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Microbial communities and nutrient dynamics in experimental microcosms are altered after application of a high dose of *Bti*

Running head: *Bti* and aquatic microbial community interactions

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Summary

1. *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) is the most widely used biopesticide against mosquitoes and blackflies, with a history of high specificity and efficacy. High doses of *Bti* have been suggested for extended vector control in some environments; however, the effects of *Bti* application on the native microfauna in the environment are poorly understood.

2. Two *Bti* (VectoBac G) treatments (high = 48.1 kg ha^{-1}; low = 0.6 kg ha^{-1}), in addition to an untreated control, were assigned to replicate 300 L microcosms.

3. *Culex* abundance, phytoplankton biomass, sestonic particulates, and nutrients were reduced significantly in the high *Bti* treatment. These changes affected other physicochemical variables in the water column during the 44-day field study.

4. Bacterial communities present in the water column were assessed by Illumina sequencing of 16S rRNA genes. The most abundant aquatic bacteria in microcosms subject to a low dose of *Bti* and untreated control, *Cyanobacteria*, *Cytophagales*, *Cyclobacteriaceae* (phylum *Bacteroidetes*) and *Sphingomonas* (class *alphaproteobacteria*), were suppressed in microcosms subject to a high dose of *Bti.* Bacteria in the high *Bti* treatment were dominated by *Mucilaginibacter*, *Sediminibacterium* (phylum *Sphingobacteria*) and *Polaromonas* (class *betaproteobacteria*), and were more diverse than in the other treatments.

5. *Synthesis and applications*. The biotic and abiotic changes resulting from a biopesticide application that significantly reduced mosquito abundance by more than 50% persisted longer than the period during which larval mosquito numbers were reduced. This warranted

further investigation into the ecosystem-level effects of *Bti* application rates used routinely for mosquito control. Application rates greater than the label rate for a *Bti* biopesticide can reduce mosquito abundance for an extended period of time and therefore lessen the operational cost of repeated application, but they could exceed manufacturer's recommendations and might violate country-specific regulations governing biopesticide applications in natural habitats. Results of our study suggest that the widespread adoption of *Bti* application above the recommended label rate should be discouraged in habitats where algal abundance and its effect on primary production, microbial communities and nutrient cycling could affect the functioning of aquatic food webs.

Keywords: nutrient dynamics, bacteria communities*, Bti, Culex* mosquitoes, outdoor aquatic microcosms, unintentional effects, phytoplankton, biopesticide, community interactions, vector control

Introduction

Bacillus thuringiensis subsp. *israelensis* (*Bti*) is a commercially successful microbial control agent for dipteran insects, which act as disease vectors (mainly mosquitoes and black flies; Lacey & Merritt 2003). The widespread adoption of *Bti* is not only due to its effectiveness and high specificity, but also its highly favourable ecotoxicological profile as compared to other synthetic pesticides. The effects of *Bti* on nontarget invertebrates and vertebrates have been intensively studied, with no known detrimental effects found to date at dosages recommended for operational mosquito control (Lagadic, Roucaute & Caquet 2014; Lagadic & Caquet 2014). However, possible interactions of *Bti* with microbial communities and effects on ecosystem-level processes associated with aquatic mosquito habitats are unexplored (Boisvert & Boisvert 2000).

Several studies demonstrated an indirect positive effect of *Bti* application on aquatic microbial communities. A 4.5-fold increase in the abundance of protozoans (i.e. *Amoeba*, *Ciliophora*, *Zoomastigophora*) was observed in *Bti*-treated wetlands (15 kg VectoBac G ha^{-1}) compared to untreated control wetlands (Östman, Lundström & Persson Vinnersten 2008). Similarly, an increase in some bacterial (e.g. *Flavobacteriaceae*) taxa in tree-hole mosquito larval habitats resulted when *Bti* was used against *Aedes triseriatus* Say larvae (Kaufman, Chen & Walker 2008; Xu *et al*. 2008). In each of these studies, the authors attributed changes in microbial communities to the removal of mosquitoes, suggesting that larval mosquitoes are responsible for "top–down" regulation of resources. Mosquito larvae are known predators of micro-organisms (i.e. protozoa, bacteria, algae and microcrustaceans) and feed on other organic matter as well (Merritt, Dadd & Walker 1992).

Contrary to the expected outcome of such a top–down hypothesis, Su & Mulla (1999) reported a significant reduction in the abundance of two microalgal species (*Closterium* sp. and *Chlorella* sp.) in microcosms treated with high doses of *Bti* to control *Culex* mosquito larvae. Algal biomass was expected to increase as a result of *Bti* application, but the opposite occurred. The authors suggested that the *Bti* treatment applications improved water quality as a result of phytoplankton suppression. However, Su & Mulla (1999) did not characterize bacterial communities or measure other key water quality indicators to support this hypothesis.

Understanding the effects of *Bti* application on microbial communities and water physicochemistry in mosquito habitats, such as treatment wetlands or other aquatic ecosystems has immense importance for several reasons. First, a reduction in autotrophs (e.g. algae) that comprise the base of many food chains might have direct or indirect effects on fish and other aquatic ecosystem inhabitants (Chen & Folt 2005; Jackson *et al*. 2013). Reductions in primary production are known to lower the abundance and production of the insect groups that are important components of wetland food webs (e.g. Hershey *et al*. 1998; Poulin, Lefebvre & Paz

2010; Lagadic, Roucaute & Caquet 2014). Secondly, microbial communities play important roles in recycling nutrients in treatment wetlands and potential algicidal and antibiotic effects of *Bti* (Su & Mulla 1999; Yudina *et al*. 2003) could have a dramatic impact on the efficacy of a treatment wetland. Thirdly, the impact of mosquito-control operations on water quality is under increasing scrutiny. The current environmental assessment protocols for biopesticides do not typically consider micro-organisms, instead focusing on a few physicochemical variables and visual inspection. Most of the existing assessment protocols monitor environments following pesticide applications for less than a week and may fail to recognize potentially important changes in aquatic ecosystem communities and processes caused by mosquito control agents. Despite studies suggesting *Bti* applications impact aquatic components (e.g. Hershey *et al*. 1998; Su & Mulla 1999; Boisvert & Boisvert 2000), knowledge is lacking about the effects of *Bti* application on microbial communities, which are important components of food webs and essential contributors to nutrient transformation in aquatic ecosystems.

In this study, we investigated the effects of a commercial *Bti* formulation used for mosquito control, VectoBac G, in replicate outdoor freshwater microcosms focusing primarily on the microbial communities and water quality variables. We duplicated the high application rate used previously by Su & Mulla (1999) and tested the null hypothesis that application rates lower or higher than the recommended label rate for VectoBac G (11.2-22.4 kg ha^{-1} in polluted environments) in field conditions have no effect on microbial community structure and water column physicochemistry.

We report an in-depth characterization of multiple environmental variables and microbiota in freshwater microcosms following a single *Bti* application to control mosquitoes. After *Culex* mosquito abundance was reduced by the high *Bti* treatment, phytoplankton biomass, sestonic particulates, and nutrients were reduced significantly. The composition and diversity of bacterial

communities in microcosms subject to high *Bti* treatment differed significantly from those in untreated controls and those subject to a very low *Bti* treatment, which did not affect the larval mosquitoes. These changes also affected other physicochemical variables in the water column. The effects on some of the environmental variables in the high *Bti* treatment persisted well after the period that mosquito abundance was reduced. Our findings suggest that high application rates of biopesticides that significantly reduce *Culex* mosquito abundance might affect key environmental variables in sensitive aquatic habitats that are subjected to mosquito control.

Material and methods

Experimental design and treatments

This study was conducted outdoors in fibreglass microcosms at the Aquatic and Vector Control Research Facility of the University of California Riverside Agricultural Experiment Station. Twelve microcosms (area = 1 m^2) were flooded to 0.3-m depth (300 L) on 28 September 2012. The microcosms were enriched with 40 g of ammonium sulphate [$(NH_4)_2SO_4$; 21% nitrogen and 24% sulphur; Lilly Miller Brands, Walnut Creek, CA)] and 50 g of rabbit pellets (17% crude protein) (Brookhurst Mill, Riverside, CA) to promote mosquito colonization (Nguyen, Su & Mulla 1999; Duguma & Walton 2014). The ammonium nitrogen concentration in the water column was 41.1 (± 0.58 SE) mg L^{-1} 24 h after enrichment. Similar nutrient levels are found in treatment wetlands in California (Popko *et al*. 2006). On 2 October 2012, two *Bti* treatments (low = 0.6 kg kg^{-} $ha^{-1}$and high = 48.1kg ha^{-1}) and an untreated control treatment were assigned to the microcosms using a completely randomized experimental design. A corncob granule formulation of *Bti* (VectoBac G; Valent Biosciences Corporation, Libertyville, IL, USA) with a toxicity of 200 ITU mg^{-1} was used. The application rates (equivalent to 12 000 ITUs and 962 000 ITUs per microcosm, respectively) were selected based on a previous study using a similar formulation (Su & Mulla 1999). The high *Bti* treatment used in the present study was approximately twice

the recommended maximum concentration (22.4 kg ha^{-1}) of VectoBac G for mosquito control in enriched habitats, whereas the low *Bti* treatment was below the minimum recommended rate (2.8 kg ha^{-1}) for mosquito control in comparatively less enriched habitats.

Phytoplankton and sestonic particulates

A single 1-L sample was taken below the water surface in the centre of each microcosm on six dates during the 44 days of the experiment reported here (Table S1). Samples were immediately placed on ice and transported to the laboratory. Phytoplankton biomass was estimated as the concentration of chlorophyll *a* in duplicate samples of 25–100-mL. Samples were filtered through 45-mm membrane filters (0.8-μm pore size) under darkened conditions in the laboratory and then frozen at -20 ºC. Filters were ground using a Teflon mortar and pestle and then pigments were extracted in 90% alkaline acetone (Wetzel & Likens 1991). After centrifugation at 262 × *g* for 5 min, pigment concentration was determined using a Biospec-1601 UV-Visible spectrophotometer (Shimadzu Scientific, Columbia, MD) following method 10200H of APHA (1995).

Sestonic particle distributions (equivalent spherical diameter [ESD]: 0.6– 224 μm) were enumerated using a Multisizer 4 Particle Analyzer (Beckman Coulter Inc., Brea, CA, USA). The particles in bulk water samples were quantified in 3–8 replicate counts of 0.5–2-mL samples using apertures of 100 μm and 280 μm. Approximately 10 mL of bulk water was filtered through a 10-μm aperture mesh and the particle distribution (0.6–10 μm ESD) in 0.1– 0.2 mL filtered water was determined in three 50-µL replicates using a 20-μm or 30-μm aperture. Blanks consisted of either unfiltered (for 100-µm and 280-μm apertures) or filter-sterilized (0.2-μm pore size; for the 20-μm and 30-μm apertures) electrolyte (ISOTON II). Particle concentration was categorized into 300 size categories across the dynamic range of each

aperture and counts were then summed across three broad size categories (small, 0.600–1.000 μ m; medium, 1.001–10.000 μ m; and large, 10.001–224 μm ESD) for statistical analyses.

Physicochemical parameters

The water sample collected for phytoplankton biomass also was used to determine the concentration of ammonium–nitrogen, nitrate–nitrogen, nitrite–nitrogen, total nitrogen, phosphate as total phosphorus, chemical oxygen demand (COD) and sulphate. These parameters were measured colorimetrically using a Hach DR 2800 spectrophotometer (TNT Plus tests, Hach Co., Loveland, CO, USA). Surface water temperature of each microcosm was recorded every 0.5 h for the entire study using a water temperature data logger (HOBO, Onset Computer Inc., Bourne, MA, USA) in one of the control microcosms (see Fig. S1 in Supporting information). Other physicochemical parameters of the water in each microcosm (i.e. pH, temperature and dissolved oxygen concentration [DO]) were measured in the morning (~08·30 h) and afternoon (~15·30 h) of each sampling date using an electronic sensor array (ICM AquaCheck, Perstop Analytical, Wilsonville, OR, USA).

Mosquitoes and other macroinvertebrates

To enumerate and identify immature mosquitoes and other invertebrates, three 350-mL dipper samples per microcosm were taken just below the water surface 24 h after the water samples were taken for nutrient analysis. Samples were taken from two corners and from the centre of each microcosm.

Bacterial communities

Two 40-mL water samples were collected in 50-mL sterile, centrifuge tubes on four dates (Table S1). The first sample was taken 24 h (on 1 October 2012) before *Bti* treatment, while the

remaining samples were taken 9, 16 and 44 days post-treatment. Total genomic DNA was extracted, and a target region of the bacterial 16S gene was amplified via PCR as previously described (Duguma *et al*. 2013). Illumina libraries were generated for each sample using NEXTflex DNA sequencing kits (and protocols) and an identifying NEXTflex DNA barcode with 6-base indices (Bioo Scientific, Inc., Austin, TX, USA). Seven of the samples collected from the field did not amplify during either PCR or Illumina preparation steps. A total of 41 samples were each normalized to 10 nm and combined to create two multiplexed samples. The samples were then subjected to 150-base paired-end sequencing on a MiSeq (Illumina) at the UCLA Sequencing and Genotyping Core Facility.

Sequence analysis, alignment and taxonomy assignment.

Managed by AXIOME version 1.6.0 (Lynch *et al*. 2013), analysis of sequence reads was carried out using QIIME (1.7.0) using PyNast version 1.2.0 (Caporaso *et al*. 2010). All paired-end sequences were assembled using PANDAseq version 2.5.0 (Masella *et al*. 2012). Clustering of similar sequences (i.e. ≥0.97 identity) to operational taxonomic units (OTUs) was carried out using cd-hit-est version 4.5.4 (Li & Godzik 2006). Chimeras were detected using UCHIME version 4.2 and discarded (Edgar *et al*. 2011). Taxonomy assignments were conducted using BLAST against the SILVA 111 reference database (Quast *et al*. 2013) via the QIIME parallel_assign_taxonomy_blast_py script with a maximum e-value of 0.001. Sequences that were assigned to *Eukaryota* or provided no BLAST hit were discarded.

Statistical analysis

Repeated-measures ANOVAs were employed to compare mosquito abundance, phytoplankton biomass, sestonic particulates and physiochemical variables using JMP version 11 (SAS Inc. 2011). Logarithmic transformation was carried out when necessary prior to analysis to assure normality of the data. One-way ANOVA followed by Tukey's test at $P<0.05$

was used to separate significantly different means on each sampling date. The *P*-values were adjusted with the Bonferonni correction before the Tukey's test was carried out.

Principal coordinate analysis (PCoA) using the Bray-Curtis dissimilarity measures and non-metric multidimensional scaling (NMDS) analysis, based on randomly sampled sequences (i.e. rarefied to 6,139 assembled sequences per sample), were carried out to determine differences in bacterial communities between samples using AXIOME version 1.6.0 pipeline (Lynch *et al*. 2013), which leverages the vegan library in R (Paradis, Claude & Strimmer 2004; Roberts 2010). A PCoA ordination using weighted UniFrac distances was also carried out using QIIME. Alpha diversity estimates were calculated through QIIME for 10 subsamplings at even sequence depth. Average estimates were compared between *Bti* treatments at each time point using ANOVA and Tukey's test through R.

Results

Phytoplankton and sestonic particulates

Phytoplankton biomass increased exponentially for the first 30 days in the low *Bti* and control treatment microcosms (Fig. 1). A similar increase was initially seen in the high *Bti* microcosms, but chlorophyll *a* concentration began to decline nine days after application ($F_{2, 9}$ =92; *P*<0.001; Fig. 1; Table1). By day 44 of the experiment, chlorophyll *a* concentration in low *Bti* and control treatments was 83-fold higher than that found in the high *Bti* treatment (Fig. 1). Chlorophyll *a* concentrations in the microcosms of the low *Bti* and control treatments did not significantly differ throughout the study. Water in the high *Bti* treatment appeared clear, compared with the dark green colour observed in the low *Bti* and control treatments 44 days after treatments (Fig. S2).

Similarly, counts of sestonic particulates in the water column were also significantly different among treatments (Table 1). The number of particles in the medium size range (1.001–10.000

μ m ESD) was significantly reduced in the microcosms treated with high *Bti* and showed a similar pattern as the chlorophyll *a* concentration (Fig. 2B). The counts of medium-sized particles and chlorophyll *a* concentration were positively correlated (r=0.75, P<0.05). Small (0.600–1.000 μ m ESD) and large (>10.000 μm) particles also varied across treatments and/or time, but without any clear pattern (Table 1; Fig. 2).

Inorganic nutrients in the water column

In all three treatments, total nitrogen decreased at a similar rate (~43% per week) over the first 17 days following *Bti* application (Fig. 3). However, by day 30, total nitrogen was significantly depleted in microcosms that received high *Bti* treatments, relative to low *Bti* and control treatments (Fig. 3; Table 1). This resulted from a reduction in nitrites and nitrates, but not ammonium (Fig. S3; Table 1).

Total phosphorus was similarly depleted in high *Bti*-treated microcosms relative to the other treatments, but for this nutrient, the difference was apparent at day 17 (Fig. 3; Table 1). Towards the end of the study, sulphate concentrations in the water column were measured twice (at 38 and 44 days after *Bti* application), and levels in microcosms that received high *Bti* treatment were suppressed relative to those in the low and control treatments (Table S2).

Other physicochemical variables

The high *Bti* dose treatment also resulted in changes to the pH and DO content of the microcosms, but those changes were only evident in measurements taken in the afternoon (Figs S4, S5; Table 1). At 17 days after *Bti* application, pH (measured at 15·30 h) was significantly lower in microcosms of the high *Bti* treatment relative to the low *Bti* and control treatments (Fig. S4; Table 1). Afternoon measurements of the DO concentration were similarly lower in the high *Bti* treatment 14 days after *Bti* application (Fig. S5; Table 1). The chemical oxygen demand in

the high *Bti* treatment was also reduced (by ~75% compared to the control treatment) at 44 days after *Bti* application (Table S2).

Culex mosquitoes and other invertebrates

Three *Culex* mosquito species (*Culex tarsalis* Coquillett, *Culex stigmatosoma* Dyar and *Culex quinquefasciatus* Say) were found in the microcosms during the study.

After the *Bti* treatment, the abundance of early (1st & 2nd) mosquito instars in the high *Bti* treatment was significantly greater than in the low *Bti* and untreated microcosms ($F_{2, 33} = 3.5$, P=0.043; Fig. 4A). In contrast, the mean number of late (3rd and 4th) instar mosquito larvae in the high *Bti* treatment was significantly reduced by 54–74% relative to the other treatments between days 3 and 30 ($F_{2, 33} = 4.2$, P=0.023; Fig. 4B). Mosquito pupae were first collected 14 days after initial flooding of the microcosms, and their abundance in the high *Bti* treatment was also initially significantly lower than in the control and low *Bti* application ($F_{2, 33} = 19.2$; P<0.001; Fig. 4C). The abundance of early instars, late instars and pupae varied across time (P<0.05), and was significantly higher in the high *Bti* treatment on the last sampling date.

Two other dipteran families (i.e. Ephydridae and Chironomidae) also occurred among the primary colonizers. Nearly 90% and 97% of the total numbers of chironomids and ephydrids, respectively, were sampled during the first week after flooding and enrichment of the microcosms, and their abundance did not differ significantly among the three treatments (P>0.05). No other invertebrate groups were collected in dip samples from these microcosms.

Bacterial communities

We generated 4 095 139 bacterial sequences resulting in 42 412 OTUs from 41 water samples taken from the microcosms on four sampling dates (i.e. once before, and three times post-treatment). Overall, 45 bacterial phyla were recovered with over 98% of bacterial

sequences classified into only seven phyla (Fig. S6): *Bacteroidetes* (35.4%), *Proteobacteria* (30.1%), *Cyanobacteria* (14.5%), *Firmicutes* (7.5%), *Actinobacteria* (7.2%), *Verrucomicrobia* (1.5%), and *Planctomycetes* (1.4%).

Pre-treatment bacterial communities

Water samples taken 3 days after initial flooding generated 26 801 bacterial OTUs containing 1 892 941 sequences. Based on rarefied sequence sets, ~99.9% of the sequences recovered on this date were classified to just eight phyla (Fig. S6): *Proteobacteria* (53.6%), *Bacteroidetes* (23.5%), *Firmicutes* (19.7%), *Cyanobacteria* (1.3%), *Verrucomicrobia* (0.8%), BD1-5 (0.7%), *Fusobacteria* (0.2%) and *Actinobacteria* (0.1%). *Proteobacteria* (primarily dominated by *Alphaproteobacteria* and *Betaproteobacteria*) accounted for 39–61% of the sequences. *Bacteroidetes,* dominated by members of *Flavobacteria,* accounted for 16–31% of the sequences per sample on this sampling date. *Firmicutes* mainly represented by members of the *Bacilli* and *Clostridia,* represented the third most abundant phylum with 9–23% of the sequences per sample. Samples from this date were significantly separated from the other dates (MRPP: A=0.307; T=-17.7; P<0.001; Fig. S7).

Alpha diversity comparisons of treatment levels

Three metrics were chosen to analyse species richness and evenness: observed species, Shannon index and phylogenetic diversity (PD). As expected, there were no significant differences between any metric for the pre-treatment sampling date. Significant differences between the high and low treatment groups were observed beginning at the second sampling date (P<0.05; Fig. S8). By the final sampling date, two metrics (observed species and Shannon index) differed significantly when comparing the high *Bti* vs. the low *Bti* and control treatment groups (Fig. S8).

Beta diversity of bacterial communities post-treatment application

Principal coordinate analysis (PCoA) using Bray-Curtis similarity distance measures revealed a separation of samples by treatment and sampling date (Fig. S9). The greatest separation of samples, as shown by PCoA 1, was by sampling date. The separation of samples by treatment was explained by PCoA 2 (Fig. S9). Samples taken 44 days after *Bti* application were differentiated significantly from samples taken on days 9 and 16 after treatment application (MRPP: A=0.1264; T=-8.9; P<0.001). Similarly, samples from high *Bti* treatments separated significantly from low *Bti* and control treatment samples (Fig. 5). The separation of the samples by sampling date and treatments was even more evident in an NMDS plot of bacterial profiles from the three treatment applications (Fig. 5). Bacterial communities from the high *Bti* treatment were significantly differentiated from the untreated control and low *Bti* treatments (MRPP: A=0.0652; T=-4.6 A; P=0.002).

PCoA of weighted UniFrac distances (Fig. 6) revealed that samples from low *Bti* and control treatments grouped together. These samples were dominated by OTUs of *Proteomonas* sp. (OTU #10, classified by SILVA as *Cyanobacteria*), *Cyclobacteriaceae* and two *Bacteroidetes* species: *Leadbetterella* and *Solitalea.* Samples from high *Bti*-treated microcosms became separated from the two other treatments in ordination space and were characterized by OTUs from *Mucilaginibacter* and *Sediminibacterium* species. The control and low *Bti* samples taken on the last sampling date grouped separately from the other two sampling dates (Fig. 6), whereas the high *Bti* samples separated poorly by date within this UniFrac-based ordination.

Overall, members of *Cyanobacteria*, *Sphingobacteriales*, *Cytophagales* and *Flavobacteria*, *Bacillales*, *Sphingomonadales* and *Burkholderiales* dominated in the low *Bti* and control treatments compared to high *Bti* treatment microcosms. We observed a slight increase in abundance in some members of *Betaproteobacteria*, *Gammaproteobacteria*,

Deltaproteobacteria, *Verrucomicrobiae*, *Actinobacteria* and *Frankiales* in the high *Bti* treated microcosms.

Discussion

Whereas a very low dose of *Bti* (0.6 kg VectoBac G ha^{-1}) did not significantly reduce the abundance of mosquito larvae and cause changes in the microbiota, nutrients and sestonic particles as compared to the untreated control, the application of a high *Bti* dose (48 kg ha^{-1}, two-fold higher than the maximum recommended rate for polluted environments) reduced *Culex* larvae abundance and concomitantly altered the microbial community, algal biomass, sestonic particle size spectra and nutrient concentrations. Contrary to expectations that reduced grazing pressure on sestonic particles following the reduction of mosquito larvae by a biopesticide would cause an enhancement of phytoplankton and bacteria, the abundance of chlorophyll-containing particles (1.001–10.000 μm ESD) was greatly reduced following the reduction of mosquito larvae. Concomitant changes in physicochemical factors associated with reduced photosynthesis as well as reductions in total phosphorus and total nitrogen occurred in microcosms in the high *Bti* treatment. As compared to the low *Bti* treatment and the untreated control, bacterial diversity increased and community composition changed significantly following the high *Bti* treatment.

Phytoplankton and particulate dynamics following high Bti application

Our study revealed that phytoplankton and particles between 1 and 10 μm ESD were significantly reduced in the high *Bti* treatments approximately two weeks after *Bti* application (Figs. 1, 2B). It is unknown whether the suppression of algal biomass or sestonic particle abundance in the water column observed in this study, or previously (Su & Mulla 1999), was

directly related to toxins or degradation products of *Bti*, proprietary components of the VectoBac G formulation or recycling of *Bti* in mosquito carcasses (Zaritsky & Khawaled 1986). It is also unknown whether the application of *Bti* indirectly favoured competitive release of heterotrophic protozoans by the removal of mosquito larvae or proliferation of bacterial taxa that may have algicidal effects.

Although the numbers of the large size particles were comparatively constant (in fact, they were lower in the high *Bti* treatment than in the other treatments, Fig. 2C), it is possible that small herbivores (i.e. protists, rotifers) were released from larval feeding pressure by the reduction of mosquitoes. The abundance of edible-sized particles and algal biomass increased when mosquitoes were present. *Anopheles* larvae are known to depress algal biomass (Kaufman *et al*. 2006), but in our study where *Culex* mosquitoes were abundant in microcosms (low *Bti* and control treatments), algal biomass was high as compared to microcosms with reduced mosquito abundance (i.e. high *Bti* treatment).

Impacts of high Bti application rate on physicochemical variables

The reduction in nutrients resulting from high *Bti* application (Fig. 3) might have been due to the reduced phytoplankton biomass associated with the high *Bti* treatments. Phosphorus and algal biomass declined at about the same time (~two weeks) after *Bti* application (Figs. 1, 3B). Total phosphorus was positively correlated (Spearman's $r = 0.68$, $P<0.001$) with algal biomass three days after *Bti* application. Differences in the water-column nutrient concentrations among the treatments persisted beyond the 30 days that mosquito abundance was reduced (cf. Figs. 3, 4) and the 44 days discussed here. Duguma (2013) found that these differences persisted for nearly three months after the *Bti* treatment.

Algal biomass is a source of organic matter and generates oxygen as a result of photosynthesis. Unlike in the morning samples where DO concentration did not differ significantly among the treatments, the DO concentration in the high *Bti*-treated microcosms

was lower than in the other treatments in the afternoon when photosynthesis by the phytoplankton was likely at its peak (Fig. S5). Chemical oxygen demand, which is a measure of potential oxygen utilization and an indicator of the amount of organic matter that is susceptible to oxidation (APHA 1995), was significantly reduced in high *Bti*-treated microcosms (Table S2).

Effects of Bti on mosquitoes and the "top–down" hypothesis

The effects of the enrichment that favoured the proliferation of *Culex* mosquitoes waned after about one month. Changes in the abundance of *Culex* early instars indicate that recruitment by mosquitoes (which was relatively higher in the high *Bti* treatment than the other treatments) into the microcosms declined across the experiment (Fig. 4A). The abundance of late instars in the control and low *Bti* treatments also declined; the abundance of late-instar larvae was comparable among the three treatments on day 30 (Fig. 4B). At the last sampling date (i.e. 44 days after *Bti* application), the mean abundance of both larvae and pupae of *Culex* mosquitoes was significantly higher in the high *Bti* treatment than in the low and control treatments (Fig. 4). It is unclear what caused this enhancement of larval and pupal abundance in the high *Bti* treatment but it coincided with the increased bacterial diversity observed in the water column on this sampling date.

Previous studies using *Bti* for mosquito exclusion experiments attributed changes in bacterial taxa in aquatic habitats to the removal of mosquitoes, agreeing with the predominant top–down population regulation hypothesis (e.g. Nguyen, Su & Mulla 1999; Kaufman, Chen & Walker 2008; Xu *et al*. 2008). Xu *et al*. (2008) reported a proliferation of *Flavobacteriaceae* in tree-hole habitats as a result of *Aedes* mosquito removal by *Bti* application. Contrary to their findings, the abundance of *Flavobacteriaceae* found in control microcosms was 3 times greater than the abundance found in high *Bti* microcosms, suggesting that this group of bacteria was suppressed as a result of *Culex* mosquito larvae reduction. Moreover, we found that the concentration of bacteria-sized small particles in high *Bti* microcosms was not significantly

increased relative to the other treatments until 30 days after *Bti* treatment (Fig. 2). Overall, the top–down regulation by mosquitoes as hypothesized by several studies (Nguyen, Su & Mulla 1999; Kaufman, Chen & Walker 2008; Xu *et al*. 2008) was not evident in our study.

Östman, Lundström, & Persson Vinnersten (2008) reported a 4.5-fold increase of the protozoan populations in wetlands treated with *Bti* compared with untreated habitats. Protozoa are known to influence bacteria populations (e.g. through predation). In our study, the abundance of the large sestonic particles (i.e. 10–224 µm ESD), which likely includes the majority of the heterotrophic protozoans, did not differ markedly among the treatments (Fig. 2C). The potential influence of protozoan grazing on bacterial communities however was not explored explicitly in the present study.

Kroeger, Duquesne & Liess (2013) demonstrated the reduction of chlorophyll in microcosms treated with *Bti* (Vectobac 12 AS) in the absence of microcrustacean colonization. Filter-feeder microcrustaceans (e.g. daphinds) are known to decrease phytoplankton in the absence of their predators. Kroeger, Duquesne & Liess (2013) found a positive correlation of chlorophyll biomass with the abundance of *Culex pipiens* mosquito larvae. In previous studies conducted in the same microcosms (e.g. Duguma and Walton 2014), microcrustaceans (e.g. cladocerans, copepods, ostracods) and immature odonates were collected in dip samples. In the current study, we did not recover microcrustaceans or predators (e.g. *Chaoborus,* odonates) in the dip samples from either the untreated control or low or high *Bti* treated microcosms.

Bacterial community dynamics in Bti treated microcosms

The bacterial communities at three days after nutrient enrichment and 24 h before the treatment application were more diverse and were comparatively similar in composition among the microcosms than on later sampling dates (Figs S7, S8). *Proteobacteria*, *Bacteroidetes* and *Firmicutes* dominated water samples on this sampling date and were probably early colonizers

of the organic matter (rabbit pellets). As the habitat aged, bacterial composition changed across sample dates (Fig. S6). Overall, bacterial diversity declined across time, but it was greater in the high *Bti*-treated microcosms than in the other treatments (Fig. S7). Bacterial diversity might be expected to increase as succession and colonization occur after inundation of newly formed habitats and then decline as interactions within the food web become more complex as the system matures. The enrichment protocol, especially the addition of carbon in the rabbit pellets, and the bacterial community present in the source water supply might have enhanced microbial diversity relative to what is found in natural ecosystems.

Previous laboratory studies showed contradictory results of the effects of *Bti* on micro-organisms (Koskella & Stotzky 2002; Yudina *et al*. 2003; Revina *et al*. 2005). Yudina *et al*. (2003) demonstrated the antibacterial activities of the endotoxins of *Bti* on six Gram-positive species of *Actinobacteria* (i.e. three *Micrococcus* spp., *Nocardia calcaea* and two *Steptomyces* spp.) in laboratory studies. Contrary to Yudina *et al*. (2003), Koskella & Stotzky (2002) reported no negative effect of *Bti* on selected species of bacteria, algae and fungi. The results of these studies were based on laboratory bioassays with very few cultivable microbial species and do not likely reveal the interaction of *Bti* with the more diverse microbial communities found in aquatic habitats.

Ordination of samples from control and low *Bti*-treated microcosms demonstrated a significant separation from bacterial communities taken from high *Bti*-treated microcosms (Figs. 5, S9). *Cyanobacteria*, *Cytophagales, Cyclobacteriaceae* (*Bacteroidetes*), and *Sphingomonas* (*Alphaproteobacteria*) were reduced in microcosms that received a high dose *of Bti* (Table S3). In contrast, *Polaromonas* (*Betaproteobacteria*) and *Candidatus* Aquiluna (*Actinobacteria*) increased in high *Bti* treatments.

Cyanobacteria occurred at about similar abundances in all treatments until day 44 after *Bti* application when it was significantly reduced in the high *Bti* treatment (Fig. S6). Nearly 86% of *Cyanobacteria* sequences were identified as *Proteomonas* (OTU #10) by the SILVA database and this taxon was significantly suppressed in the high *Bti* treatment, occurring in significantly greater average proportions per sample in both low (47±6.9%; n=4) and control (26±8.3%; n=4) treatments than in samples from high (1.7±0.3%; n=4) treatments on the last sampling date (Table S3). *Proteomonas* is classified as *Cryptophytaceae* in the NCBI database, and the correct position of this taxon is currently unresolved.

The suppression of some bacterial taxa and increased diversity that occurred in the high *Bti* treatments might be linked to the inhibited growth of algae. An increase in primary production has been associated with increased bacterial abundance but reduced overall bacterial diversity (Horner-Devine *et al.* 2003). Bacteria and algae are also known to interact indirectly with one another depending on the availability of resources (e.g. carbon:nutrient ratios) and the presence or absence of bacterial predators (Cole 1982; Hulot, Morin & Loreau 2001; Amin, Parker & Armbrust 2012). Carbon-rich resources are generally considered favourable for bacterial proliferation whereas nutrient-rich (e.g. nitrogen, phosphorus) media are thought to encourage algal growth (Cole 1982; Danger *et al*. 2007).

The fate of Bti formulations in the water column

In our study, we did not recover any *Bti* sequences from the water column by using bacteria-specific 16S rRNA gene primers nine days after application and we did not attempt to recover *Bti* from the substrate. We have, therefore, not ruled out whether the larval mosquito mortality for nearly a month after *Bti* application was due to the toxins recycling in the water column or reduction of food resources in the high *Bti* treated microcosms. *Bti* formulations rapidly disappear from the water column soon after application (Ohana, Margalit & Barak 1987). The

primary cause of the loss of the toxicity of *Bti* was due to the immediate settling and binding of the spores with the soil or particulate substrates and toxicity was restored after three weeks by subsequent stirring and filtering of the substrates (Ohana, Margalit & Barak 1987).

Our study demonstrates a strong and significant effect of a high application rate of a granular-formulation of *Bti* (VectoBac G) on microbial communities in aquatic microcosm habitats. *Bti* (Vectobac WG and Vectobac WDG) application rates higher than the recommended rates were previously shown to prolong control of container-dwelling mosquitoes (e.g. *Aedes* spp.) in experimental studies and thereby reduce application costs and the potential resistance development of repeated low dose applications (Ritchie, Rapley & Benjamin 2010; Farajollahi *et al*. 2013). Although the risk is limited in containers or in situations where larval mosquito companion fauna and primary production is absent, this deviation from authorized practices cannot be recommended for widespread adoption as it may eventually impact the environment. In our microcosm study, the abundance of *Culex* mosquitoes was reduced for approximately one month by a very high *Bti* application rate and concomitant changes were observed at other trophic levels in the food web for up to 44 days. However, it is currently unknown what aspect of the *Bti* application impacted microbial community composition, increased bacterial diversity, and reduced nutrient and algal biomass in the water column. The consequences of these biotic and abiotic changes to natural or agricultural aquatic ecosystems, where other filter feeding organisms coexist with mosquitoes, warrants investigation.

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Data Accessibility

All sequence data were submitted to European Nucleotide Archive with accession numbers PRJEB6267. http://www.ebi.ac.uk/ena/data/view/ERP005772.

All experimental data are uploaded as online supporting information.

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Figure legends

Fig. 1. Phytoplankton biomass in the water column. Phytoplankton biomass (total chlorophyll: mean ± SE; *n* = 4) in the water column of microcosms assigned to two application rates of larvicide treatments and an untreated control. Points on each sampling date are offset horizontally to facilitate illustration.

Fig. 2. **Sestonic particulates in the water column**. Sestonic particle concentration (mean ± SE) in microcosms assigned to two application rates of mosquito larvicide treatments and an untreated control. The y-axis represents counts of small (0.600–1.000 μm, Panel A), medium (1.001–10.000 μm, Panel B), and large (10.001–224 μm, Panel C) particles.

Fig. 3. Total nitrogen and total phosphorus in the water column. Total nitrogen concentration (mg N L^{-1}: mean ± SE) (Panel A) and total phosphorus (mg PO_4 L^{-1}: mean ± SE) (Panel B) in the water column of microcosms assigned to two application rates of a mosquito larvicide and an untreated control.

Fig. 4. *Culex* mosquito abundance. Abundance of first and second instars (Panel A), third and fourth instars (Panel B), and pupae (Panel C) in microcosms assigned to two application rates of mosquito larvicide treatments and an untreated control.

Fig. 5. Nonmetric multidimensional scaling plot of post-treatment samples. NMDS ordination of post-treatment bacterial communities by sampling date (Panel A) and treatments (Panel B) based on a Bray-Curtis distance matrix.

Fig. 6. UniFrac distance PCoA. A PCoA ordination based on a weighted UniFrac distance matrix comparing bacterial communities in water column samples from three treatments. Panel A) biplot of the 10 most dominant taxa associated with sample placements in the PCoA ordination, B) PCoA ordination of samples coloured by treatment and C) PCoA ordination of samples coloured by sample date.

Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1. Sampling schedule of microcosms

Table S2. Sulphate and chemical oxygen demand concentration (mg L^{-1})

Table S3. Sequence abundance (percentage per sample) of 11 bacterial taxa

Fig. S1. Temperature

Fig. S2. Colour of water

Fig. S3. Bioavailable nitrogen species

Fig. S4. pH level

Fig. S5. Dissolved oxygen concentration in water column

Fig. S6. Major bacteria phyla found in water column

Fig. S7. Nonmetric multidimensional scaling plot

Fig. S8. Alpha diversity comparisons

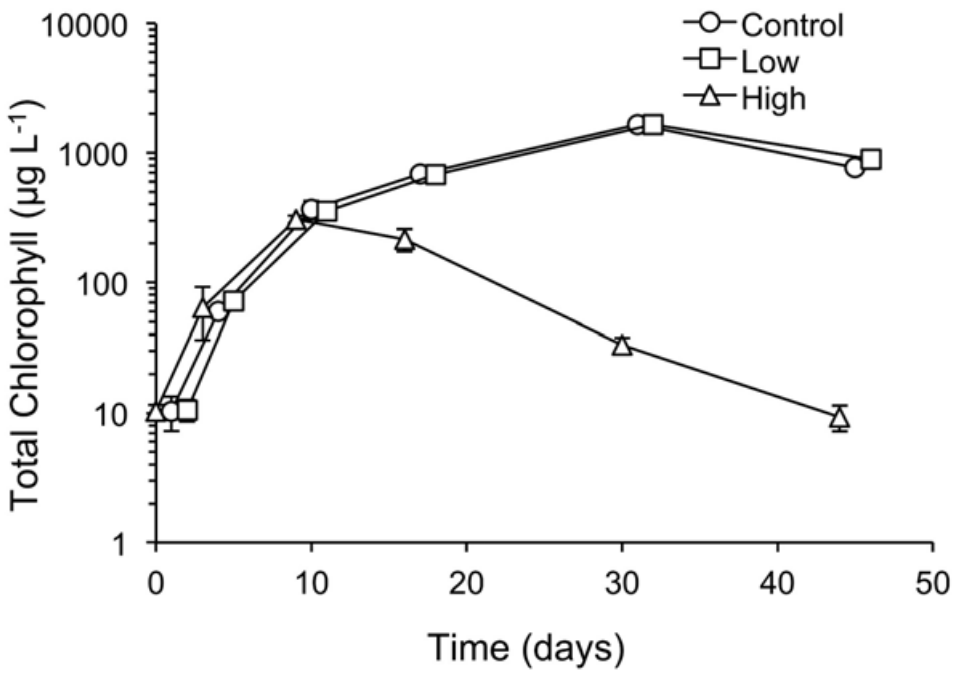
Fig. S9. Principal coordinate analysis

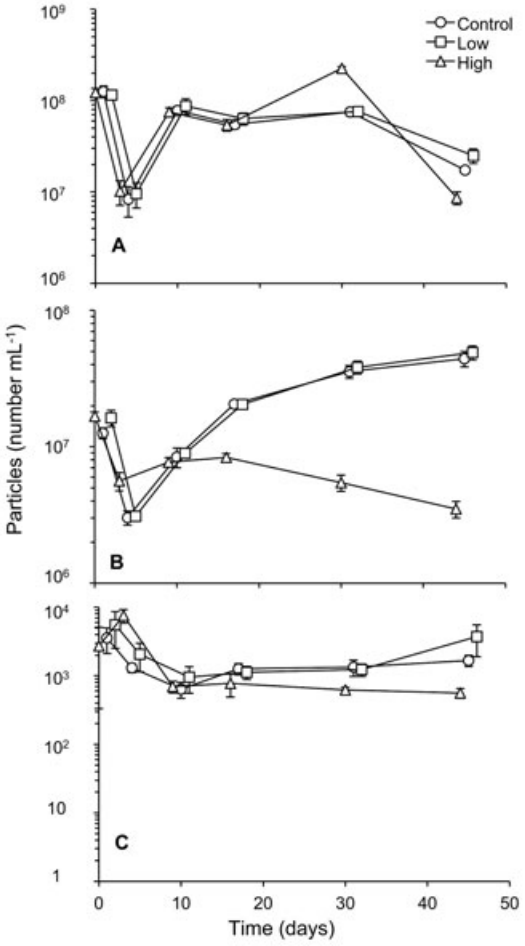
Table 1. Repeated-measures analysis of variance of water quality parameters

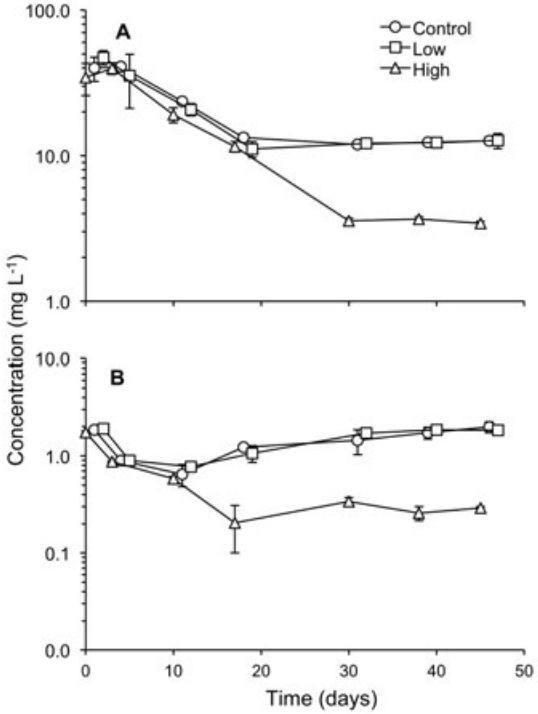
Sources	Treatments	Time	Treatment × Time
Total chlorophyll μg mL^{-1}	*F* 92 (2,9)	131.97 5,45)	38.9 (10,45)
	P 0.0001	<0.0001	<0.0001
Particles (0.6–1.0 μm)	*F* 16.4 (2,9)	90.2 (5,45)	16.4 (10,45)
	P 0.0022	<0.0001	<0.0001

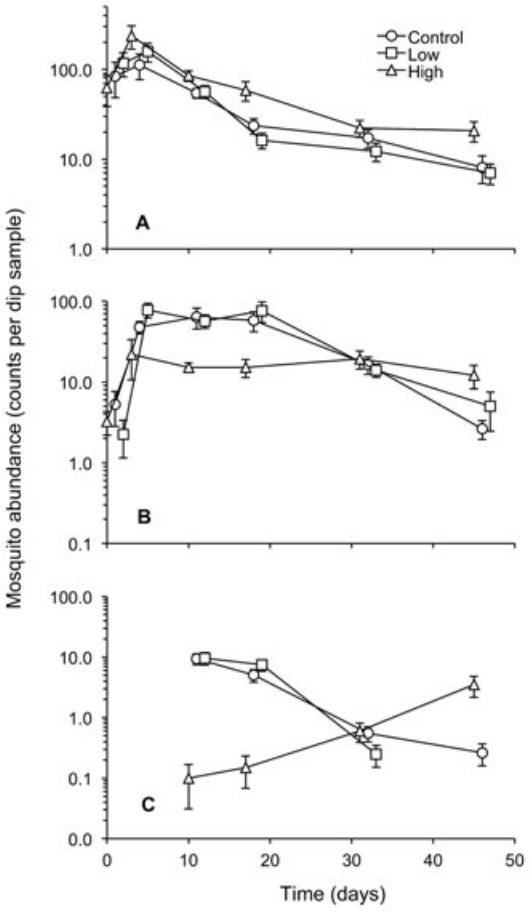
Particles (1.001–10 µm)	*F*	36.0 (2,9)	63.7 (5,45)	23.3 (10,45)
	P	0.0001	<0.0001	0.0205
Particles (10–224 µm)	*F*	0.4 (2,9)	5.0 (5,45)	2.6 (10,45)
	P	0.6626	0.0011	0.0127
Total N (mg L^{-1})	*F*	19.8 (2,9)	41.8 (6,54)	4.9 (12,54)
	P	0.0005	<0.0001	<0.0001
NO_3-N (mg L^{-1})	*F*	16.6 (2,9)	13.5 (5,45)	1.9 (10,45
	P	0.001	<0.0001	0.0687
NO_2-N (mg L^{-1})	*F*	15.4 (2,9)	17.8 (5,45)	5.2 (10,45)
	P	0.0012	<0.0001	<0.0001
NH_4-N (mg L^{-1})	*F*	0.4 (2,9)	15.9 (5,45)	2.2(10,45)
	P	0.6917	<0.0001	0.7959
Total P (mg L^{-1})	*F*	21.0 (2,9)	32.9 (6,54)	11.8 (12,54)
	P	0.0004	<0.0001	<0.0001
pH (time, 08·30 h)	*F*	6.4 (2,9)	52.2 (7,63)	1.7 (14,63)
	P	0.0183	<0.0001	0.0852
pH (time, 15·30 h)	*F*	53.3 (2,9)	103.4 6,54)	7.9 (12,54)
	P	<0.0001	<0.0001	<0.0001
DO (time, 08·30 h)	*F*	14.1 (2,9)	42.9 (6,54)	5.4 (10,45)
	P	0.0017	<0.0001	<0.0001
DO (time, 15·30 h)	*F*	14.1 (2,9)	11.8 (5,45)	5.4 (10,45)
	P	0.0017	<0.0001	<0.0001

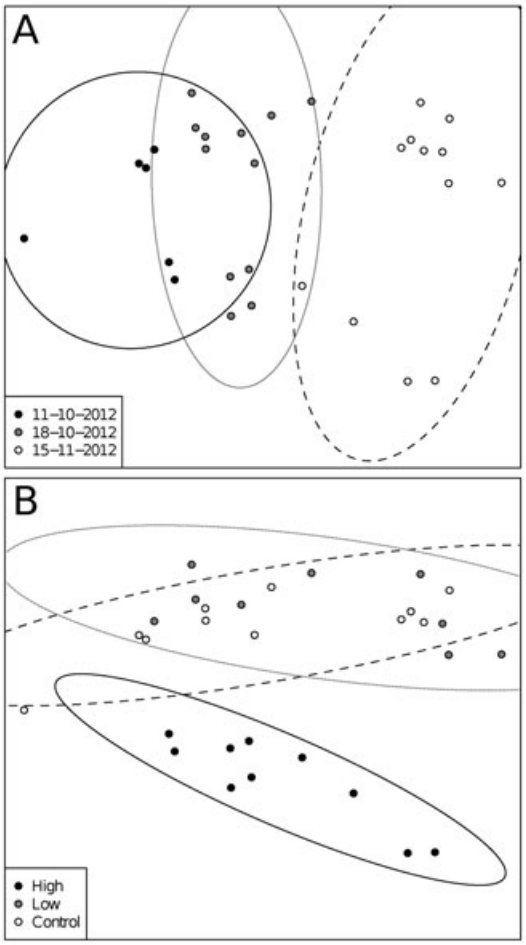
[1]*F* values from within subjects are univariate unadjusted epsilon values unless otherwise stated. A *P* value < 0.003 is significant. Numbers in the brackets are degrees of freedom.

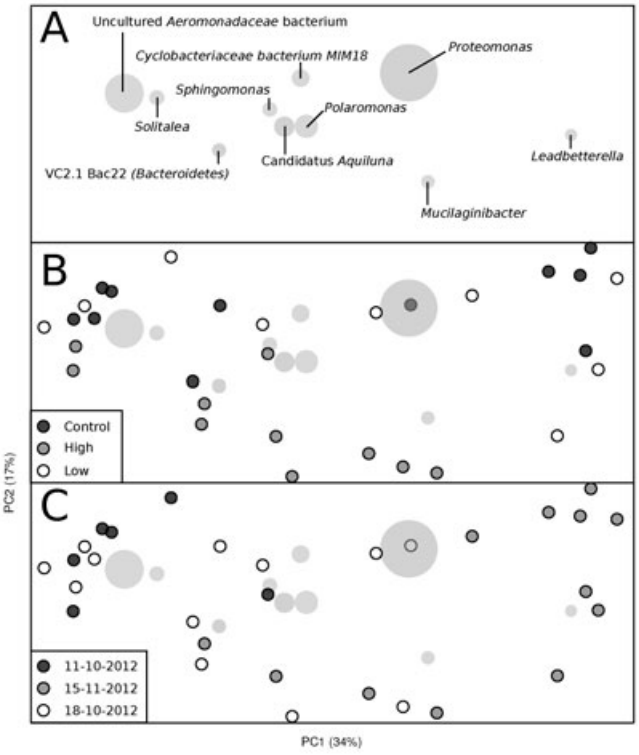












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