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A multi-year study following BACI design reveals no short-term impact of *Bti* on chironomids (Diptera) in a floodplain in Eastern Austria

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Abstract Short-term impacts of aerial application of *Bacillus thuringiensis israelensis (Bti)* on Culicidae and Chironomidae were investigated over several years in temporary waters of the Dyje and Morava floodplains in Eastern Austria. The sampling followed a Before-After-Control-Impact (BACI) approach with sampling dates immediately before and shortly after the application and was repeated for 3 years. To test for effects of the *Bti* treatment on the two Diptera families, linear mixed-effects models were used. Data analysis included the factors Before-After and Control-Impact as fixed effects, while general temporal and spatial variables were random effects. One hundred sixteen taxa of chironomids were identified. Abundance varied between 2 and 1125 larvae per m^2, while culicid densities reached values of several 100 ind. per liter. Total culicid abundance significantly decreased after the *Bti* treatment, whereas no significant effects were found on the abundance of total chironomids and dominant chironomid subfamilies, tribes, and genera, on relative proportions of chironomid feeding guilds, diversity, and species composition. Further studies from this area are needed to extend the investigation over a period of several weeks in order to reveal possible delayed effects of the larvicide application.

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Introduction

Since its discovery in the 1970s, the crystal protein *Bacillus thuringiensis israelensis (Bti)* has become the most widely used biocide against mosquitoes (Culicidae) and black flies (Simuliidae) worldwide (Becker and Zgomba 2007; Minnesota Department of Health 1999; Fillinger et al. 2003).

Due to a complex mode-of-action and several *Bti* proteins involved (Ben-Dov 2014), it is almost exclusively these two Diptera families against which *Bti* unfolds its toxic effect. No direct negative impact of *Bti* has been found on plants, mammals, birds and most aquatic organisms (Boisvert and Boisvert 2000; McKie and Goedkoop 2010).

However, in some studies, *Bti* was shown to affect other families within the order of Diptera, such as non-biting midges (Chironomidae), crane flies (Tipulidae) or horse-flies (Tabanidae), though usually in much higher concentrations than commonly used against mosquitoes and black flies (Boisvert and Boisvert 2000; I. P. Vaughan et al. 2008; Lacey and Merritt 2003).

While the aspect of specific direct effects of *Bti* on non-target species is well documented (Boisvert and Boisvert 2000), little is known about indirect and long-term effects (Poulin 2012; McKie and Goedkoop 2010). To some extent, this is due to the temporal and spatial variability of the habitats where *Bti* is applied, such as inundated floodplains and marshes.

Up to now, only a few studies have dealt with possible environmental impacts of *Bti* over several years. Hershey et al. (1998) investigated wetlands in Minnesota over 3 years and found a significant decline of chironomids and other non-target organisms as well as changes in diversity and dominance structure, which they assigned to the application of the *Bti* formulation VectoBac® G. Further studies, however, could not repeat and prove these results (Read et al. 1999; Balcer et al. 1999).

In the Camargue, Jakob and Poulin (2016) revealed significant lower species richness among dragonflies in areas where *Bti* was applied as compared with control areas. Poulin and Lefebvre (2016) pointed mainly at the reduction of food resources for birds. By using a modeling approach the authors aimed at proving earlier studies by Poulin et al. (2010) and Poulin (2012) who found an evidence of indirect negative impacts of *Bti* on the house martin *(Delichon urbicum)*.

Based on a 4-year-study in French coastal and continental wetlands, Lagadic et al. (2016) did not see any risk of *Bti* for the environment and for non-target species, at least if the recommendations for the application (concentration, areal dosage) are met. Also, Lundstrom et al. (2010) did not find negative effects of *Bti* (VectoBac® G) on chironomid diversity and secondary production during their 6-year-study at the Swedish river Dalälven.

The objective of the present study is to investigate the impact of *Bti* on diversity and abundance of chironomids in a floodplain in Eastern Austria at the border to Slovakia. This is an area where regular floods allow mosquitoes (mainly *Aedes* and *Ochlerotatus* sp.) to develop very large populations. Considered a nuisance by the local population in 11 municipalities along the rivers Dyje and Morava, the mosquitoes are controlled with *Bti*, which has been applied locally since about 14 years and on a larger scale by helicopter since 2013.

The permission for applying *Bti* in the floodplains of the Dyje and Morava Rivers according to the Austrian Water Law of 2010 required an accompanying ecological monitoring over several years in order to document possible side effects of the *Bti* application on other insect groups. The results of this monitoring are presented in this study. We tested the null hypothesis that there is no short-term effect of *Bti* treatment on culicid and chironomid larvae in inundated meadows and backwaters of the Morava River over a period of several years.

Materials and methods

Study area

The floodplains of the river Morava and its tributary Dyje are situated in Eastern Austria at the border to Slovakia and Czech Republic (Fig. 1). The inundation area between the river and the right-hand dyke on the Austrian side is up to 2 km broad and includes oxbows, ditches, and swamps. Numerous depressions are inundated almost annually and become water bodies for a few days to up to several weeks. From the Dyje-Morava basin, flood events arise mainly during the snow melting period in spring, while the Danube typically has a high water level in early summer. Dyje-Morava floods affect mainly the northern part of the floodplain, while high water from in the Danube regularly runs into the Morava valley and causes floods in the southern part of the study area. The inundated area covers a high variety of habitats including forests, meadows and reed beds (Figs. 2 and 3).

Bti application

Bti has been applied in the study area for about 10 to 15 years, but only locally by using backpack sprayers. In 2011, the municipalities along the Dyje-Morava floodplain founded an association to pool their resources for controlling the mosquitoes. Based on permission according to the Austrian Water Law, the association has organized aerial spraying by helicopter in areas, which are too large for *Bti* application from the ground.

In each year, potential inundation and thus mosquito breeding sites are controlled weekly by employees and volunteers of the local municipalities. *Bti* is applied, when the densities of culicid larvae exceed a threshold of 20 individuals per liter. Depending on the number and area of the inundated water bodies, the *Bti* application is carried out from the ground (backpack sprayers) or by helicopter. The first helicopter mission took place in 2013. Since then, *Bti* was applied by helicopter two to four times per year, but local application by backpack sprayers has continued in addition to the aerial spraying.

The *Bti* formulation used during the study period in the backpack sprayers was VectoBac® AS (aqueous suspension, 0.5–1 l per hectare, $0.64–1.28 \times 10^9$ ITU/ha) and VectoBac® WDG (powder suspension,

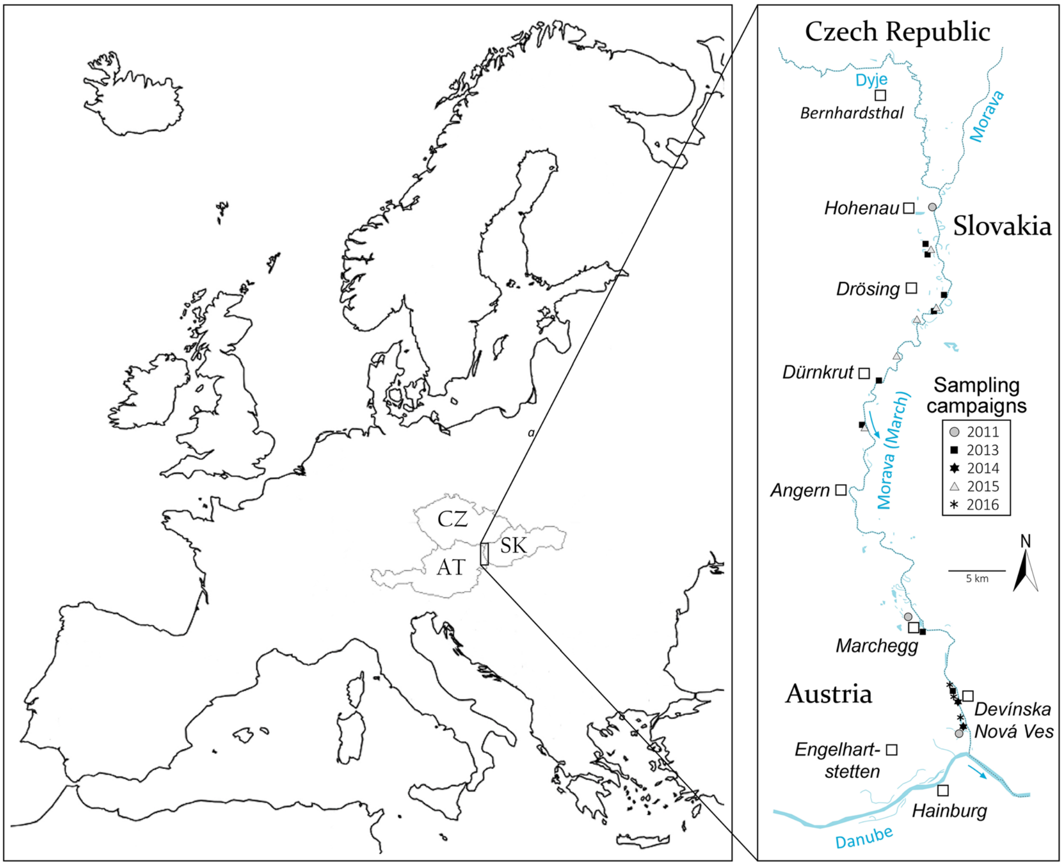


Fig. 1 Map of the floodplains of Morava and Dyje at the Austrian-Slovakian border

up to 400 g/ha or 1.2×10^9 ITU/ha). For the aerial application, a *Bti* granulate (VectoBac® G, 200 ITU/mg) was used at a dosage of 10–12 kg/ha ($2.0–2.4 \times 10^9$ ITU/ha).

Fig. 2 Photo from inundated grassland in the Morava floodplain (photo: P. Wenzl)

Fig. 3 Photo from an inundated willow forest in the Morava floodplain (photo: P. Wenzl)

Sampling dates, sampling method, and sample processing

Sampling was carried out in 2011 and from 2013 to 2016, in total for 5 years. The sampling campaign was suspended in 2012, when no large-scale *Bti* application took place because of the lack of inundations. During each sampling campaign, 6 sites were sampled at 3 dates, the first before and the second and third sampling date after the *Bti* application. Three sites each were defined as control (no *Bti* application) and impacted sites (*Bti* application), respectively.

The sudden beginning of the Bti spraying in 2011 and 2014 prevented us from carrying out a first sampling previous to the *Bti* application. The data from these years had thus to be excluded from a part of the analysis. Besides, due to year-to-year differences in hydrology, the time between the second and the third sampling date varied from a few days to several weeks. The data set resulting from the three-date-sampling design turned out to be too heterogeneous for a thorough statistical analysis. Therefore, we decided to restrict the BACI analysis to a data set with two sampling dates per campaign. The final data set used in the statistical analyses comprised 3 years (2013, 2015, and 2016) with 2 dates (before and after *Bti* treatment) at 6 sampling sites each (3 impact and 3 control sites, resp.) (Table 1). The data from the third sampling date are regarded only for the descriptive analysis of diversity.

As a result of the spatial heterogeneity of the Morava floodplains and the stochastic nature of the flood events, it was not possible to sample identical sites over the whole study period. It was rather necessary to select sites on a very short term, depending on the time and extent of the flood, the spatial distribution of inundated areas within the Morava floodplain and the areas where *Bti* was applied. In consequence, the location of impact and control sites was identical within each year, but varied between years (Fig. 1).

The sampling protocol at each site included site description, photo documentation, sampling of water for chemical analyses, and sampling of chironomid and culicid larvae.

The sampling sites were classified in the field according to the main habitat type (inundated willow forest,

Table 1 Data set used for the statistical analysis showing the origin of the flood event, the villages closest to the sampling sites (see Fig. 1) as well as the dates of *Bti* application and the sampling campaigns

Campaign	2013	2015	2016
Main flood event	Danube	Morava	Morava
Sampling sites	Drösing Dürnkrut Marchegg	Drösing Dürnkrut Angern	Engelhartstetten Marchegg
Sampling date 1	13 Jun	17 Apr	19 May
Bti application	14 Jun	18 Apr	20 May
Sampling date 2	18 Jun	21 Apr	23 May

meadows, reed fringe etc.). For the statistical analyses, only two habitat types (Forest and Meadow) were distinguished.

Selected physico-chemical parameters (temperature, conductivity, oxygen, pH) were measured in the field with a multi-probe and datalogger (Hydrolab, MS5). Other parameters were analyzed following common standards (general examination: DIN 38402, phosphorus: EN ISO 6878, ammonium: ISO 7150-1, nitrate: EN ISO 10304-1, nitrite: EN 26777, calcium, magnesium, total hardness: EN ISO 14911, chlorophyll-a: DIN 38412 – L 16).

Chironomid larvae were sampled by using a handnet (25 × 25 cm, mesh-size 500 μm). At each site, 10 subsamples were taken in all representative habitats (e.g., inundated grass, reed, dead wood, etc.), thus following the multi-habitat sampling (MHS) approach as commonly used for wadable rivers (Hering et al. 2003) and in the littoral of lakes (Urbanic et al. 2012). The total area sampled was 0.625 m^2. In the laboratory the larvae were sorted under a stereoscopic binocular microscope at 50x magnification and identified under a light transmission microscope at up to 400x magnification to species/genus level using the following keys: Andersen et al. (2013), Contreras-Lichtenberg (1986, 1999), Hirvenoja (1973), Janecek (1999), Kalugina (1963); Kalugina (1975), Klink et al. (2002), Langton (2003), Moller Pillot et al. (1997), Schmid (1993), Vallenduuk et al. (1997), Vallenduuk and Moller Pillot (2007), Wiederholm (1983). Abundance of chironomids is expressed in individuals per m^2.

Culicid larvae were sampled with a ladle (330 mL) fixed on a long stick. The ladle was dipped into the water at different places of each sampling site ten times. In each catch, the number of culicids was counted in the field. The mean of the 10 catches was taken as the abundance of mosquito larvae per site, expressed as individuals per liter.

Data analysis

For general comparison of samples based on the chironomid species composition in different years, a non-metric multidimensional scaling (nMDS) procedure was performed using the R package *Vegan* (Simpson et al. 2011) of R version 3.4.2 (R Core Team 2017). The nMDS technique ranks samples on the basis of the similarity (Bray-Curtis distances) and produces a two-dimensional plot of the sample distribution, with short distances indicating high similarity between sites. A numerical measure of the fit between the similarities in the two-dimensional plot and the original data is given as the stress index. Using nMDS allowed us to illustrate the data structure, and thus provide graphical support for our interpretation of the interrelations and possible effects of different factors.

To test for effects of the *Bti* treatment on the Diptera families Culicidae and Chironomidae we used linear mixed-effects models (LMM) (Zuur et al. 2009) as available in the *lme4* package (Bates et al. 2015) (Method: Restricted Maximum Likelihood, REML). The sampling design followed the idea of Before-After, Control-Impact (BACI), as proposed by Green (1979) and Underwood (1993).

The variables *Site_Class* (with the factors Control and Impact, *CI*) and *Period* (with the factors Before and After, *BA*) were included as fixed effects, while *Site*, *Year* and *Habitat* were random effects. The hypothesis was tested by creating a linear mixed-effects model in the form of:

$$\text{Response} \sim \text{BA}^{*}\,\text{CI} + (1|\text{Site}) + (1|\text{Year}) + (1|\text{Habitat})$$

We used the following variables as response:

- Abundance of total Culicidae and total Chironomidae
- Abundance and relative proportion of the dominant chironomid tribe Chironomini and subfamily Orthocladiinae
- Abundance and relative proportion of the dominant chironomid genera
- Scores and relative proportion of the feeding guilds among the Chironomidae
- Chironomid diversity expressed as taxa richness S and Shannon-Wiener index of diversity H′

Dominant chironomid genera were defined as those occurring at least in one third of the samples or with a relative proportion of total chironomid abundance > 20% at least in one sample. These were *Corynoneura*, *Cricotopus* (mainly *C.* cf. *tricintus* and *C. sylvestris* group), *Hydrobaenus (H. lugubris)*, *Limnophyes*, *Pseudosmittia*, *Chironomus* (various species), and *Polypedilum* (mainly *P. sordens* group and *P. nubeculosum*). Scores of feeding guilds (grazers, miners, shredders, gatherers, filterers, and predators) were calculated as the product of the abundance and the guild valences as defined in Moog (1995, 2002). The valences were developed in order to characterize the taxa based on their feeding traits. For each taxon, 10 points were distributed over the six above-mentioned feeding guilds depending on their preferences, e.g., *Corynoneura:* grazers = 7 points, gatherers = 3 points. For taxa, for which Moog (1995, 2002) does not provide information on the feeding guilds, we defined the valences by expert judgment and based on the autoecology of the taxa as described in the literature.

For the linear mixed-effects models, the abundance data of chironomids were log-transformed before the analysis (to satisfy the assumption of normality), while we used the logit link function for percentage data (Baum 2008). The two diversity measures were used without transformation and calculated in Microsoft® Excel.

In addition, we performed a correspondence analysis (CA) on the (untransformed) chironomid abundance on genus/species level data using the *ca* function of the *ca* package. CA is a multivariate statistical technique for categorical data (here: individuals per m^{-2}). It allows the reduction and summation of a multi-dimensional data set (i.e. the species composition at several sampling sites and on several dates) in two-dimensional form. While the purpose of CA is usually to graphically display the data set, we used the standard coordinates of the first two axes as additional response variables, in order to test, whether *Bti* treatment affects species composition.

Particular interest lies on the interaction (Before/After * Control/Impact), which, if significant, implies that the culicid or chironomid populations respond differently at sites, where *Bti* was spread (Impact), and at control sites.

The coefficients and standard errors of the fixed effects and the variance of the random effects were calculated using the *lmer* function in the *lme4* package (Bates et al. 2015). This function allows the fitting and analysis of linear mixed-effects models. The *p*-values of the coefficients were calculated using the *lmerTest* package (Kuznetsova et al. 2016), with Satterthwaite approximation for degrees of freedom. The *rand* function of the same package was used to test the significance of the random effects. It performs likelihood ratio tests (LRT) comparing the full model with a reduced model that did not include the specific components.

Results

Chemical properties of the sampled water bodies are summarized in Table 2. They highlight a pronounced variability and a high range of water quality indicators. Electrolyte concentrations were medium to high (calcium 56–429 mg L^{-1}, magnesium 14–53 mg L^{-1}), with electric conductivity reaching values of up to 1547 µS cm^{-1}. The high median values of total phosphorus concentration (169 µg L^{-1}) and orthophosphate (57 µg L^{-1}, analyzed as soluble reactive phosphorus) indicate eutrophic to hypertrophic conditions, while nitrogen concentrations remained low in most cases (median below detection limit) as a result of denitrification processes. The range of chlorophyll-a concentrations span over three orders of magnitude from 1.3 to

Table 2 Chemical properties (minimum, maximum, 10th and 90th percentile, median) at the sampling sites (before/after *Bti* application) in the years 2013, 2015, and 2016

	N	Min	10th perc.	Median	90th perc.	Max
Water temperature [°C]	36	10.1	11.4	17.4	23.9	26.1
pH	36	6.9	7.2	7.6	8.0	8.8
Oxygen concentration [mg L^{-1}]	36	1.3	4.3	7.6	15.4	16.9
Oxygen saturation [%]	36	12	44	81	162	206
Electric conductivity [µS cm^{-1}]	36	482	512	673	910	1547
Soluble reactive phosphorus [µg L^{-1}]	36	< 1	4	57	370	785
Total phosphorus [µg L^{-1}]	36	25	54	169	574	1210
Nitrate (as N) [µg L^{-1}]	36	< 10	< 10	< 10	1739	2815
Ammonium (as N) [µg L^{-1}]	36	< 10	15.3	31.4	174	2064
Chlorophyll-a [µg L^{-1}]	36	1.3	2.1	13.7	66.2	104

104 µg L^{-1}, which underlines the high productivity of the studied waters.

The number of chironomid taxa varied between 1 and 23 per sample and summed up to 28–70 over the whole season (minimum in 2016, maximum in 2015). In total, 116 taxa of non-biting midges were identified over 5 years from 2011 to 2016 (Table 3). Taxa which reached high abundance were *Hydrobaenus lugubris*, *Limnophyes* sp., *Corynoneura* sp., *Chironomus* sp., *Cricotopus sylvestris* gr., *Cricotopus tricinctus*, *Pseudosmittia* sp., *Polypedilum nubeculosum*, *Acricotopus lucens*, and *Psectrotanypus varius*. Several other taxa such as *Einfeldia pagana*, *Glyptotendipes glaucus/pallens*, and *Paralimnophyes hydrophilus* were found regularly (occurrence > 15%), but mostly in low numbers.

Chironomid abundance varied between 2 and 1078 larvae per m^2 at control sites and between 5 and 1125 larvae per m^2 at impact sites. Culicid density was 8 to 500 ind. per liter at control and impact sites before *Bti* application but dropped to zero (only in 1 case to 14 ind. per liter) after *Bti* had been applied (Fig. 4).

Using non-metric multi-dimensional scaling, it was not possible to graphically distinguish between chironomid communities from *Bti* sites and non-*Bti* sites. However, highlighting in the same plot the 3 years in different colors allows separating samples, and thus chironomid assemblages from different sampling seasons (Fig. 5).

This pattern was supported by the results of the mixed-effects model (Tables 4 and 5). A significant and positive coefficient associated with the BA * CI interaction was found only for the total density of Culididae larvae, while none of the chironomid taxa and the metrics derived from them showed any significant response to the *Bti* application (Table 4). A slight increase was found for gatherers (expressed as product of abundance and guild valences), but the coefficient for the BA * CI interaction was not significant ($p = 0.094$, for relative proportions of gatherers: $p = 0.0727$). Among the fixed factors in the BACI analysis, the spatial variable *Site* was significant in all analyses, which corresponds to a high similarity of samples from the same sampling station independent of time, habitat and impact.

Discussion

One of the challenges of assessing the impact of *Bti* on the ecological integrity of floodplains is their extraordinary spatial-temporal variability (Umweltbundesamt 1999; Tockner et al. 1999; Chaparro et al. 2018). The diversity of inundated forests, reed stands and meadows goes hand in hand with a high physico-chemical and structural variability of the temporary water bodies, which often develop a high productivity. As such they can serve as ecological hot spots in an otherwise desolate and monotone cultivated landscape in Central Europe (Schindler et al. 2016; Tockner et al. 2008). This is also true for the Dyje and Morava River floodplains, which comprise a diverse and complex mosaic of mesotrophic, macrophyte-dominated backwaters and hypertrophic, turbid inundation areas with varying persistence and connection to the main river, as is typical for natural lowland river ecosystems (Hein et al. 2015; Tockner et al. 2008). As a consequence of the abiotic variability, the Morava floodplains provide habitats for a highly diverse biological community and are inhabited by more aquatic species than any other river landscape in Austria. They are home to 70% of amphibian species and about 75% of breeding birds known from Austria (Umweltbundesamt 1999; Zuna-Kratky et al. 2000). A high diversity characterizes also the entomofauna along the Morava river (Lechthaler 1993), as confirmed by the high chironomid taxa richness found in the present study (> 100 taxa).

Chironomids often dominate the invertebrate community in temporary waters and wetlands in terms of species richness (Moller Pillot and Buskens 1990; Dettinger-Klemm and Bohle 1996; Cranston 1995; Wolfram et al. 1999) and usually form a significant proportion of the total invertebrate biomass and secondary production (Lindegaard 1989; Wolfram 1996; Lundström et al. 2009; Williams 2006). As r-strategists, they can establish large individual numbers within short time (Armitage and Cranston 1997; Dettinger-Klemm and Bohle 1996). This strategy is especially pursued by several members of the tribe Metriocnemini (subfamily Orthocladiinae) such as *Hydrobaenus lugubris*, *Limnophyes asquamatus* and *Pseudosmitia* spp., which dominated in the samples in this study along with typical representatives of eutrophic waters such as *Polypedilum nubeculosum* und *Chironomus*. Lechthaler (1993) showed that the Metriocnemini taxa, which prevailed also in emergence traps in his study, developed high abundance in freshly inundated meadows, but decreased in number with ongoing inundation, while other chironomid species became dominant only after a few weeks and at a certain age of their environment.

Table 3 List of chironomid taxa found between 2011 and 2016

	Aug/Sep 2011	Jun/Jul 2013	Aug 2014	Apr 2015	May 2016
Tanypodinae					
Ablabesmyia longistyla	•		•		•
Ablabesmyia monilis		•			
Ablabesmyia phatta			•		
Ablabesmyia sp.			•		
Clinotanypus nervosus	•		•		
Macropelopia sp.					•
Monopelopia tenuicalcar	•		•		
Pentaneurini Gen. sp.	•				
Procladius sp.	•	•	•	•	•
Psectrotanypus varius	•	•			
Tanypodinae Gen. sp. juv.		•			
Tanypus kraatzi	•		•		
Tanypus punctipennis	•		•		
Tanypus sp.	•		•		
Tanypus vilipennis	•				
Thienemannimyia group				•	
Xenopelopia nigricans		•			•
Xenopelopia sp.	•				
Orthocladiinae					
Acricotopus lucens		•		•	•
Bryophaenocladius sp.		•			
Chaetocladius sp.					•
Corynoneura sp.	•	•	•	•	•
Cricotopus cf. *annulator*					•
Cricotopus cf. *tricinctus*		•			•
Cricotopus intersectus			•		
Cricotopus obnixus			•		
Cricotopus ornatus				•	
Cricotopus reversus	•				
Cricotopus sp.		•	•	•	•
Cricotopus sylvestris	•				
Cricotopus sylvestris group	•	•	•	•	•
Diplocladius cultriger					•
Gymnometriocnemus sp.	•				
Hydrobaenus lugubris				•	•
Hydrobaenus sp.		•			
Limnophyes sp.	•	•	•	•	•
Nanocladius bicolor			•		
Orthocladiinae COP sp.		•			•
Orthocladiinae Gen. sp. juv.	•	•	•	•	•
Orthocladius oblidens					•
Orthocladius sp.				•	
Paracladius conversus					•
Parakiefferiella sp.	•				
Paralimnophyes hydrophilus	•	•		•	•

Table 3 (continued)

	Aug/Sep 2011	Jun/Jul 2013	Aug 2014	Apr 2015	May 2016
Psectrocladius cf. *octomaculatus*		•			
Psectrocladius limbatellus group		•	•	•	•
Psectrocladius obvius		•		•	•
Psectrocladius sp.		•			
Pseudosmittia cf. *simplex*		•			
Pseudosmittia sp.	•	•		•	•
Smittia aquatilis group			•		
Smittia sp.	•	•			
Thienemannia cf. *gracilis*					•
Chironominae Chironomini					
Chironomini Gen. sp. juv.	•	•	•		•
Chironomus aberratus/sororius			•	•	
Chironomus annularius agg.	•	•			•
Chironomus annularius/cingulatus		•			
Chironomus cf. *melanescens*				•	
Chironomus commutatus	•				
Chironomus dorsalis		•		•	•
Chironomus luridus/pseudoth./uliginosus	•	•		•	•
Chironomus nuditarsis	•				•
Chironomus plumosus	•		•		
Chironomus riparius/piger	•	•		•	•
Chironomus sp.	•	•	•	•	•
Chironomus type *acidophilus*	•				
Cladopelma cf. *viridulum*			•		
Cladopelma laccophila group	•				
Cladopelma lateralis group	•				
Cryptochironomus sp.			•		
Cryptotendipes sp.	•				
Dicrotendipes modestus	•		•		
Dicrotendipes nervosus	•	•		•	
Dicrotendipes sp.	•		•		
Dicrotendipes tritomus		•			
Einfeldia pagana	•			•	•
Endochironomus albipennis	•				
Endochironomus tendens	•		•	•	
Glyptotendipes barbipes	•		•		
Glyptotendipes cf. *glaucus*	•	•	•	•	•
Glyptotendipes cf. *pallens*	•				
Glyptotendipes sp.	•	•	•	•	
Harnischia sp.	•		•		
Kiefferulus tendipediformis	•		•		
Lauterborniella agrayloides	•				
Microchironomus tener	•				
Microtendipes chloris group	•				
Microtendipes pedellus group			•		
Parachironomus arcuatus group	•			•	•

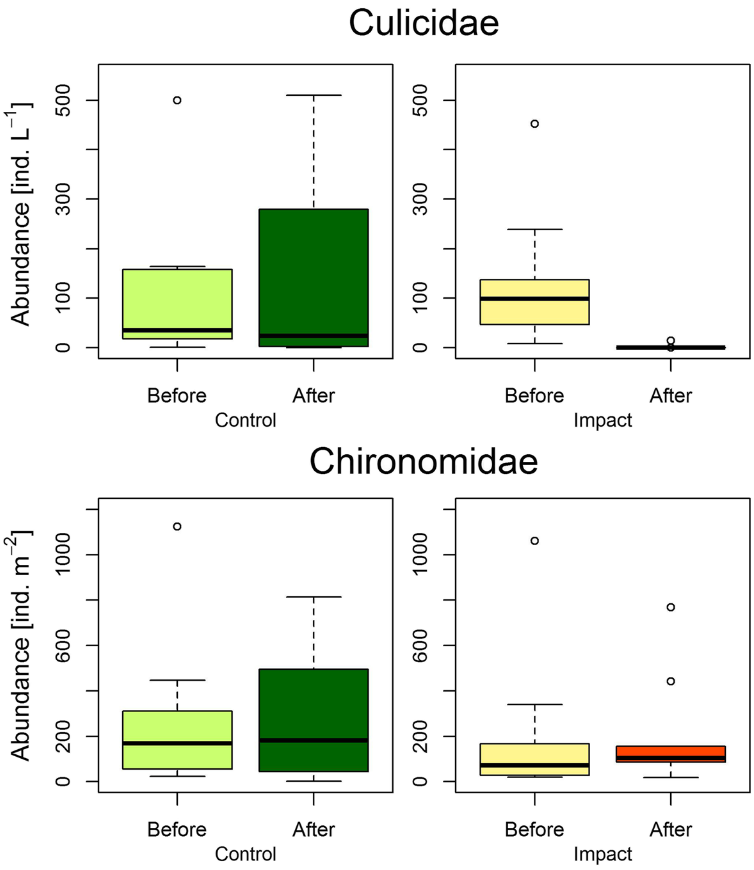
Table 3 (continued)

	Aug/Sep 2011	Jun/Jul 2013	Aug 2014	Apr 2015	May 2016
Parachironomus sp.	•	•	•		
Paratendipes albimanus	•	•	•	•	•
Phaenopsectra sp.		•	•	•	
Polypedilum bicrenatum	•				
Polypedilum cf. *cultellatum*	•				
Polypedilum cf. *tritum*				•	
Polypedilum cf. *uncinatum*	•	•			
Polypedilum nubeculosum	•	•	•		•
Polypedilum scalaenum group	•		•		
Polypedilum sordens group	•	•	•	•	
Polypedilum sp.	•	•	•	•	•
Polypedilum uncinatum					
Zavreliella marmorata			•		
Chironominae Tanytarsini					
Cladotanytarsus mancus group	•				
Cladotanytarsus sp.			•		•
Micropsectra sp.					•
Paratanytarsus austriacus		•			
Paratanytarsus inopertus	•		•		
Paratanytarsus laetipes	•				
Paratanytarsus lauterborni		•			•
Paratanytarsus sp.	•	•			•
Paratanytarsus tenuis group					•
Tanytarsini Gen. sp.	•				
Tanytarsus cf. *ejuncidus*			•		
Tanytarsus cf. *fimbriatus*				•	
Tanytarsus mendax	•				
Tanytarsus sp.	•	•	•	•	

Mosquitoes are opportunists as well and can develop extremely high numbers in freshly inundated areas of the lowland river floodplains (Becker et al. 2010). This is especially true for the so-called inundation mosquitoes such as *Aedes vexans* and *Ochlerotatus sticticus*, which account for 90% of all mosquitoes along the Morava River (unpublished data from CO_2 trap counts). The fact that many mosquitoes and chironomids share their habitats and survival strategy has raised concerns about possible impacts of *Bti* on the non-biting midges. Besides, as both dipteran groups are important prey items for other insects (Corbet 1999; Medlock and Snow 2008), insectivorous birds (Buchanan et al. 2006; Sánchez et al. 2006), bats (N. Vaughan 1997; Gonsalves et al. 2013; Flavin et al. 2001) and amphibians (Cicort-Lucaciu et al. 2005; DuRant and Hopkins 2008), several authors have pointed at the risk of indirect effects via the food web (Jakob and Poulin 2016; Kreuziger 1998).

Our study showed that while the *Bti* application significantly reduced the culicid population, there was no evidence that chironomid diversity and abundance were negatively impacted by *Bti* shortly after the aerial spraying. This is in line with most studies on the impact of *Bti* on chironomids, at least when the dosage remains at levels as recommended for controlling mosquitoes (Duchet et al. 2008; Persson Vinnersten et al. 2009; Lagadic et al. 2013). Bioassays have proven that non-biting midges are susceptible to *Bti* though usually at concentrations which lie one or two orders of magnitude higher than those recommended for the use against mosquitoes (Boisvert and Boisvert 2000; Lacey and

Fig. 4 Boxplots showing the abundance of Culicidae and Chironomidae at control and impact sites before and after the *Bti* treatment (data from the years 2013, 2015, and 2016)



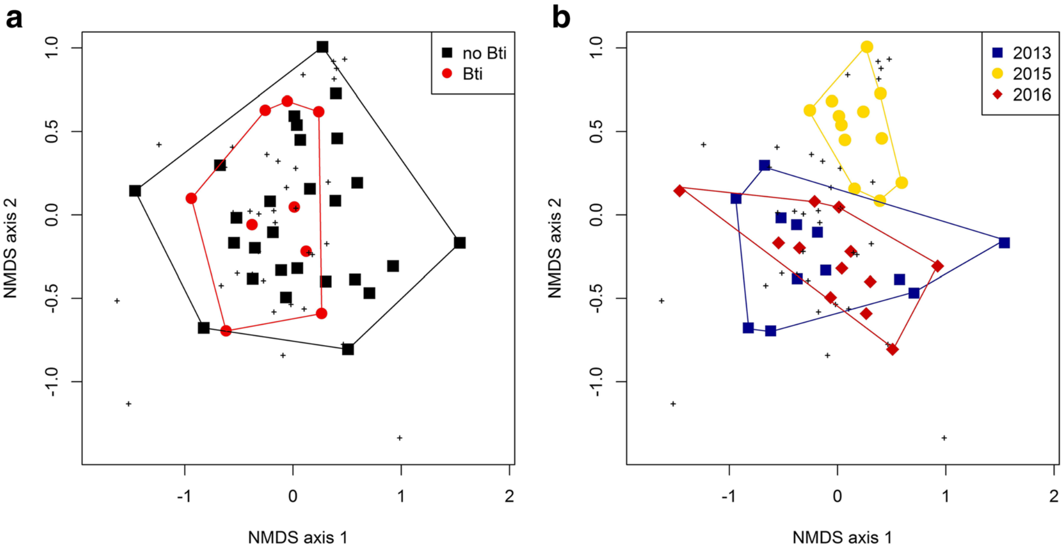


Fig. 5 Non-metric multi-dimensional scaling (nMDS) of log-transformed chironomid abundances on genus level grouped by *Bti* treatment (**a**) and year (**b**), showing samples as large colored symbols and taxa as small crosses. The "no *Bti*" sites in the left plot (black squares) include impact sites before the *Bti* spraying (1st date only) as well as control sites from both sampling dates. Stress 0.184

Table 4 BACI analysis with the parameter estimates and significance tests from linear mixed-effects models fitted to the log-transformed abundance of Culicidae, Chironomidae und chironomid tribes and subfamilies. SE: standard error, P: significance level, Var: Variance

Response	Test	Coefficient	SE	*P*		Var	*P*
Culicidae	Intercept	1.5233	0.2620	*0.0003*	Site	0.2973	*0.001*
	Before vs After = *BA*	− 0.1585	0.1809	0.3924	Year	0.0577	0.429
	Control vs Impact = *CI*	0.4033	0.3143	0.2135	Habitat	0	1.000
	BA * *CI*	− 1.6247	0.2558	*< 0.0001*	Residual	0.1472	
Chironomidae	Intercept	2.1362	0.2250	0.1234	Site	0.1920	*0.005*
	Before vs After = *BA*	− 0.1354	0.1585	0.4044	Year	0.0398	0.621
	Control vs Impact = *CI*	− 0.1870	0.2684	0.5094	Habitat	0.0120	0.954
	BA * *CI*	0.2816	0.2242	0.2251	Residual	0.1124	
Chironomini	Intercept	1.0515	0.2036	*0.0001*	Site	0.2323	*0.004*
	Before vs After = *BA*	0.2978	0.1769	0.1094	Year	0	1.0
	Control vs Impact = *CI*	− 0.1579	0.2879	0.5881	Habitat	0	1.0
	BA * *CI*	0.0617	0.2501	0.8080	Residual	0.1408	
Orthocladiinae	Intercept	2.0459	0.2992	*0.0238*	Site	0.2179	*0.01*
	Before vs After = *BA*	− 0.2202	0.2890	0.4577	Year	0.0752	0.53
	Control vs Impact = *CI*	− 0.2931	0.1874	0.1351	Habitat	0.0452	0.66
	BA * *CI*	0.3420	0.2650	0.2131	Residual	0.1580	

Significant coefficients ($P < 0.05$) are emphasized in italics

Merritt 2003). Rodcharoen et al. (1991) showed that chironomid abundance in mesocosms and in three man-made residential-recreational lakes (8–22 ha) significantly decreased after *Bti* application. However, the authors used various *Bti* formulations and dosage with a final *Bti* concentration varying between 2.7 and 34.8 × 10^9 ITU/ha, which is higher than the dosage recommended along the river Rhine by Becker (2003) (0.75 × 10^9 ITU/ha) or by Russell et al. (2003) (0.38–1.54 × 10^9 ITU/ha). I. P. Vaughan et al. (2008) studied the effects of *Bti* on larval chironomids from eight experimental treatments, over 3 years, on a newly created eutrophic, urban lake of 200 ha and reduced chironomid abundance by up to 35%. The formulation and dosage used corresponds to a concentration of 18 × 10^9 ITU/ha, which is also much higher than the concentration used against mosquitoes. No evidence of negative effects of larvicide treatment on non-target species was found by Hershey et al. (1995) in their study on the effects of *Bti* in a divided pond experiment in Minnesota. Neither density nor biomass of any invertebrate group was decreased, nor was there a treatment-related decrease in richness of benthic invertebrate taxa under drought conditions in 1989. Further studies between 1991 and 1993 revealed a transient decrease of chironomid abundance (Hershey et al. 1998); however, a follow-up study a few years later demonstrated that, after intensive and continuous use of *Bti* for 8 years, total chironomid abundance and biomass remained unaffected (Balcer et al. 1999). No negative effects of *Bti* application on chironomid diversity and secondary production were also found in temporary wetlands of the River Dalälven by Lundstrom et al. (2010).

Poulin et al. (2010) found a lower intake of Nematocera by house martin *(Delichon urbicum)* at treated sites in the Camargue and presumably assigned this finding to a locally lower abundance of insects. However, the study lacks a direct prove of a decrease in chironomid abundance or biomass. Lagadic et al. (2013) criticized environmental discrepancies between treated and control areas (viz. the distance to the sea), which may have been the reason for differences found by Poulin et al. (2010). The same holds true for the study on the impact of *Bti* on non-biting midges sampled by passive traps by Jakob and Poulin (2016).

A recent study by Theissinger et al. (2018) revealed a 65% reduction of chironomid emergence in the Upper Rhine Valley at sites with *Bti* application (1.44 × 10^9 ITU/ha) as compared with control sites, while species composition remained unchanged. In this study, sampling by emergence traps was carried out over 13 weeks, but started *after* the *Bti* application by helicopter. Since

Table 5 BACI analysis with the parameter estimates *(coefficient)* and significance level *(P)* for the interaction *BA* * *CI* from linear mixed-effects models fitted to the (logit-transformed) relative proportions of dominant genera, subfamilies/tribes and feeding guilds, two diversity measures and the standard coordinates of the first two axes of the correspondence analysis (CA, calculated for the whole chironomid community on species/genus level)

Response variables	Coefficient	*P*
Chironomid genera		
% *Pseudosmittia*	0.3675	0.6573
% *Hydrobaenus*	− 0.1321	0.8504
% *Corynoneura*	− 1.3382	0.1966
% *Limnophyes*	1.1706	0.1784
% *Cricotopus*	− 0.7661	0.3752
% *Polypedilum*	− 0.5235	0.4869
% *Chironomus*	0.4750	0.6046
Chironomid subfamilies and tribes		
% Chironomini	− 0.7870	0.3531
% Orthocladiinae	0.7443	0.2828
Feeding guilds		
% Grazers	− 0.8254	0.0727
% Miners	− 1.1523	0.2775
% Shredders	− 0.1837	0.8810
% Gatherers	0.6758	0.2339
% Filterers	− 0.3582	0.6622
% Predators	− 0.5504	0.6431
Chironomid diversity		
Taxa richness *S*	− 0.1111	0.9527
Shannon-Wiener index of diversity *H′*	− 0.1371	0.6829
CA (chironomid community)		
Standard coordinates 1st axis	0.2242	0.1554
Standard coordinates 2nd axis	0.4201	0.3875

the sampling design of this investigation did not follow a BACI design, it is unclear whether the treatment with the larvicide or other factors (location, habitat) was responsible for lower densities of adult chironomids.

Although so far, no field study could demonstrate significant negative side effects of mosquito control with *Bti* on non-target species such as chironomids with sufficient confidence, we cannot exclude such impacts on a local scale or for selected species. The lack of evidence may rather reflect methodological weaknesses and the difficulty to identify single stressor impacts in a complex environment, which is affected by numerous natural and anthropogenic factors. In our study, we pursued a strict BACI design, which is recommended to evaluate natural and human-induced perturbations on ecological variables when treatment sites cannot be randomly chosen (Conner et al. 2016). With the variables Site, Year and Habitat, we integrated time and space in order to avoid a bias from factors other than *Bti*.

The concern about local effects of *Bti* application on non-target species nourishes from doubts that the dosage of *Bti* in the field cannot be sufficiently controlled, since numerous factors such as the wind and water depth can affect the dispersal and the final concentrations of *Bti* in the treated waters. Only few authors took, such as Russell et al. (2003), water depth into account when recommending a dosage for *Bti* application. Becker (2003) suggested to use double dosage in deeper waters or in case that mosquito larvae have reached the 4th instar. This of course raises the risk of side effects on non-target species, since the distribution of *Bti* within a water body can hardly be predicted and a decrease of the water level of temporary waters may naturally lead to higher *Bti* concentrations.

An issue not addressed in this study is the possible impact of *Bti* on early larval instars of chironomids. Since a net with a mesh-size of 500 μm was used, most chironomids identified and counted belonged to 4th, 3rd, and partly 2nd instar, whereas 1st instar larvulae were largely lacking in the samples. Therefore we cannot exclude side effects on young chironomid larvae even at the dosage used to control the culicids, as several studies have proven that the sensitivity of non-biting midges to *Bti* decreases from 1st to 4th instar (Kästel et al. 2017; Hughes et al. 2005; Stevens et al. 2004; Lei et al. 2005).

In our study, due to the high variability of the floodplains, only short-term effects on culicid and chironomid larvae were analyzed. We have no sound data on the overall reduction of adult mosquitoes (and other Dipteran families) in the Morava floodplains.

Long-term effects (at least over several weeks) on culicid and chironomid larvae could not be analyzed with sufficient statistical confidence as a result of a rapidly changing environment. The unpredictive way of draining and drying out of the temporary waters (some of which totally disappearing after a few days or weeks only) did not allow defining unambiguous additional explanatory factors and made it necessary to exclude the data from the 2nd sampling date after the *Bti* applications. The data available from this date, however, do not indicate any decrease of abundance, which might arise from the above-mentioned enhanced sensitivity of early instar larvae against *Bti*. This methodological limitation underlines the need of further studies on long-term effects, in order to reveal possible delayed effects, which may become more relevant as new *Bti* formulations are developed by genetic engineering techniques in order to increase the efficacy and persistence in the environment (Federici et al. 2007; Park et al. 2005; Mittal 2003).

Further emphasis should also be placed on identifying possible impacts on non-target organisms during different times of the year. Although the sampling seasons of our study covered a rather narrow time window, with campaigns in April (2015), May (2016), and June/July (2013), there were obvious differences in the taxonomic composition of the chironomid assemblages. The data from April 2015 clearly deviated from those of the other 2 years (Fig. 5), mainly as a result of the dominance in the 2015 samples of *Hydrobaenus lugubris*, which is a typical representative of the spring fauna in temporary waters (see Steinhart (2000)). This finding underlines the high temporal variability of the species assemblage and makes clear the need for caution in extrapolating from results based on a few sampling occasions.

To minimize hidden or indirect effects on predators of mosquitoes and chironomids ((Kreuziger 1998; Jakob and Poulin 2016) we recommend to establish and maintain a high level of documentation, control and regular training, when *Bti* is applied over a wide area.

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