomid prey abundances in the controls may have facilitated the coexistence of newts and dragonflies by suppressing intraguild predation due to the increased availability of valuable alternative prey. Both predators reduced overall chironomid emergence suggesting that chironomid larvae were not only eaten preferably by newts but also by dragonfly nymphs. Additionally, the increased growth rate of dragonfly nymphs in the control (Fig. 4) indicated that they were well-fed which facilitates the coexistence with newt larvae. Bti-affected body sizes were already found in *Erythemis simplicicollis* nymphs which were smaller after repeated Bti applications (Painter et al. 1996). Furthermore, the correlation between the presence of more preferable alternative prey and decreased predation rates was recently shown for amphibian larvae and a mosquitofish predator in outdoor mesocosms (Preston et al. 2017).

The much higher intra-population variability in the isotopic niche of Bti-treated *A. cyanea* reflects a high diet diversification and trophic diversity that may be the result of the predation on larval newts. The estimation of dragonflies' diet proportions was not possible because their isotopic composition was lower than the one of their potential prey (Table 2). In terms of isotopic composition a high trophic level is displayed in enriched $\delta^{15}N$ values relative to the respective prey, while $\delta^{13}C$ values depend on the source of dissolved carbon in the water and change little among the food chain (Peterson and Fry, 1987). Late-instar dragonfly nymphs presumably did not assimilate enough tissue during the experiment and thus reflect the signature of putative prey in the permanent pond of their origin. Moreover, Seifert and Scheu (2012) proposed that large-bodied Odonata larvae integrate temporal variations in prey $\delta^{13}C$ signatures including periods with depleted prey signatures, especially in autumn and winter. Hence, dragonfly nymphs in the late-instar would need more time to incorporate isotopic signals prevailing in our mesocosms.

4.5 Direct effect of Bti on amphibians

The exposition to Bti as the only stressor did not lead to acute toxic effects on newts which is consistent with the majority of studies on vertebrates (Boisvert and Boisvert, 2000; Lacey and Merritt, 2003). Nevertheless, Lajmanovich et al. (2015) recently detected increased mortality of a South American frog after applying a liquid Bti formulation (Introban®). On the contrary, ten times the field rate of VectoBac®WG did not reveal mortality in tadpoles of the common frog *Rana temporaria* (Allgeier et al., 2018). However, Bti exposure induced behavioral changes in tadpoles that resulted in decreased swimming activity (Junges et al., 2017) which has already been detected with other insecticides (Relyea and Edwards, 2010). While activity reduction is a common behavioral response to avoid short-term predation (Relyea, 2001), a reduced activity induced by contaminants has no clear benefits for tadpoles but rather long-term implications such as less foraging time, smaller sizes and longer time to metamorphosis (Relyea, 2007). Moreover, sublethal effects induced by contaminants often increase susceptibility to predation in amphibians for instance by inhibiting the development of an antipredator behavior (Hayden et al., 2015; Polo-Cavia et al., 2016; Reeves et al., 2011). Changes in in larval newts' behavior induced by Bti would be responsible for the reduced dietary intake and the higher vulnerability to predation.

4.6 Importance for wetlands

Natural wetlands can be packed with additional stressors for amphibian larvae not predicted from our experiment such as several predators, competition or various chemical contaminants that often have interactive effects (Boone et al., 2007; Rohr et al., 2004). Thus, the observed effects on newt survival can even be more pronounced when Bti further decreases essential chironomid food resources as they often represent the most dominant taxa in wetlands(Allgeier et al., 2019; Hershey et al., 1998; Leeper and Taylor, 1998). Adult and larval amphibians largely contribute to habitat interconnectivity due to their biphasic life cycle and to several ecosystem services including the biological control of mosquitoes, pests and related diseases (Hocking and Babbitt, 2014). Hence, less amphibian metamorphs leaving Bti treated ponds will have implications on entire ecosystems for instance by reducing the transfer of biomass and energy across ecosystem boundaries (Gibbons et al., 2006).

Our results are of particular concern because large parts of Bti treated areas all across Europe are situated in national and international nature conservation sites (RAMSAR, Natura 2000) (Allgeier et al., 2019; Lagadic et al., 2014; Land and Miljand, 2014). These areas intrinsically focus on the preservation of biodiversity and the conservation of species particularly worthy of protection such as amphibians. Moreover, wetlands are valuable parts of our landscape as they largely contribute to ecosystem services and freshwater biodiversity (Biggs et al., 2017; Mitsch et al., 2015) and should be managed in a sustainable manner especially in light of the upcoming biodiversity extinction crisis (Ceballos et al., 2015).

5. Conclusion

Despite the increasing use of Bti in mosquito control (van den Berg et al., 2012), adverse effects on wetland ecosystems are still a matter of debate especially when it comes to long-term and indirect effects on the non-target biodiversity (Land and Miljand, 2014; McKie and Goedkopp, 2010). This study highlights the crucial role of non-target chironomids in the food webs of seasonal ponds where Bti increased vulnerability of newt larvae to predation. Besides from using Bti in mosquito control, the bacterium Bt is also extensively applied over forests to eradicate other pests such as phytophagous caterpillars (UBA and BfN, 2018) resulting in an ubiquitous occurrence of Bt in many amphibian habitats. Taking into account that amphibians suffer from worldwide population declines (Stuart et al., 2004), Bt-based pest control may pose an additional threat to amphibian populations already faced with multiple stressors in their habitats.

In future, the control of vector mosquitoes will most definitely gain in importance with regard to human health aspects. Since most vectors are container-breeding species their control requires small-scale strategies rather than large-scale Bti treatments of wetlands often integrated in nature protection areas. Thus, the application of Bti in habitats relevant for amphibian and nature conservation such as the Camargue (France) and the Upper Rhine Valley (Germany) should be reconsidered.

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Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Appendix VII: Mosquito traps as alternative mosquito control

Introduction

Several mosquito traps are commercially accessible for the public to decrease the risk of mosquito-borne illness and to reduce mosquito nuisance (Lühken et al., 2014). Trapping devices require attractants to appeal female mosquitoes. Carbon dioxide is the most used attractant to fulfil this goal (Kröckel et al., 2006) because it simulates human respiration, a key process of attracting blood-feeding insects. Biogents GmbH, a spin-off company of the Institute of Zoology at the University of Regensburg, Germany has developed and patented several mosquito traps including one using CO_2 (Kröckel et al. 2006). BG-Mosquitaire CO_2 is an outstanding and eco-friendly tool to catch nearly all mosquito species. Moreover, these traps have been designed to be placed in gardens and were tested worldwide proving their efficiency (Biogents AG, 2017a). Non-CO_2-based traps were already shown to be effective in catching tropical mosquitoes such as *Aedes aegypti* in Brazil (Kröckel et al., 2006) and *Ae. albopictus* in Italy (Englbrecht et al., 2015). No study so far examined their effectiveness of controlling the floodwater mosquito *Ae. vexans* which responsible the nuisance in the local population (Becker, 1997; Becker et al., 2018). The efficacy of the BG-Mosquitaire CO_2 traps was determined in a small village next to the river Rhine that offers perfect breeding habitats for several mosquito species (floodwater and house mosquitoes). Berg/Pfalz is located on the southern edge of Rhineland Palatinate, next to the river Lauter and France in the south, the river Rhine to the east and the Bienwald forest to the north-west. The aim of the study was to evaluate the efficiency of the BG-Mosquitaire CO_2 traps against all local mosquito species with a special focus on *Ae. vexans*.

Material and methods

Biogents-Mosquitaire CO_2

The BG-Mosquitaire CO_2 traps are made of sturdy plastic with a diameter of 40 cm and require little care, energy and maintenance (www.biogents.com; Fig. 1). The principle of capturing mosquitoes is mainly comprised out of two functions: attraction and suction. First, the trap itself has a contrast between the light surface and the dark center which is an attractive signal for the mosquitoes. Besides, there is a scent dispenser inside the trap which supplements the air stream with small quantity of substances like those emanated by the human skin. To improve and maximize the attractiveness and capture rates of mosquitoes,

CO_2 is supplied from a nozzle and added to the air stream. The rate of emission of the gas simulates the respiration emission rate from an infant (0.5 kg/day). The CO_2 sources are 10 kg bottles (Linde AG, Pullach) and its consumption was regulated via the BG-CO_2 timer, which allows controlling when CO_2 is released. The second function consists in the trapping capability of the mosquitoes. Therefore, a single fan sucks the attracted mosquitoes into a catch bag below the suction column of the trap. The air stream is deviated and leaves the trap through the white upper surface together with the odor of the dispenser.

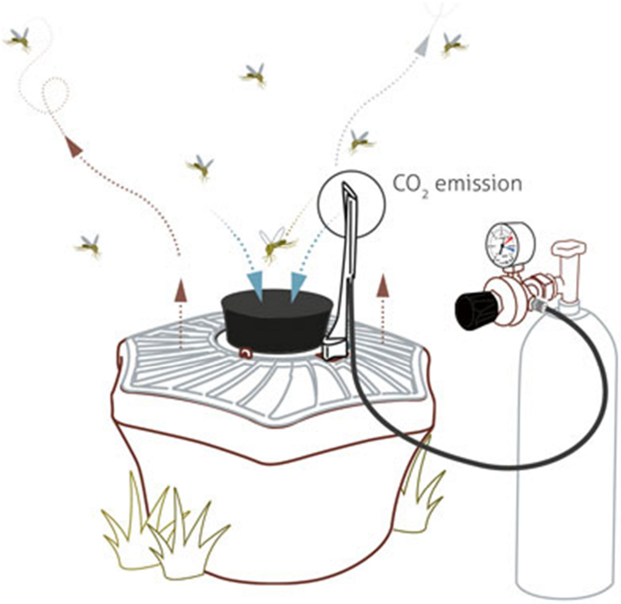

Figure 1: Scheme of the BG-Mosquitaire CO_2 trap (www.biogents.com)

Study design

The study was conducted between June and September 2017. In total, 38 BG-Mosquitaire CO_2 traps were placed on the private properties of selected households. Traps were located in shady, wind-protected moist places near breeding sites of midges (bushes or standing water). Three experimental setups were applied (Fig. 2):

- Single houses: five houses were selected on the edges of the village. In total, 13 traps were located on the property of these houses, Römerring 36a (2 traps), Holzhof 4 (2), Ludwigstraße 10d (3), Ludwigstraße 64 (3) and Obertalstraße 3 (3).
- Wall: 7 traps were placed along properties in Bruchbergstraße 10, 14, 16, 16a, 18, 18a and 23.
- Cluster: 18 traps were located around seven houses in Rosengärtenstraße 11, 12, 13, 14, 15, 16 and 17.

The amount of traps was selected according to the size of the property and the amount of shrubberies that adult mosquitoes would use as resting habitats. Additionally, one control area was chosen close to each single household and for the wall and the cluster respectively. The CO_2 emission was set to run between 4:00 and 11:00 pm.

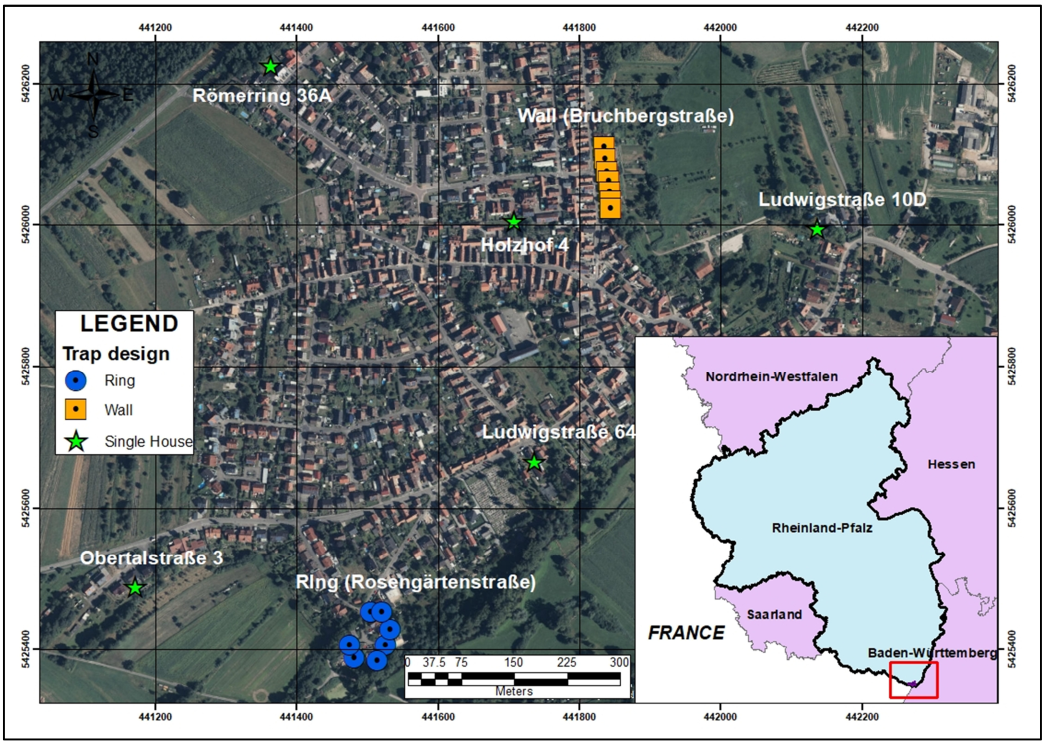

Figure 2: Satellite image with the locations of the traps in Berg according to the experimental designs.

The human landing rate (HLR) was measured weekly by exposing an arm or a leg for 15 minutes either in a control zone (without trap) or an intervened zone (with trap). During this time, the number of landing approaches was assessed. When possible, approaching mosquitoes were caught for further identification. Measurements started three weeks before the traps were activated and lasted until five weeks after activation, when CO_2 flasks were empty. To distinguish the origin of the mosquitoes, the catch bags of the traps were collected weekly and stored in a freezer (-20ºC) for further determination. The determination of the mosquito genus was performed following the key in Becker et al. (2010).

For HLR, statistical differences in intervention and control sites were analyzed using Wilcoxon signed-rank test with paired alternative. The data on HLR of the single houses were analyzed explicitly using a generalized linear mixed model (GLMM) with negative binomial distribution and a random factor for measurement and house.

Results & Discussion

Traps caught 1575 mosquitoes during the entire sampling period. Most of the individuals determined belonged to the genus *Culex* (93.9%), followed by *Aedes* (3%), *Anopheles* (2.5%), *Culiseta* (0.4%) and *Coquillettidia* (0.1%). Within the genus *Culex* the majority of individuals were *Culex pipiens*. Moreover, *Ae. japonicus* was found to occur in Berg. *Aedes japonicus* is an invasive vector mosquito from Southeast Asia that is already widely distributed in Central Europe (Jansen et al., 2018).

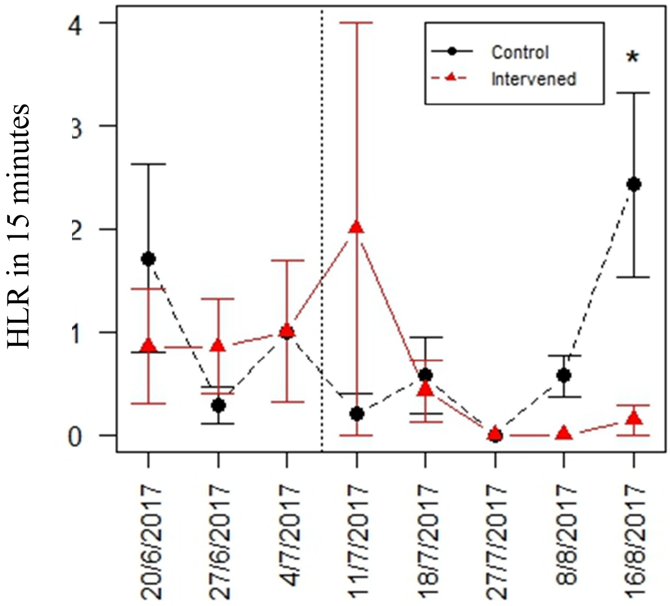

Figure 3: Weekly means of HLR measurements in intervention (red) and control sites (black) within 15 minutes. Error bars represent the 95% confidence intervals. The vertical dash line Implements the activation of the traps. Asterisk* indicate statistical significant differences (p<0.5)

The HLR measurements revealed no difference in mosquito levels between control and intervention sites in the period before the traps were activated and during the first weeks after activation (Fig. 3). Traps reduced mosquito approaches starting four weeks after their activation (Wilcoxon signed-rank test, 8/8/2017: v=10, p-value=0.07; 16/8/2017: v=21, p-value=0.03). One of the reasons for this delay in catching efficiency might be that new traps first have to run for some time to lose their new smell. Much more important, however, is that the traps continuously catch mosquitoes and the cumulative effect can only appear somewhat delayed. Thus, it is to be assumed that the effect would have been shown further clearly with a progress of the study.

According to the model output of the GLMM on HLR measurements, 8.7-times more mosquitoes were approaching single houses in the control areas (z=-2.63, p=0.009, Table 1). Thus, 88.5% less mosquito biting attempts occurred in the intervention areas with mosquito traps.

Table 1: HLR measurements before and after activation of traps in control and intervention areas of the different experimental set-ups.

Experiment	Period	Treatment	n	sum	mean	sd
single houses	before	control	11	8	0.73	1.27
		intervention	15	10	0.67	1.11
	trap activation	control	23	19	0.83	1.67
		intervention	23	2	0.09	0.29
wall	before	control	2	0	0.00	0.00
		intervention	3	1	0.33	0.58
	trap activation	control	5	4	0.80	0.84
		intervention	5	0	0.00	0.00
cluster	before	control	2	7	3.50	3.54
		intervention	3	8	2.67	2.52
	trap activation	control	5	3	0.60	0.89
		intervention	5	12	2.40	4.34
all			102	74	0.73	1.60

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Appendix VIII: Declaration

I, the undersigned author of this work, hereby declare that I independently wrote this thesis with the title “**Mosquito control based on Bacillus thuringiensis israelensis (Bti) - Ecological effects on wetland food chains and public acceptance of control alternatives**”. All used resources and references are specified in this work. Contributions of coworkers and coauthors have been clearly identified.

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

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

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

Landau, 18.12.2019

………………………………………. ………………………………………………..

Place, Date Stefanie Allgeier

Appendix IX: Curriculum Vitae

PERSONAL DATA

Name	Stefanie Allgeier
Date of birth	31 August 1988
Nationality	German
E-Mail	stefanie.allgeier@gmail.com

SCIENTIFIC EDUCATION

Since 02/2015	**Dissertation in natural sciences (PhD)** Ecotoxicology & Environment, University of Koblenz-Landau, Campus Landau Thesis: *Mosquito control based on Bti - Ecological effects on wetland food chains and public acceptance of control alternatives*
10/2011 - 09/2014	**Master program "Ecotoxicology" (M.Sc)** University of Koblenz-Landau, Campus Landau Thesis: *Indirect effects of mosquito control using Bti on newt larvae in temporary wetlands* Graduation grade: 1.4
08/2010 - 01/2011	**Semester abroad** Lund University, Sweden
10/2007 - 08/2011	**Bachelor program "Management of natural resources" (B.Sc)** Martin-Luther University of Halle-Wittenberg, Halle (Saale) Thesis: *Untersuchungen zum Einsatz der SPME-Technik in der Umweltanalyse anhand von Bodenproben aus Röblingen* Graduation grade: 1.6
09/1999 - 07/2007	**Secondary school, Abitur** Gymnasium mit Schülerheim Pegnitz Graduation grade: 1.8

EXPERIENCE

02/2015 - present	**Research associate** DBU Project (Az 32608/01) and AufLand research initiative in the project "MOSCOFEE"

	Ecotoxicology & Environment, University of Koblenz-Landau, Campus Landau
11/2014 - 01/2015	**Research assistant** AufLand research initiative, project "MOSCOFEE" Ecotoxicology & Environment, University of Koblenz-Landau, Campus Landau

PUBLICATIONS AND PRESENTATIONS

Peer-reviewed publications (included in thesis)

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, 13565. DOI: 10.1038/s41598-017-14019-2

Allgeier, S., **Kästel, A., Brühl, C.A. (2019).** 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. DOI: 10.1016/j.ecoenv.2018.11.050

Theissinger, K., Kästel, A., Elbrecht, V., Makkonen, J., Michiels, S., Schmidt, S., Allgeier, S., Brühl, C.A. (2018). Using DNA metabarcoding for assessing chironomid diversity and community change in mosquito controlled temporary wetlands. *Metabarcoding and Metagenomics*, *2*: e21060. DOI: 10.3897/mbmg.2.21060

Theissinger, K., Röder, N., Allgeier, S., Beermann, A., 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. DOI: 10.1111/mec.15214

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. DOI: 10.1016/j.envres.2018.01.010

Allgeier, S**., Friedrich, A., Brühl, C.A. (2019).** Mosquito control based on Bacillus thuringiensis israelensis (Bti) interrupts artificial wetland food chains. *Science of the Total Environment* 686: 1173-1184.

Further articles

Brühl, C. A., **Allgeier, S.** (2019). Letter to the editor: Kommentar zum Artikel „Konfliktfeld Stechmücken und Naturschutz" von Kiel et al. (2019) in Natur und Landschaft, 2, 52-58. https://www.natur-und-landschaft.de/de/extra/leserbriefe-7

Brühl, C. A., Després, L., Frör, O., Land, M., Patil, C.D., Poulin, B., Tetreau, G., **Allgeier, S**. *(in process)*. Environmental and socioeconomic effects of mosquito control using the bacterio-insecticide Bti. Review article.

Reports

Allgeier, S., Brühl, C. A., Frör, O. (2019). Entwicklung eines naturschutzkonformen Konzeptes zur Stechmückenbekämpfung am Oberrhein. Abschlussbericht des DBU Projektes Az 32608/01. https://www.dbu.de/OPAC/ab/DBU-Abschlussbericht-AZ-32608_01-Hauptbericht.pdf

Contributions to scientific conferences

2018

Allgeier S., Kästel, A., Brühl, C.A. (2018). You are what you eat: Does mosquito control affect predators by altering wetland food chains? Oral presentation. Jahrestagung der Gesellschaft für Ökologie (GfÖ), Wien, Austria.

Allgeier S., Kästel, A., Brühl, C.A. (2018). European common frog (Rana temporaria) larvae show subcellular responses under field-relevant Bacillus thuringiensis var. israelensis (Bti) exposure levels used in mosquito control. Poster presentation. Jahrestagung der Gesellschaft für Ökologie (GfÖ), Wien, Austria.

Allgeier S., Kästel, A., Brühl, C.A. (2018). European common frog (Rana temporaria) larvae show subcellular responses under field-relevant Bacillus thuringiensis var. israelensis (Bti) exposure levels used in mosquito control. Poster presentation. SETAC Europe Meeting, Rome, Italy.

Allgeier S., Kästel, A., Brühl, C.A. (2018). Effects of regular mosquito control using Bti on amphibians? Oral presentation. Conference on ecological and socioeconomic impacts of mosquito control using Bti - International scientific workshop DBU-Project Az 32608/01, Annweiler, Germany.

2017

Allgeier S., Kästel, A., Brühl, C.A. (2017). Stechmückenbekämpfung mit Bacillus thuringiensis israelensis - Auswirkungen auf die Ökosysteme des Oberrheins? Oral presentation. SETAC GLB, Neustadt/ Weinstraße, Germany.

Allgeier S., Kästel, A., Brühl, C.A. (2017). Is mosquito control using Bacillus thuringiensis israelensis environmentally safe? Oral presentation. YES Meeting, Stockholm, Sweden.

Kästel, A., **Allgeier S**., Brühl, C.A. (2017). Underestimated risk of mosquito control with Bacillus thuringiensis israelensis? Oral presentation. YES Meeting, Stockholm, Sweden.

2016

Allgeier S., Kästel, A., Brühl, C.A. (2016). Stechmückenbekämpfung mit Bacillus thuringiensis israelensis - mögliche direkte und indirekte Auswirkungen. Oral presentation. 23. DGL Jahrestagung, Wien, Austria.

Kästel, A., Elbrecht, V., Makkonen, J., Michiels, S., **Allgeier S**., Leese, l., Schmidt, S.I., Brühl, C.A., Theissinger, K. (2016). Chironomid diversity and species richness in temporal wetlands estimated with DNA metabarcoding. 23. DGL Jahrestagung. Wien, Austria.

2015

Allgeier S., Kästel, A., Brühl, C.A. (2015). Indirect effects of Bti-based mosquito control on exemplary wetlands: A mesocosm study. Oral presentation. Young Academic Conference, AufLand -Land-Water-Interactions. Klingenmünster. Germany.

Kästel, A., **Allgeier S**., Brühl, C.A. (2015). Moscofee - mosquito control food chain effects. Oral presentation. Young Academics Conference, AufLand - Land-Water-Interactions. Klingenmünster. Germany.

Presentations at further meetings/events

Brühl, C.A., **Allgeier, S.** (2018). Umweltauswirkungen der Schnakenbekämpfung mit Bti (*Bacillus thuringiensis israelensis*) am Oberrhein – mit Vorschlägen zur Entwicklung eines naturschutzkonformen Behandlungsschemas. Oral presentation. Information event BUND & Pollichia. Haßloch, Germany.

Brühl, C.A., **Allgeier, S.**, Frör, O. (2018). Stechmückenbekämpfung in Deutschland – Umwelteffekte, Fallenmethode, Sicht der Bevölkerung. Oral presentation. AufLand final conference. Landau, Germany.

Allgeier, S., Frör, O., Brühl, C.A. (2018). Entwicklung eines naturschutzkonformen Konzeptes zur Stechmückenbekämpfung am Oberrhein. Poster presentation. SERIOR Transferveranstaltung. Landau, Germany.

Allgeier S. (2017). Umweltverträgliche Stechmückenbekämpfung? Ökologische und sozioökonomische Betrachtungen. Oral presentation. POLLICHIA Themenabend „Stechmücken“. Speyer, Germany.

Allgeier S., Göttler, S. (2017). Stechmückenreduktion im häuslichen Umfeld. Oral presentation. Information event on Biogents-Mosquito trap study. Berg, Germany.

Brühl, C.A., Frör, O., Kästel, A., **Allgeier, S.** (2016). Stechmückenbekämpfung in Deutschland – Bewilligungsverfahren, Bewertungsgrundlagen, Handlungsbedarf? Oral presentation. National workshop DBU Project Az 32608/01.Landau, Germany.