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

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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 nontarget 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 nontarget 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 versus 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.

KEYWORDS

Bacillus thuringensis var. *israelensis*, biodiversity loss, community metabarcoding, nonbiting midges, operational taxonomic units, species turnover

1 | INTRODUCTION

The Upper Rhine Valley is one of 30 "hotspots of biodiversity" in Germany (Ackermann et al., 2012). The river Rhine is the largest river in Germany, which 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 (Lukács, Sramkó, & Molnár, 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; Lukács 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, nonbiting midges (Diptera: Chironomidae) are one of the most dominant taxa (Milošević et al., 2013; Puntí, Rieradevall, & Prat, 2009), 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 (Lindegaard & Brodersen, 1995; Milošević et al., 2013; Rossaro, Lencioni, Boggero, & Marziali, 2006), 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; Pfitzner, Beck, Weitzel, & Becker, 2015; Poulin, Lefebvre, & Paz, 2010; Stav, Blaustein, & Margalit, 2005). 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; Allgeier, Kästel, & Brühl, 2019; Theissinger et al., 2018). Although Bti is considered environmentally friendly, the nonbiting midges are the most Bti-sensitive nontarget 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, Gill, & Soberon, 2011). Several other studies have previously shown Bti nontarget effects on chironomids with abundance reductions ranging from 35%–80% (Hershey, Shannon, Axler, Ernst, & Mickelson, 1995; Jakob & Poulin, 2016; Liber, Schmude, & Rau, 1998; Poulin et al., 2010; Rodcharoen, Mulla, & Chaney, 1991; Vaughan, Newberry, Hall, Liggett, & Ormerod, 2008). 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; Kästel, Allgeier, & Brühl, 2017; Ping, Wen-Ming, Shui-Yun, Jin-Song, & Li-Jun, 2005; Treverrow, 1985), and with different sensitivities among species (Yiallouros, Storch, & Becker, 1998). In a mesocosm study with seminatural conditions Liber et al. (1999) 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 predatory 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 (Beermann, Zizka, Elbrecht, Baranov, & Leese, 2018; Carew, Pettigrove, Metzeling, & Hoffmann, 2013; Theissinger 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 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 or groundwater, respectively, and therefore the soil is still moist, even when the wetland has dried out. (i) We thus hypothesized 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 were 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 three independent 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 a 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) was sampled in spring and summer 2016. This floodplain was 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) were sampled in spring 2016. Here, 12 temporary ponds were sampled of which some had been regularly treated with Bti for 20 years ($N = 4$), whereas others had 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 1.44×10^9 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 versus 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, Appendix S1). 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 1,270 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, Appendix S1). Consequently, this resulted in 17 forest samples for metabarcoding. A detailed list of all samples per site and number of individuals pooled for metabarcoding is in Appendix S1. 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

	Meadow		Floodplain		Forest		
	Bti	Control	Bti	Control	Bti	Control	Unknown
Bti history	20 years	4 years ago[a]	First year	Never	20 years	Never	NA
N ponds	1	1	1	1	4	5	3
N emergence traps	12	12	12	18[b]	12	15	9
Chironomid abundance	1,138	3,527	542	923	354	1,522	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[a]	4	10[c]	3

Note: 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.
[a]Four years since first Bti intermittence after 20 years of continuous Bti application.
[b]Floating emergence traps ($N = 6$) not included in Bti effect analyses.
[c]One sample that was split in six subsamples.

2.2 | Laboratory methods

Pooled chironomid samples for all sites and treatments (N = 119) were dried for at least 24 hr at 60°C. Specimens were grinded using the Tissue Lyser II (Quiagen) at 30 Hz for 3 × 1 min using two metal beads (3 mm, Hobbyfix, Opitec) 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. Fifty µl of DNA extract were treated with 0.55 µl RNAse (10 mg/ml, Roth) and incubated at 37°C for 30 min followed by purification using a MinElute Reaction Clean up Kit (Qiagen) according to manufacturer's instructions. The DNA concentration was measured using Nanodrop spectroscopy and concentrations of all samples were adjusted to approximately 20 ng DNA/µl. For DNA concentrations per technical replicate see Appendix S1.

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), 1 µl DNA/amplicon template under the following cycling profile: 94°C for 3 min, 25 cycles (15 cycles in second PCR) of 94°C for 30 s, 50°C for 30 s, 65°C for 120 s and ended with 65°C for 5 min. 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). The library was purified and size selected (retaining fragments of >300 bp) with left size selection of magnetic beads (SpriSelect, Beckmann Coulter, ratio: 0.76×). Purified PCR products were pooled into a library proportional to the number of specimens in each sample (see Appendix S1) to ensure all specimens are sequenced with comparable sequencing depth. The library was sent to an external laboratory (GATC) for 2 × 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).

2.3 | Bioinformatic analysis

Raw data were processed with R JAMP (https://github.com/VascoElbrecht/JAMP, last accessed on 06/08/18, R script available in Appendix S2). 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).

2.4 | Statistical analysis

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 (Bærholm Schnell, Bohmann, & Gilbert, 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 (Appendix S3). To estimate whether the read depth was sufficient to cover all chironomid OTUs in our samples we calculated an octave plot according to Edgar and Flyvbjerg (2018), where the number of OTUs were plotted against the (binned) read abundances (for more details see Appendix S3). All statistical analyses were conducted in R (R Core Team, 2017).

2.4.1 | 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., 2016).

2.4.2 | 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 pretransformation, 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., 2016).

The OTU presence or absence in pooled Bti versus 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 an 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 Jaccard matrix of presence absence community data between samples and 999 permutations, using the command adonis in the R package VEGAN v. 2.5-2 (Oksanen et al., 2016). 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 Appendix S4) at Bti-treated versus 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 (Appendix S3). The octave plot (Figure S3, Appendix S3) 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 (five OTUs). Technical replicate read abundances were in the same order of magnitude for all samples (Appendix S3), indicating reliable results. Negative controls showed only few reads in some samples for especially high abundant OTUs (Appendix S3) 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 (a) similarities were <97% to the best matching BOLD sequences; (b) only the genus was provided in BOLD; or (c) 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, Appendix S4). 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, Appendix S4). 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 (Appendix S4). 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 (Appendix S4; Moog, 1995, 2002).

TABLE 2 Operational taxonomic units (OTU) presence at different sites (meadow, floodplain, forest) and treatments (Bti vs. control) across 57 samples [Colour table can be viewed at wileyonlinelibrary.com]

OTU	Genus	Species	Meadow		Floodplain		Forest	
			Bti *N* = 12	Control *N* = 12	Bti *N* = 12	Control *N* =1 2	Bti *N* = 4	Control *N* = 5
OTU_1[a]	*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[a]	*Chironomus*	NA	0	17	50	58	50	0
OTU_6	*Chironomus*	NA	8	83	0	8	0	0
OTU_7[a]	*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[a]	*Xenopelopia*	*nigricans*	25	58	67	67	25	20
OTU_13	*Chironomus*	*melanotus*	0	8	42	83	0	0
OTU_15[b]	*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[b]	*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[a]	*Limnophyes*	*minimus*	0	8	8	8	100	80
OTU_46	*Paratanytarsus*	*tenellulus*	0	17	50	25	0	0
OTU_48[b]	*Procladius*	*fuscus*	0	17	0	0	0	0
OTU_49[b]	*Limnophyes*	NA	0	17	0	0	0	0
OTU_50[a]	*Limnophyes*	sp. 14ES	50	83	42	33	75	40
OTU_51[b]	*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[b]	*Procladius*	sp. ES02	0	17	0	0	0	0
OTU_66	*Paratanytarsus*	*grimmii*	0	0	25	25	0	0
OTU_67[a]	*Limnophyes*	*asquamatus*	8	0	0	8	75	40
OTU_68	*Limnophyes*	NA	0	33	0	0	0	0
OTU_69	*Pseudosmittia*	sp. BOLD:AAG6458	0	0	0	0	50	0
OTU_70	*Chironomus*	*acidophilus*	0	0	8	0	0	0
OTU_74[b]	*Zavrelimyia*	*barbatipes*	0	17	0	0	0	0
OTU_76[b]	*Chironomus*	*melanescens*	0	0	8	0	0	0
OTU_77[a]	*Kiefferulus*	*tedipediformis*	0	8	17	17	0	20
OTU_78[b]	*Limnophyes*	NA	8	8	0	0	0	0
OTU_79	*Corynoneura*	*carriana*	8	17	0	0	0	0
OTU_80[b]	*Micropsectra*	NA	0	17	0	0	0	0
OTU_82	*Tanytarsus*	*usmaensis*	0	17	25	0	0	0
OTU_84[b]	*Limnophyes*	*pentaplastus*	0	8	0	0	0	20
OTU_85	*Cricotopus*	*sylvestris*	33	25	8	17	0	0
OTU_88[b]	*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[b]	*Corynoneura*	*coronata*	8	0	0	0	0	0
OTU_99	*Psectrotanypus*	*varius*	17	25	0	0	0	0
OTU_105[b]	*Pseudosmittia*	sp. BOLD:AAM6263	0	0	0	0	25	0

(Continues)

TABLE 2 (Continued)

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[b]	*Paratendipes*	*albimanus*	0	0	8	0	0	0
OTU_116	*Polypedilum*	*tritum*	0	8	8	8	0	0
OTU_119[b]	*Tanytarsus*	*heusdensis*	0	8	0	0	0	0
OTU_122	*Chironomus*	*pseudothummi*	0	0	0	0	0	0
OTU_126[b]	*Synendotendipes*	*impar*	0	0	8	0	0	0
OTU_128[b]	*Polypedilum*	*cultellatum*	0	8	0	0	0	0
OTU_129[b]	*Paratanytarsus*	*dissimilis*	0	8	0	0	0	0
OTU_132[b]	*Micropsectra*	NA	0	8	0	0	25	0
OTU_133	*Microtendipes*	*chloris*	0	17	0	0	0	0
OTU_134[b]	*Macropelopia*	*nebulosa*	0	8	0	0	0	0
OTU_135[b]	*Polypedilum*	*cultellatum*	0	8	0	0	0	0
OTU_136[b]	*Glyptotendipes*	sp. 2sc	0	0	0	0	0	0
OTU_137[b]	*Phaenopsectra*	*flavipes*	0	8	0	0	0	0
OTU_138	*Micropsectra*	*atrofasciata*	0	0	0	0	0	40
OTU_140[b]	*Metriocnemus*	*eurynotus*	0	8	0	0	0	0
OTU_141[b]	*Micropsectra*	*lindrothi*	0	8	0	0	0	0
OTU_150[b]	*Zavrelimyia*	*barbatipes*	0	8	0	0	0	0
OTU_156[b]	*Polypedilum*	NA	0	8	0	0	0	0
OTU_157[b]	*Tanytarsus*	*eminulus*	0	8	0	0	0	0
OTU_158[b]	*Georthocladius*	sp. BOLD:ACD9509	0	0	0	0	0	20
OTU_160[b]	*Polypedilum*	NA	0	8	0	0	0	0
OTU_171	*Xenopelopia*	NA	8	8	0	0	0	20
OTU_178[b]	*Procladius*	NA	0	17	0	0	0	0
OTU_181[b]	*Paratanytarsus*	*laccophilus*	0	8	0	0	0	0
OTU_184[b]	*Limnophyes*	*natalensis*	0	0	0	0	25	0
OTU_188[b]	*Tanytarsus*	*volgensis*	0	0	8	0	0	0
OTU_198[b]	*Chironomus*	*pseudothummi*	0	0	0	0	0	20
OTU_205[b]	*Smittia*	*edwardsi*	0	0	0	17	0	0
OTU_206[b]	*Smittia*	NA	0	0	8	0	0	0
OTU_220	*Smittia*	sp. 8ES	0	0	0	0	0	40
OTU_233[b]	*Polypedilum*	NA	0	8	0	0	0	0
OTU_237[a]	*Limnophyes*	sp. 14ES	33	58	8	25	25	0
OTU_250[b]	*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_283[b]	*Polypedilum*	NA	0	0	0	0	0	40
OTU_295[b]	*Chironomus*	NA	0	8	0	0	0	0
OTU_296[a]	*Polypedilum*	*tritum*	42	83	8	0	50	60
OTU_298[b]	*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_317[b]	*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

Note: 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. Colour intensity corresponds to the frequency of an OTU across *N* samples.

[a]OTUs shared among all three sites.

[b]Rare OTUs: present in only one or two samples.

3.3 | Chironomid community composition at different study sites

The chironomid communities were characterized by a high spatial heterogeneity within each site, i.e., emergence samples differed greatly in their OTU composition between the traps. At the meadow site, 76 OTUs were detected 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). 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 the most frequent OTUs were 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.

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; AppendixS3). In contrast, out of the 108 chironomid OTUs, 44 OTUs were recorded in only one or two of all samples (Table 2; Appendix S3), and thus 40.7% of the detected OTUs can be classified as rare taxa in this study. 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).

3.4 | Bti effects on chironomid community composition

For the meadow site, overall we detected 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 versus 10 and 7 versus 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).

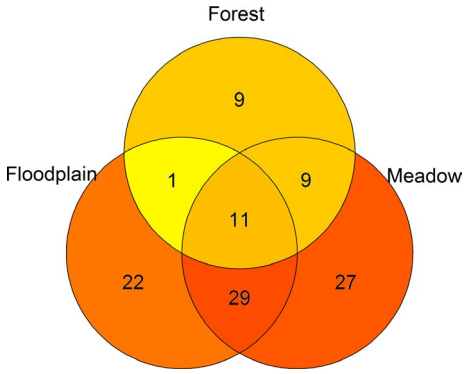

FIGURE 1 Venn diagram showing number of detected and shared operational taxonomic units (OTUs) per site across all samples analysed [Colour figure can be viewed at wileyonlinelibrary.com]

The correspondence analysis (Figure 5) depicts, with a total explained variation of 16%, the constrained ordination of the community composition in terms of OTU distribution for the three sites and treatments (Bti vs. control). The model showed that there was a significantly different distribution of OTUs across sampling sites and treatments (envfit: R^2 = 0.89; p = .001). There was a slight ellipsoid overlap across sites, and a stronger overlap between Bti and control samples per site (Figure 5).

The Sørensen pairwise 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 = .0009) but not for floodplain (p = 1.0) and forest (p = .9013; Appendix S3). 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 = .002).

When focusing on the feeding strategy of the species, the OTU presence of the 19 predatory taxa (Appendix S4) was lower in 17 detections (63.0% of all detections, Table 4) and higher in four detections (14.8% of all detections, Table 4) at Bti-treated versus the respective control samples across all study sites. Similarily, of the 26 filterer taxa the OTU presence was lower in 24 detections (64.9%) and higher in eight detections (21.6%; Table 4).

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 apparent 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

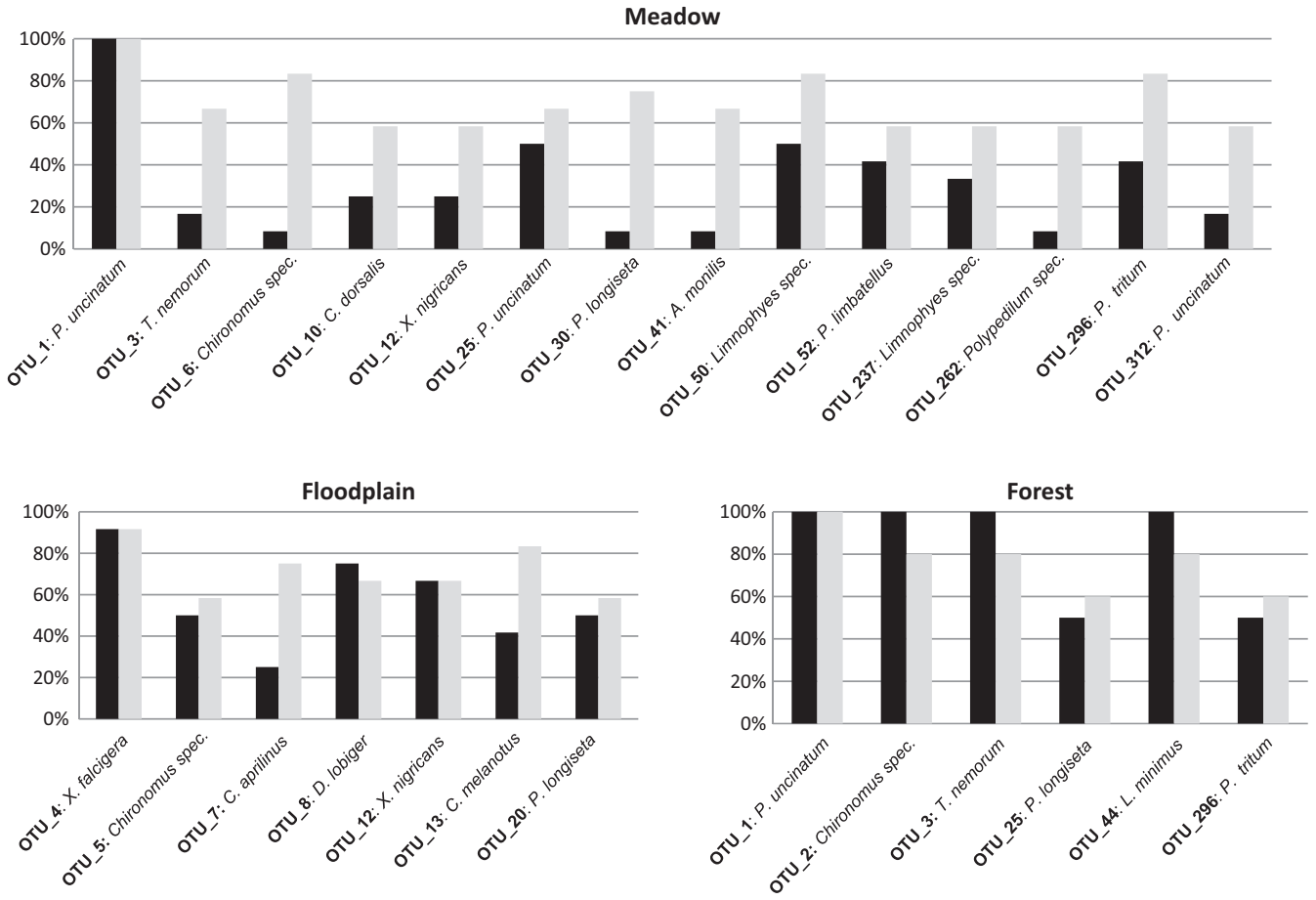

FIGURE 2 Most frequent operational taxonomic units (OTUs) per site and treatment (Bti-treated, black; control, light grey). Given is the OTU presence across all samples in %

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).

4 | DISCUSSION

In this study, we investigated the impact of mosquito control actions with the biocide Bti on the community composition of the nontarget 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 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 first or second 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 a 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 recolonization 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 Appendix S3). Out of the 55 chironomid

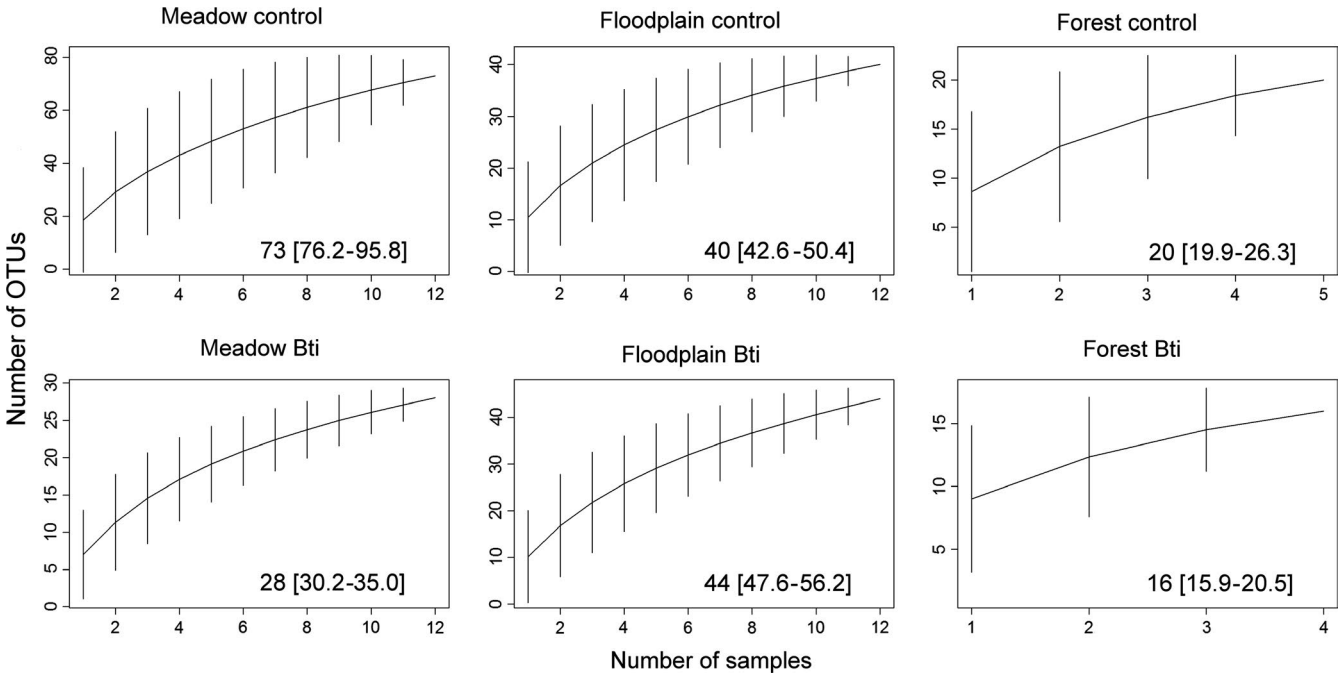

FIGURE 3 Exact site-based species accumulation curves based on operational taxonomic units (OTUs) for all sites separated by treatment. Given are the number of detected OTUs and the expected range of OTU numbers (bootstrap value ± *SE*) per site

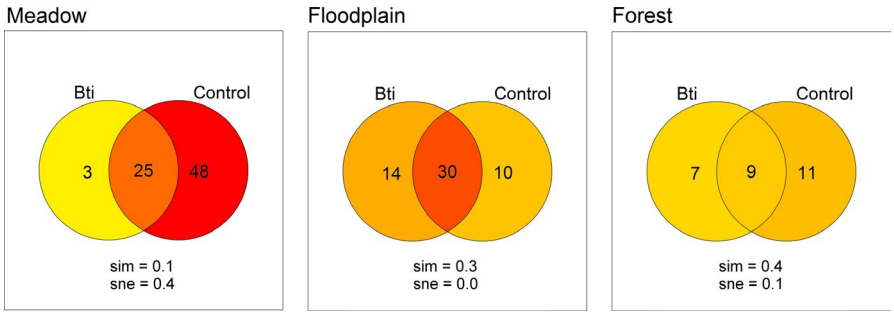

FIGURE 4 Venn diagram showing the numbers of detected and shared OTUs per site and treatment (Bti vs. control). Given are all the Simpson dissimilarity index (sim) as the OTU turnover component of the Sørensen dissimilarity, and the nestedness-resultant fraction of the Sørensen pairwise dissimilarity (sne) as measure for an OTU reduction, based on pooled communities per site and treatment [Colour figure can be viewed at wileyonlinelibrary.com]

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). In particular, *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 in the chironomid communities among the traps within each site, which was most likely due to 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 diverse chironomid communities across the three study sites provided a good basis for testing potential Bti-induced nontarget effects across a wide range of chironomid OTUs in all three mosquito-control relevant wetland types.

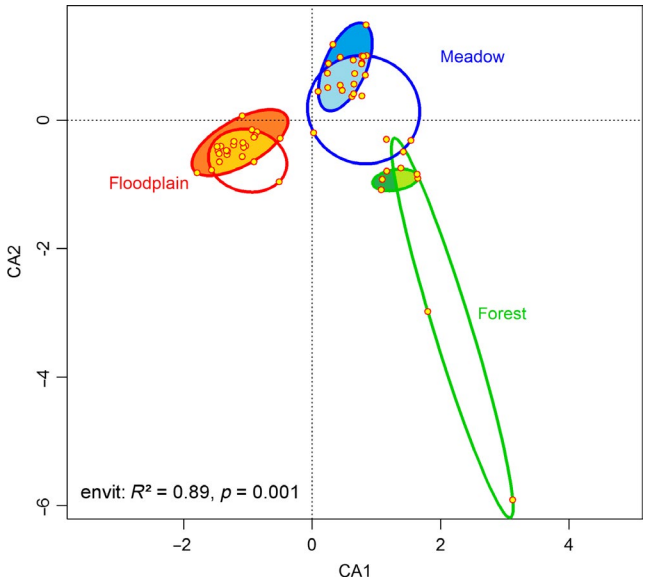

FIGURE 5 Correspondence analysis. Dots represent sample, ellipsoids represent the significant best fit of OTU composition on the environmental samples and enclose all points in the group. Filled ellipsoids: Bti-treated samples; transparent ellipsoids: control samples [Colour figure can be viewed at wileyonlinelibrary.com]

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 versus control samples (Figure 4). The correspondence analyses (Figure 5) showed that the sites, including hydraulic and other biotic and abiotic differences, had the biggest influence on species composition. However, Bti treatment also might have an effect as indicated 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 (Appendix S3, 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, Appendix S3). This species turnover within the chironomid community might also have cryptic effects on ecosystem functioning through altered trophic interactions (Benke, 1998).

TABLE 3 Results from the PERMANOVA on the effect of treatment at the three study sites

	df	Sum of squares	Mean squares	*F* model	R^2	*p*
Meadow						
Treatment	1	0.892	0.892	3.179	0.126	.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	.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	.450
Residuals	7	1.450	0.207		0.877	
Total	8	1.655			1.000	

Note: F model, *F* statistic of the respective submodel.
Abbreviation: *df*, degrees of freedom.
Significance theshold: $p < .05$

TABLE 4 Operational taxonomic units (OTU) presence of predatory and filter feeding taxa at Bti-treated samples as compared to controls across all samples (P) per 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

Note: Higher, $P_{Bti} > P_{control}$; equal, $P_{Bti} = P_{control}$; lower, $P_{Bti} < P_{control}$; absent, OTU not present at this site.

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 (Allgeier et al., 2019; Liber et al., 1998) it is conceivable that predatory chironomids might be both directly and indirectly affected through the food chain: Tanypodinae in the first 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 first instar larvae can be assumed due to probably the same Bti receptors as in the digestive system of Tanypondinae. As second instar larvae Tanypodinae then switch to the predatory feeding type and feed on first and second 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 second 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 has already been 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, second instar larvae are half as sensitive compared to the most sensitive first 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 nontarget 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 (Jakob & Poulin, 2016; Poulin et al., 2010). 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 (Carew, Pettigrove, Cox, & Hoffmann, 2007, 2013; Cranston, 2000; Marzali et al., 2010; Nicacio & Juen, 2015; Pettigrove & Hoffmann, 2005). 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

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

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	*Coronate*	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[a]	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

(Continues)

TABLE 5 (Continued)

2016			2013		
Genus	**Species**	**Bti/control?**	**Genus**	**Species**	**Bti/control?**
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		

Note: 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 as not available (NA).
[a]Species was present in the data set with two operational taxonomic units (OTUs): OTU_116 was only present in C, OTU_296 was present in C + B.

and suggest avoiding applications especially in nature protection reserves to enhance ecological resilience and prevent an ongoing biodiversity loss.

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AUTHOR CONTRIBUTIONS

Sampling: A.F., S.A.; Laboratory work: N.R., A.J.B.; Bioinformatic data analyses: N.R.; K.T.; Biological data analyses: K.T., N.R., S.A., S.M.; Multivariate statistics: N.R.; Study design and supervision: K.T., C.A.B., K.S.; Manuscript writing: K.T. All authors edited and commented on the manuscript draft.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

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

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