

Effects of mosquito larvae removal with *Bacillus thuringiensis israelensis* (Bti) on natural protozoan communities

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Abstract The protein crystals produced by *Bacillus thuringiensis israelensis* (Bti) are used against the larvae of pestiferous flood-water mosquitoes in ephemeral wetlands. Although mosquito larvae are considered important predators on protozoans and bacteria, it is not known how a distinct reduction of mosquito larvae density in natural wetlands caused by application of Bti may indirectly affect these microbial communities. Here we show, in a large scale experiment in six natural wetlands, that the densities of heterotrophic protozoans was on an average 4.5 times higher in wetland areas treated with Bti than in control areas. In addition, the taxonomic richness of heterotrophic protozoans increased on an average of 60% in areas with Bti application compared to control areas. The increase in protozoan density and richness was fairly consistent among sites of different

wetland habitats. We discuss the potential implications of our results for other parts of the ecosystem.

Keywords *Aedes* · *Ochlerotatus sticticus* ·
Protozoa · Mosquito control · Top-down control

Flooding that cause temporary high water levels during the summer season (April–August) can provide favorable conditions for egg hatching and larval survival of pestiferous flood-water mosquito species, and adults can reach extreme densities (Becker et al., 2003). One way to decrease adult densities of these mosquito species is to treat the larval sites in ephemeral wetlands with the crystalline pre-toxins produced by the bacteria *Bacillus thuringiensis israelensis* (Bti). Such treatment is highly efficient against mosquito larvae, causing almost 100% mortality, and has been shown to have very limited direct effect on other organisms when applied at the appropriate dose for control of mosquito larvae (Boisvert & Boisvert, 2000).

Mosquito larvae feed on microorganisms (bacteria, protozoans, algae), detritus, and parts of dead invertebrates. Depending on species, the larvae feed at the water-air interface, in the water column, in the aquatic plant root zone, and on mineral and organic surfaces under water (Merritt et al., 1992). As predators on aquatic microorganisms mosquito larvae can affect lower trophic levels, and in small and relatively closed aquatic communities such as water-

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filled pitcher plants, and tree-holes, as well as in laboratory experiments, mosquito larvae in general show a strong effect on density, richness, and composition of lower trophic levels including protozoan (Addicott, 1974; Cochran-Stafira & von Ende, 1998; Kneitel & Miller, 2002, 2003; Kneitel & Chase, 2004). However, the effect of mosquito larvae on their prey has not been as well investigated in larger and more open communities such as ponds, lake edges, and temporary flooded wetlands. Flood-water mosquito larvae can occur in very high densities in temporary flooded wetlands, and given the distinct reduction of these mosquito larvae caused by application of Bti, we anticipate strong indirect effects on the community structure of the prey organisms utilized as food by mosquito larvae. Our aim in the present study is to quantify this potential indirect effect of Bti-based mosquito larval control on a lower trophic level, protozoans, in temporary flooded natural wetlands.

We studied the effect of mosquito larvae on protozoan communities in temporary flooded wetlands around Lake Färnebofjärden (66°85' N, 15°56' E), which is situated in the lowlands of the River Dalälven catchment area (26,000 km²) in central Sweden. Spring floods occur almost every year in the lowlands, while summer floods occur more irregularly (every second to fourth year). These floods eventually cover several thousand hectares of open and trigger hatching of flood-water mosquitoes: *Ochlerotatus sticticus*, *Aedes rossicus*, *A. cinereus*, and *A. vexans* (Schäfer et al., 2008). These are aggressive day-active species that commonly feed on humans and especially *Ochlerotatus sticticus* occurs in pestiferous numbers after the floods. Due to the massive nuisance problems to humans and livestock caused by these mosquito species, a mosquito control program was implemented in 2000, and since 2002 aerial application of VectoBac G[®] (Bti-coated corn cob granules) is used to control these mosquitoes after floods (www.mygg.se). A long-term assessment of the potential effects of this mosquito control on insects in the temporary wetlands was initiated in 2002, and is based on comparing the insect fauna composition and production in three wetlands with and three without Bti-based control of mosquito larvae. Treatment areas and control areas are paired; areas of similar habitat and size close to each other. In these six areas we studied how the reduction of

mosquito larvae density by Bti-treatments indirectly affected the protozoan community.

The wetland study areas were the meadows along the streams at Laggårbo (Bti-treated area) and Fågle (control area), the alder swamp forests along semi permanent rivulets (dries out in summer) at Valmbäcken (Bti) and Koversta (control), and the wide and shallow river shores at Nordmyra (Bti) and Lusmyren (control). In early May 2006, flooding of the River Dalälven lowlands triggered the hatching of larvae of the flood-water mosquito species, mainly *O. sticticus* but also *A. cinereus*. Control of the mosquito larvae by aerial application of VectoBac G[®] (Bti-coated corn cob granules) was performed on the 11th and 12th May in temporary wetlands around the Lake Färnebofjärden. The material was spread at a dose of 15 kg per hectare, about one granule per square decimeter, and was applied over 5 ha at Valmbäcken, 68 ha at Laggårbo, and 270 ha at Nordmyra. Assessments for mosquito larvae a few hours before Bti-treatment and again 24 h after the treatment showed a 97–100% reduction in mosquito larvae density (personal observation, J. O. Lundström). We sampled protozoans at the Bti-treatments sites and their respective control sites on the 26th May 2006. The two weeks between Bti-treatment against mosquito larvae and sampling for protozoans provides the protozoan communities a reaction time after removal of predators. However, the time lag also allows mosquitoes in second to third instar larvae in the control areas to develop and leave the water as adults, and among control sampling sites mosquito larvae were found in only 10 out of 15 sites.

Within each area, in a 1 ha plot, we sampled protozoan communities at five different sites varying from small isolated pools of water to larger more or less permanent water. We took water samples containing the protozoan communities using a 100 ml plastic cup, and initially stored each sample in a cooling bag for a few hours and then in a refrigerator at 8°C for up to two days. In order to assess the presence of mosquito larvae, and other potential predators on protozoans we swept the immediate surrounding water (radius about 0.5 m) with a plankton net, (mesh size 65 µm, diameter 12 cm). The net was moved up and down in the water column (if possible), and approximately the same water volume was sampled at all sites except in very shallow water and/or sites with much vegetation. For

each of these sweep net collections we noticed presence or absence of mosquito larvae, copepods and cladocerans.

In order to see if the connectance between the sampled water and nearby flowing water, i.e., movements of protozoans to and from outside, influenced protozoan communities we categorized the water-vegetation interface in four categories on a square meter scale around each of the five sampling points in each of the six study areas: 1. Small puddles isolated from each other by tussocks, 2. Small puddles connected to each other but with clear tussocks above the water line, 3. Continuous water surface with only plants above the surface, and 4. More or less permanent water with no terrestrial plants. If protozoan communities changed consistently between different categories this would suggest connectance to flowing water is important for protozoan communities.

We analyzed the live samples for protozoans during the two days following sampling. The water samples were allowed to reach room temperature (20°C) to assure that the protozoans had similar activity in all samples. We then gently stirred the water, extracted 1 ml using a sterilized pipette, and transferred the sub-sample into a Sedgewick rafter counting cell. Individual protozoans were identified to taxonomic units (e.g., genus) and counted under a microscope using standard methods (e.g., Kneitel & Miller, 2003; Kneitel & Chase, 2004; Östman et al., 2006). We counted all eukaryote organisms (body size $> \sim 10 \mu\text{m}$), but we here refer only to obligate heterotrophic taxa (Amoeba, Ciliophora, Zoomastigophora) as protozoans, to avoid the complex distinction between protozoans and algae.

In order to analyze the effect of Bti-treatment on protozoan richness and density we first performed three-way ANOVAs with Bti-treatment, pairs of areas, and water category (see above) as independent variables and all two-way interactions, but interaction terms were removed if $P > 0.1$. As Bti-treatment was not perfectly associated with mosquito presence, we repeated the ANOVAs with mosquito presence as a category variable instead of Bti-treatment. We performed a second set of ANOVAs with Bti-treatment, water category, and presence/absence of the potential predators copepods and cladocerans (category variables) as independent variables (no interaction terms were included), to check that the results were not

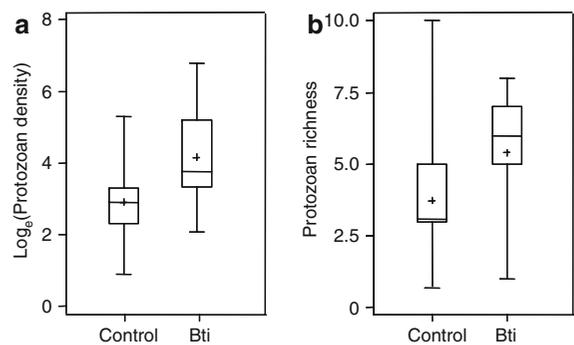


Fig. 1 The effect of reduced predation, after Bti-treatment against mosquito larvae, on protozoan richness and density. The difference in (a) protozoan richness and (b) protozoan density between three wetlands with Bti-treatment against flood-water mosquito larvae (Bti-areas) and three without such treatment (control areas). Plus denotes arithmetic mean, horizontal line represents median, box length represent interquartile range, and whiskers minimum and maximum values

confounded by the presence of other predators than the mosquito larvae. We added density as a covariate in an ANCOVA of protozoan richness because richness can be strongly dependent on sample size. Density, the equivalent of sample size in our study, was \log_e -transformed before use in the analysis.

Both protozoan density and richness were higher in areas with Bti-treatment (Density: $F_{1,23} = 7.6$, $P = 0.01$, Richness: $F_{1,23} = 6.6$, $P = 0.02$, $N = 30$ for both, Fig. 1a, b). Density was on an average 4.5 times higher in Bti-treatment areas than control areas (on an average 127 vs. 28 protozoa/ml), and richness on an average 60% higher in Bti-treatment areas compared to control areas (on an average 5.6 vs. 3.5 taxa/ml). If the analysis is based on mosquito larvae presence instead of Bti-treatment, the mosquito larvae had even larger effects on both protozoan density and richness (Density: $F_{1,23} = 8.9$, $P = 0.007$, Richness: $F_{1,23} = 11$, $P = 0.003$, $N = 30$ for both). Protozoan richness tended to differ between pairs of areas ($F_{2,23} = 2.9$, $P = 0.07$, $N = 30$), being lowest in the meadows and highest in the swamp forest. Neither protozoan density nor richness differed among water categories (Density: $F_{3,23} = 0.4$, $P = 0.8$, Richness: $F_{3,23} = 0.8$, $P = 0.5$, $N = 30$ for both), and no interaction term had $P < 0.15$. The higher protozoan richness in Bti-treatment areas was, however, mainly a result of the higher protozoan densities. Protozoan richness and density was positively correlated ($r^2 = 0.39$, $P < 0.001$, $N = 30$). When

protozoan density was entered as a covariate, protozoan richness did not differ between Bti-treatment areas and control areas ($F_{1,22} = 0.5$, $P = 0.5$, $N = 30$). Instead, protozoan density explained most of the variation in protozoan richness ($F_{1,22} = 9.5$, $P = 0.005$, $N = 30$).

Relative presence of other predators on protozoans BTI-treatments explained 21% of the variation in protozoan density (partial- r^2). Presence of cladocera explained another 5.2% ($F_{1,21} = 1.9$, $P = 0.2$, $N = 30$) and copepods <0.5% ($P = 0.8$) of the variation in protozoan density. Thus, protozoan density was mainly influenced by mosquito larvae density and was not confounded by the additional predator groups.

The results suggest that Bti-treatment, by strongly reducing the density of mosquito larvae, had indirect effects on the protozoan community and the effect on protozoan communities was even larger when we considered mosquito larvae presence instead of Bti-treatment. Thus, the results of the present study show that mosquito larvae can affect protozoan communities also in large and open natural communities. This translation from small isolated communities to larger natural systems also validates the use of microcosms as research tools in aquatic community ecology. The higher protozoan density and richness in areas with Bti-treatment were expected given the high densities of mosquito larvae in control areas. We could not find any interactions between Bti-treatment and habitat (pair) or local water-terrestrial interface suggesting the distinct effects on protozoan communities are not highly site or context specific, but fairly similar over an environmental gradient.

Most protozoan genera had lower densities in control areas where mosquito larvae were more abundant, although the largest difference was found for rather common protozoa genera (Table 1). Furthermore, the main mechanism for the observed higher protozoan richness in Bti-treated area seemed to be the higher protozoan density in these areas, because there was no significant difference in protozoan richness between Bti-treated areas and control areas when protozoan richness was controlled for. Thus, as protozoan density increases, samples are more likely to contain locally rare species. This suggests that the larvae of *O. sticticus*, and other flood-water mosquito species, in this study were generalist predators on protozoans without distinct

Table 1 The effect of reduced predation, after Bti-treatment against mosquito larvae, on protozoan genera

Genera	Bti-treatment	Untreated control
<i>Amoeba</i>	0.4	0
<i>Aspedisca</i>	8.9	5
<i>Bodo</i>	16.9	14.9
<i>Colpidium</i>	7.8	0.2
<i>Caolpoda</i> ^a	65.2	1.6
<i>Cyclidium</i>	13.6	4.3
<i>Euplotes</i>	0	0.3
<i>Halteria</i>	10.1	1.47
<i>Litinosus</i>	0.9	0.7
<i>Paramecium</i>	0.7	1.9
<i>Stichotrichia</i>	1.2	0.4
<i>Tetrahymena</i>	10.1	1.1
<i>Uronema</i>	0	1.4
<i>Vorticella</i>	0.7	0.8
Unidentified ciliate	0	0.3
Unidentified flagellate	0.3	0

Mean density (numbers per ml) of protozoan genera in samples from natural flooded wetlands in central Sweden—three wetlands with Bti-treatment against flood-water mosquito larvae (Bti-treatment) and three without such treatment (untreated control)

^a The density of *Colpoda* was skewed—one site had a very high density (874 individual/ml) while the mean was 11 individual/ml for the other sites it occurred in

preference for a specific group. A decrease in general protozoan richness with increasing mosquito larvae density have been observed in most of the previous studies (Addicott, 1974; Kneitel & Miller, 2002, 2003; Kneitel & Chase, 2004), although some genus, e.g., *Bodo*, appear to have benefit from the co-occurrence with mosquito larvae (Cochran-Stafira & von Ende, 1998; Kneitel & Miller, 2003).

The Bti effect observed here on the protozoan community may also extend to other parts of the food web. Both the mosquito larvae and heterotrophic protozoans feed on bacteria, the use of Bti against mosquito larvae may therefore, also indirectly affect the bacteria community. Experiments in small closed system have shown that mosquito larvae tend to increase bacterial densities by reducing the densities of protozoans that feed on bacteria (Cochran-Stafira & von Ende, 1998; Kneitel & Miller, 2002). Thus, a negative indirect effect of Bti on bacterial density could therefore, be expected. Rotifers also consume some protist genera, but rotifers can also be

consumed by mosquito larvae (Kneitel & Miller, 2002, 2003), and may therefore also be indirectly affected by Bti treatments. In order to conclude, we have for the first time shown that large scale Bti-treatment in naturally flooded wetland areas, through the strong reduction in mosquito larvae density, have significant and distinct positive effects on protozoan communities. Protozoan densities increased 4.5 times, and this was coupled to a 60% increase in richness, in areas where the mosquito larvae were temporarily whipped out by the Bti-treatment.

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