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Does mosquito control by *Bti* spraying affect the phytoplankton community? A 5-year study in Camargue temporary wetlands (France)

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Abstract – Mosquitoes are both vectors of disease and a hindrance to outdoor activities. Since its discovery in 1976, the larvicide *Bacillus thuringiensis* var. *israelensis* (*Bti*) has proven its effectiveness in controlling mosquito populations, with negligible environmental impact. We performed an integrated, 5-year study on the potential ecological direct and indirect effects of mosquito control agent *Bacillus thuringiensis* var. *israelensis* (*Bti* applied as VectoBac® WG) on phytoplankton communities in the Camargue temporary wetlands of the French Mediterranean coast. Mosquito larvae are considered major algae predators, so a distinct reduction in mosquito larva density (> 80%) in natural wetlands due to *Bti*-treatment could be expected to indirectly affect these phytoplankton communities. Physical parameters and phytoplankton were sampled in the water of three temporary oligohaline pools between 2006 and 2011 in the following order: T0 = 1 day before treatment (control) – T2 = 2 days after treatment – T5 = 5 days after treatment – T11 = 11 days after treatment. No negative effects on phytoplankton densities and diversity could be attributed to *Bti*-treatment: no plytoplankton proliferation was observed in these treated pools. However, changes in the density of phytoplankton taxonomical groups were observed: the Diatoms group was reduced and replaced by other algal groups typical of temporary wetlands and able to tolerate drought. These results suggest that the phytoplankton community's evolution in total density, density of phytoplankton taxonomical groups and community structure is largely driven by natural environmental factors and by the ecological complexity of these temporary wetlands.

Key words: Phytoplankton / density / diversity / *Bacillus thuringiensis* var. *israelensis* / wetlands

Introduction

Bacillus thuringiensis var. *israelensis* serotype H14 (*Bti*) is a biological larvicide increasingly used worldwide for selective control of larval mosquito populations (Lacoursière and Boisvert, 2004; Becker, 2006; Wegner, 2006; Östman *et al.*, 2008; Després *et al.*, 2011). This bacterial larvicide is applied via aerial spraying over large areas in Northeastern Spain, along the French Mediterranean coast (Duchet *et al.*, 2010) and also in the French Atlantic coastal wetlands (Caquet *et al.*, 2011; Lagadic *et al.*, 2014). All these regions contain temporary aquatic ecosystems, habitats propitious to the massive emergence of mosquitoes with major nuisance potential (Becker *et al.*, 2003; Duchet *et al.*, 2010; Lagadic *et al.*, 2014). Living near wetlands often entails serious health hazards for humans and animals due to increased risk of mosquito-borne diseases (Russell, 1999; Walton, 2002; Kirkman *et al.*, 2011; Lagadic *et al.*, 2014). Today, *Bti* represents the best alternative to chemical insecticides for mosquito control (Després *et al.*, 2011) and is described as non-toxic to humans, mammals, birds, fish, plants and most aquatic organisms (Boisvert and Boisvert, 2000; Lacey and Merritt, 2004). *Bti* is the only larvicide used in Europe following implementation of the EU Biocidal Products Directive 98/8/EC (Lagadic *et al.*, 2014). Findings from numerous studies indicate that *Bti* can be considered as a larvicide with low environmental toxicity, even when used long-term in repeated treatments (from 3 to 7 years). Most of these long-term studies were conducted on invertebrate populations (under the assumption of direct effects). In a 4-year study (1998–2001) by Lagadic *et al.* (2002) in Morbihan, France, the health, number and abundance of non-target aquatic

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invertebrates present in mosquito breeding sites treated annually with *Bti* (VectoBac®12AS) were monitored. No significant effect was observed on either of the sentinel species *Nereis diversicolor* and *Chironomus salinarius*. Even analysis at community level showed that environmental fluctuations had the greatest impact on community structure, with no *Bti* effect detectable. Other studies were conducted both over the shorter- and long-term in the field (Caquet *et al.*, 2011), to analyze the effects of *Bti*-based mosquito control on non-target aquatic invertebrates in wetlands. These concluded that the evolution of invertebrate communities in terms of taxa abundance, richness and diversity is, in the main, driven by natural environmental factors, rather than by *Bti*-treatment (*e.g.,* Hershey *et al.*, 1998; Russell *et al.*, 2009; Caquet *et al.*, 2011; Lagadic *et al.*, 2014). Temporal fluctuations in numbers of invertebrate taxa within a given area are often much greater in magnitude than any difference between control and treated pools, as stated in Lagadic *et al.* (2014). None of the long-term studies currently available was able to show *Bti*-induced alterations of invertebrate community structure. The innocuity of *Bti* for micro- and macro-invertebrates, fish, batracians and other vertebrates sharing the same habitats as mosquito larvae is well established for the dosage rates used at the operational scale (Lacoursière and Boisvert, 2004; Lacey and Merritt, 2004; Després *et al.*, 2011).

The Camargue temporary wetlands are ecological niches favorable to the development of mosquito species. Each *Bti*-treatment causes almost 80% mortality of phytophagous mosquito larvae. Algicidal properties of *Bti* are unknown, but some indirect effects are documented. Several studies revealed an indirect positive effect of *Bti* application on certain taxa of aquatic microbial communities. A 4.5-fold increase in abundance of protozoans (size > 10 µm: Amoeba, Ciliophora, Zoomastigophora) was observed in *Bti*-treated wetlands as compared with untreated control wetlands (Östman *et al.*, 2008). Similarly, a significant increase in some *Bacteria* (*e.g., Flavobacteriaceae*) taxa in treehole mosquito larval habitats resulted from the exclusion of *Aedes triseriatus* (*Ochlerotatus triseriatus*) larvae by *Bti* (Kaufman *et al.*, 2008; Xu *et al.*, 2008). These changes in microbial communities were attributed directly to the removal of mosquitoes (Kaufman *et al.*, 2008; Östman *et al.*, 2008; Xu *et al.*, 2008) and support the hypothesis of "top-down" regulation of resources by larval mosquitoes. Mosquito larvae are known to be predators of microorganisms (Protozoa, *Bacteria*, algae) and to feed on other organic matter as well (Merritt *et al.*, 1992). Contrasting with the expected outcome according to the "top-down" hypothesis, Su and Mulla (1999) reported a significant reduction in abundance of two microalgae species (*Closterium* sp. and *Chlorella* sp.) in mesocosms treated with *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* applications improved water quality by eliminating phytoplankton.

The importance of algae in the primary production and nutrient cycling of wetlands is increasingly recognized (Goldsborough and Robinson, 1996; Robinson *et al.*, 1997; Wu and Mitsch, 1998; Weilhoefer and Pan, 2007; Hagen, 2009). In wetlands, phytoplankton cells constitute a trophic resource and are a significant dietary resource for many kinds of mosquito larvae that feed opportunistically on microorganisms (Merritt *et al.*, 1992). The larvae may filter algae from the water column, scape them from the substrate surface or scoop them from the bottom of the aquatic habitat where mosquitoes breed. Mosquito biologists find that algae are generally represented in the gut in proportion to their abundance among the microflora where mosquito larvae feed (Marten, 2007).

Our study monitored the effects of *Bti*-treatment on phytoplankton of treated wetlands, seeking to determine whether mosquito control could induce modifications in food-web structure. The effects were tested on total densities, on the densities of different phytoplankton taxonomical groups and on community structure (diversity and evenness). It can be hypothesized that phytoplankton might be subject either to direct toxic effects, or to indirect effects linked to decreased grazing pressure from mosquito larvae.

Our hypothesis was that the decrease in the mosquito larvae population would induce phytoplankton proliferation in our wetlands immediately after *Bti*-treatment.

Materials and methods

Study sites

The French Mediterranean coast includes a large number of temporary wetlands characterized by wide spatial and temporal variations in many environmental parameters (Nuccio *et al.*, 2003). The Camargue Rhone Delta region contains a great variety of natural and human-modified ecosystems, including temporary ponds and agricultural fields. The hydrological cycle of these ecosystems results both from autumnal precipitation and from artificial irrigation (rice-growing), inducing an alternating flooding/drought cycle. To reduce mosquito nuisance, an experimental control program encompassing 2500 of the 25 000 ha of potential larval biotopes was assessed through impact studies on the non-target flora and fauna by Parc Naturel Régional de Camargue (Poulin *et al.*, 2010). This mosquito larva control has systematically been carried out since 2006 by aerial spraying of *Bti* (aqueous solution of VectoBac® 12AS at 2.5 $L.ha^{-1}$). Expressed as International Toxic Units (ITU), the recommended application rate of VectoBac® 12AS (0.371×10^9–1.59×10^9 $ITU.ha^{-1}$ corresponding to 0.29–1.24 $L.ha^{-1}$).

The study investigated three shallow Mediterranean temporary oligohaline wetlands located in Southern France (Clos d'Armand = site 1 (43°21′56″N; 4°48′59″ E), Belugue = site 2 (43°25′27″N; 4°40′35″E) and Mourgues = site 3 (43°30′16″ N; 4°16′04″E)). All three sites were located in areas treated in winter, spring and summer after

Table 1. Causes of flooding, and sampling chronology before and after Bti-treatment (T0 = 1 day before treatment and considered as control – T2, T5 and T11, respectively, 2, 5 and 11 days after treatment) at three sites: Clos d'Armand = site 1; Belugue = site 2; Mourgues = site 3.

			T0	T2	T5	T11
Wetland	Sampling campaign	Cause of flooding	Control	Post-treatment dates		
Site 1	August 2006	Irrigation	+	+	+	Dried
	September 2006	Precipitation	+	+	+	+
	May 2008	Irrigation + precipitation	+	+	+	+
	August 2008	Irrigation	+	+	+	+
	June 2009	Irrigation	+	+	+	+
	September 2009	Precipitation	+	+	+	+
	April 2010	Precipitation	+	+	+	Dried
	May 2010	Irrigation	+	+	+	+
Site 2	May 2007	Irrigation	+	+	+	+
	June 2008	Irrigation	+	+	+	Dried
	May 2009	Irrigation + precipitation	+	+	+	+
	July 2010	Irrigation	+	+	+	Dried
	April 2011	Irrigation + precipitation	+	+	+	+
	June 2011	Precipitation	+	+	+	+
Site 3	February 2010	Precipitation	+	+	+	+
	May 2010	Precipitation	+	+	+	Dried
	September 2010	Precipitation	+	+	Dried	Dried
	April 2011	Precipitation	+	+	+	+
	July 2011	Precipitation	+	+	+	+

flooding by precipitation or irrigation. Treatments of the wetlands were performed by technicians from the "Entente Interdépartementale pour la démoustication du littoral méditerranéen" (EID méditerranée = French public organization responsible for mosquito control and survey in Southern France).

Phytoplankton community and physico-chemical parameters

Analysis of phytoplankton dynamics was conducted over 19 campaigns spread over 5 years. For each campaign, total phytoplankton densities, densities of different phytoplankton taxonomical groups and community structure were analyzed before and after each treatment in the following order: T0 = 1 day before treatment and considered as control, T2, T5 and T11 = 2, 5 and 11 days, respectively, after treatment. Samples at T11 were not systematically taken due to drying out of the wetlands (Table 1).

In the wetlands where phytoplankton was sampled, water depth was measured to the nearest 1 mm using a graduated aluminum gauge at the same point on every sampling date. Concurrently, water temperature, conductivity and salinity were measured at 5 cm below the water surface, using a portable probe (Wissenschaftlich-Technische-Werkstatten—WTW). Measurements were always made between 9:00 a.m. and 12:00 p.m. to ensure consistency among data relative to possible circadial influence.

Nitrites ($N–NO^{-2}$), nitrates ($N–NO^{-3}$) and soluble reactive phosphate (SRP) were measured at the beginning of each campaign (control T0), because these parameters play a key role in eutrophication of surface waters (OECD, 1982; Reavie *et al.*, 1995). High *P* concentrations have been linked to increasing rates of plant growth, changes in species composition and proliferation of planktonic and epiphytic and epibenthic algae, resulting in shading of higher plants (Mainstone and Parr, 2002). Nitrites and nitrates were analyzed from water samples filtered in the laboratory using Whatman GF/C glass-fiber filters according to APHA (1989). SRP, a measure of monomeric inorganic phosphorus (orthophosphate) in solution, was measured spectrophotometrically following the formation of phosphomolybdic acid.

For each date and site, three phytoplankton samples (pseudo-replicates) were realized, making a total of 12 samples per mosquito control campaign. Three water samples were collected using PVC sterile 1 L bottles at the water sub-surface. These three phytoplankton samples were taken at points far enough apart to represent the entire wetland surface. All in all, 205 phytoplankton samples were preserved with formaldehyde solution (35%). Under an "Olympus" inverted microscope (× 400 magnification), phytoplankton taxa were identified and the number of cells were counted for each species, allowing densities to be estimated for each species. Quantification of phytoplankton total cell number (density) was provided via standard counting techniques (APHA, 1989). Digested Bacillariophyta samples were mounted in a highly refractive medium, Naphrax, accentuating the frustular details used in taxonomy. Most taxa were identified using the diverse band of the *Süsswasserflora von Mitteleuropa* (Krammer and Lange-Bertalot, 1986, 1988, 1991a, 1991b; Komàrek and Anagnostidis, 1999, 2005). Euglenophyta was identified using the atlas of Wotowski and Hindàk (2005).

Table 2. Values and range of variation (minimum to maximum) of environmental parameters measured in water for the three wetlands from 2006 to 2011. (Site 1 $n = 30$; Site 2 $n = 22$; Site 3 $n = 15$). SRP are Soluble Reactive Phosphate.

Wetlands		T (°C)	Depth (cm)	Salinity ($g.L^{-1}$)	$N–NO^{-2}$ ($mg.L^{-1}$)	$N–NO^{-3}$ ($mg.L^{-1}$)	SRP ($\mu g.L^{-1}$)
Site 1	Mean	21.1	28	2.3	< 0.01	< 0.1	< 0.01
	Min	16.3	5	0.4	< 0.01	< 0.1	< 0.01
	Max	23.1	47	3.1	< 0.01	< 0.1	0.01
Site 2	Mean	21.3	35	1.7	< 0.01	0	< 0.01
	Min	17.4	6	0.1	< 0.01	0	< 0.01
	Max	25.2	56	2.5	< 0.01	< 0.1	0.04
	Mean	17.8	30	2.1	< 0.01	0	< 0.01
Site 3	Min	9.4	7	0.6	< 0.01	0	< 0.01
	Max	25.9	43	3.7	< 0.01	< 0.1	0.01

Statistical analysis

For each site and campaign, between-campaigns ($n = 19$) and between-dates comparisons were analyzed for total density values measured at T0 (control) and at T2, T5 and T11 (post-treatment dates), respectively. These values were Log-transformed and compared using the Wilcoxon non-parametric test (R package pgirmess).

The response of each phytoplankton taxonomical group to *Bti*-treatment was analyzed using generalized linear mixed models (GLMM), with time and sites as fixed effects (due to the low number of sites; Bolker *et al.*, 2009) and dates and replicates nested in dates as random effects. To account for overdispersed count-data, we used negative binomial distribution as error distribution using the R software (version 3.1.2, R Core Team 2014) and the glmmADMB package (Fournier *et al.*, 2012). These GLMMs estimated the average responses of phytoplankton abundance to *Bti*-treatment over time, the between-dates variability of these responses and the variability among replicates.

Dissimilarity of phytoplankton communities was assessed using the Bray–Curtis distance calculation based on phytoplankton species cell counts. Between pseudo-replicates dissimilarities were calculated for each campaign and each date. Between-dates distances were calculated between T0 and T2, T0 and T5, T0 and T11, for each campaign. Between-sites differences were estimated for campaigns performed during the same season and for the same dates (T0, T2, T5 and T11). Finally, between-years changes were calculated between 2 years, respectively, 1, 2, 3 or 4 years of treatment apart.

Shannon's diversity index and Pielou's evenness index were calculated for the control (date T0) and after VectoBac® applications (mean values of post-treatment dates T2, T5 and T11). The calculations were performed with the R package Vegan, and were compared using the Wilcoxon non-parametric test (R package pgirmess).

Results

Water quality and nutrients

Water temperature varied from 9.4 to 25.9 °C (February–July) and the mean depth of these sites never exceeded 56 cm (Table 2). Salinity varied slightly according to the source of flooding and the impact of the salt ground water during the decrease in water level (Table 2).

Chemical parameter values indicate the very limited effect of nutritive enrichment in the three study sites: Nitrite ($N–NO^{-2}$), nitrate ($N–NO^{-3}$) and SRP values were very low, never exceeding 0.01, 0.1 and 0.04 $g.L^{-1}$, respectively. Nutrient values indicated oligotrophic conditions at all three study sites.

Phytoplankton communities

Total density analysis

At sites 1 and 2, between-campaigns changes were observed in all total phytoplankton densities (Figs. 1(a) and (b)). At site 1, total densities were similar in 2008 and were significantly lower than densities registered in 2006, 2009 and 2010. These total densities were significantly higher for the two campaigns of 2009. At site 2, total phytoplankton densities were significantly higher in 2009 than in any other campaigns, and they were significantly lower in April 2011 than in any other campaigns. Algal cell densities at these two sites were highest in 2009. At site 3, a non-significant low temporal variation was observed between campaigns (Wilcoxon test, $P > 0.05$).

The dynamics of total phytoplankton densities (between T0 and T11) differed from site to site and campaign to campaign. Density patterns varied widely with cases both of increase (12 cases as follows: site 1: August and September 2006, May and August 2008 and April 2010. site 2: May 2007, June 2008, May 2009, June 2010. Site 3: February and May 2010 and April 2011) and of decrease (seven cases) between the control date (T0) and the first post-treatment date (T2). Nevertheless, between-dates comparison (T0/T2, T0/T5 and T0/T11 for a single site and a single campaign) revealed the differences to be non-significant (Wilcoxon test, P-value > 0.05), Thus, no tendency to phytoplankton proliferation was observed. Despite this inter-annual variability, total phytoplankton densities did not increase systematically after *Bti*-treatment.

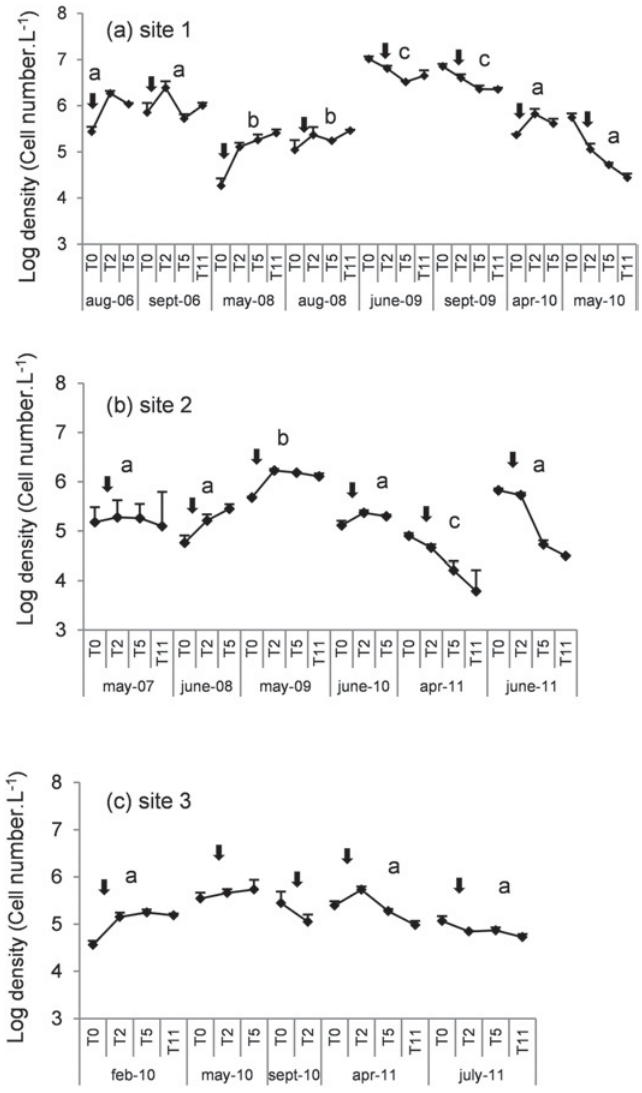

Fig. 1. Changes in mean ± SE ($n = 3$) phytoplankton cell density (cell.L^{-1}) log-transformed at site 1 (a), site 2 (b) and site 3 (c) in the control (date T0) and after VectoBac® application (T2, T5 and T11, post-treatment dates). Different letters indicate significant differences according to the Wilcoxon test ($P < 0.05$). Small arrows indicate the VectoBac® applications (between T0 and T2) in the treated area.

Density of different phytoplankton taxonomical groups

Five phytoplankton taxonomical groups were identified: Bacillariophyta (Diatoms), Chlorophyta, Cyanobacteria, Euglenophyta and Dinophyta.

The GLMM showed Diatoms to be the only group with a significant average decrease in density over time (Wald test, P-value < 0.001). Diatoms group density showed a significant decrease after T0.

No significant relationships between density and time were observed for the four other groups. The between-dates variability (random effect, Table 3) in the relationships between phytoplankton group density and times (intercept and slopes) was always significant (likelihood ratio test, P-value < 0.001). Notably, the between-dates variability on the slopes was always high (greater than the standard deviation on the fixed effect). No pattern emerged, since almost all possible responses of densities to time could be observed. In contrast, the variability between replicates for a given date was very low, a thousand times lower than between dates (Table 3) and never significant (likelihood ratio test, P-value > 0.05). For the Euglenophyta, this variability was even too low to be estimated.

Phytoplankton community structure

Between-pseudo-replicates dissimilarity was quite low, and did not change during a given campaign. The median varied between 17% (post-treatment T5) and 22.5% (control T0) (Fig. 2(a)), suggesting similar phytoplankton densities in the pseudo-replicates at all three sites. Between-dates dissimilarity values were quite high, and fluctuated during a given campaign (Fig. 2(b)). The median of the Bray–Curtis index varied between 67.4% (T0/T2) and 87.1% (T0/T11). The highest values were usually observed when the wetland began to dry up, suggesting that the impact of drought may have been greater than that of the VectoBac® applications.

Between-sites dissimilarity (Fig. 2(c)) was very high, with the medians of the Bray–Curtis index varying between 77.9% (T2) and 96.7% (T0 control), confirming the hypothesis of higher phytoplankton community variability linked to inputs of water and origin of irrigation, and thus random phytoplankton colonization. Moreover, between-years dissimilarity (Fig. 2(d)) was high, the median varying from 53.3% (after 1 year of treatment) up to more than 80% (after, 2, 3 and 4 years of treatment), suggesting that each new water input engendered a new algal colonization leading to increasing between-years differences in communities.

A total of 88 phytoplankton taxa were identified over 19 treatment campaigns. Diatoms dominated, with 47 Taxa. Chlorophyta (green algae) were represented by 21 taxa, Euglenophyta by 14 taxa, Cyanobacteria by 13 taxa and Dinophyta by 4 taxa.

The Shannon–Wiener diversity values measured at the three sites for 19 phytoplankton sampling campaigns (Fig. 3(a)) showed high variability between sampling campaigns. We noted a greater variability at T0 (control) for each phytoplankton sampling campaign than immediately after VectoBac® applications (post-treatment dates). At site 1, the highest Shannon diversity index value was observed at control T0 in April 2010 (IS = 3.37) and the lowest at control T0 in September 2009 (minimum = 0.042). At site 2, the highest Shannon diversity index value was observed at control T0 in June 2010 (IS = 3.33). In June 2008, only one phytoplankton species was detected in samples, explaining the value of IS = 0. At site 3, the highest value was measured at control T0 in February (IS = 3.04), and the lowest value was measured post-treatment (April 2011; IS = 0.44). No

Table 3. Effect of time (fixed effect) on algae abundances (slope on the link scale), and between-dates and between-replicates variability on intercept and slopes (random effect, SD). The significant effects are in bold (Wald test for fixed effect and likelihood ratio test for random effects, $\alpha = 5\%$).

	Fixed effect		Random effect			
			Date		Replicates	
	Intercept	Time	Intercept	Time	Intercept	Time
Diatoms	**7.5426 (0.6662)**	**−0.3061 (0.0775)**	1.459	0.2961	7.8×10^{-5}	4.6×10^{-5}
Chlorophyta	**6.5817 (0.8877)**	0.0674 (0.0977)	2.0157	0.3474	1.1×10^{-4}	4.6×10^{-5}
Cyanobacteria	**9.5716 (0.4096)**	− 0.0288 (0.0437)	0.8533	0.1349	1.3×10^{-4}	5.3×10^{-5}
Euglenophyta	**6.4881 (1.1538)**	− 0.0145 (0.0325)	2.7579	0.0985		
Dinophyta	1.1515 (2.3225)	0.5078 (0.3283)	4.709	1.2814	2×10^{-4}	5.9×10^{-5}

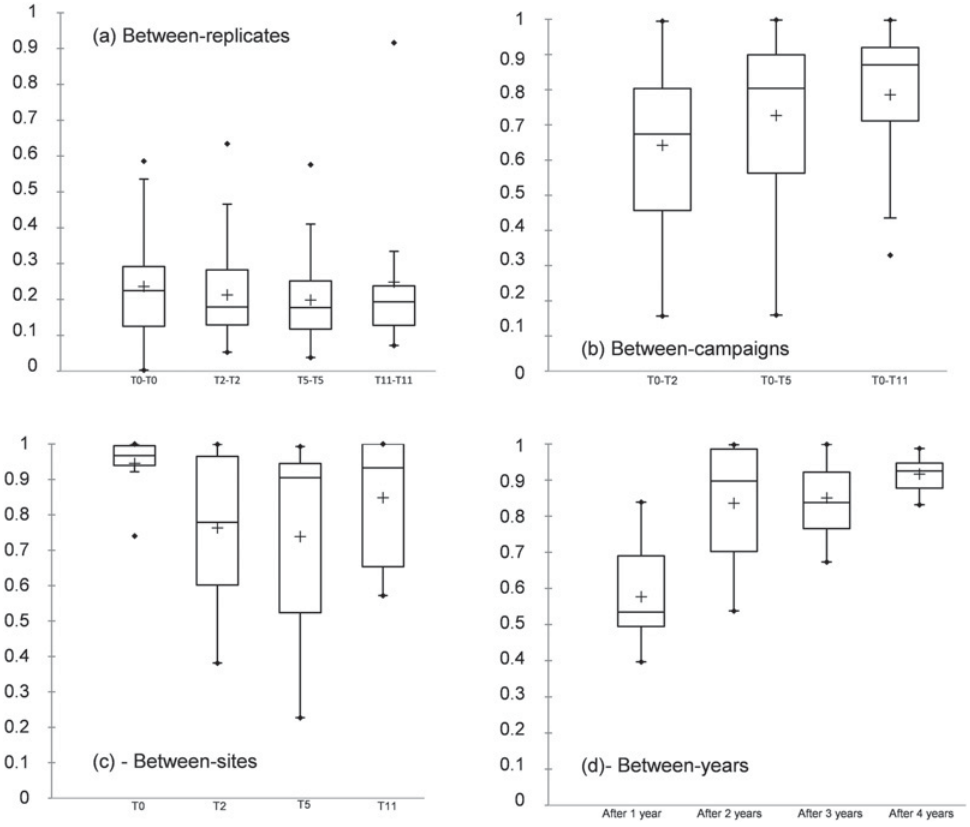

Fig. 2. Box plots for dissimilarity of phytoplankton communities (median and minimum–maximum values of Bray–Curtis distance). (a) Between pseudo-replicates distance calculated for each campaign and each date. (b) Between-dates distances calculated between T0 and T2, T0 and T5, T0 and T11, for each campaign. (c) Between-sites distances were estimated for the same dates (T0, T2, T5 and T11) and for campaigns performed in the same season. Between-years distances were calculated between 2 years, respectively, 1, 2, 3 or 4 years of treatment apart.

significant differences in Shannon's diversity index appeared between control T0 and post-treatment dates for any site (Wilcoxon test, $P > 0.5$ at the three sites). Pielou's evenness values (Fig. 3(b)) evolved in the same way at the three sites, and evenness index values were not statistically significant. Like phytoplankton densities, community structure descriptors showed large temporal variations, and did not systematically decrease after VectoBac® spraying. Actually, diversity and evenness varied widely, with cases of both increase and decrease, except for site 3. These results indicate marked temporal changes in the three pools, without any real pattern.

Discussion

Phytoplankton may be ideal indicators in aquatic systems because of their ubiquitous distribution and rapid response to variable environmental stresses (Hutchinson, 1967; Lowe and Pan, 1996). Our findings indicate that phytoplankton community variability in total density, taxonomical group density and community structure in these Camargue treated wetlands is driven by natural fluctuations in the environmental conditions of each site. No trends toward phytoplankton proliferation or density increase were observed under VectoBac® spraying over the

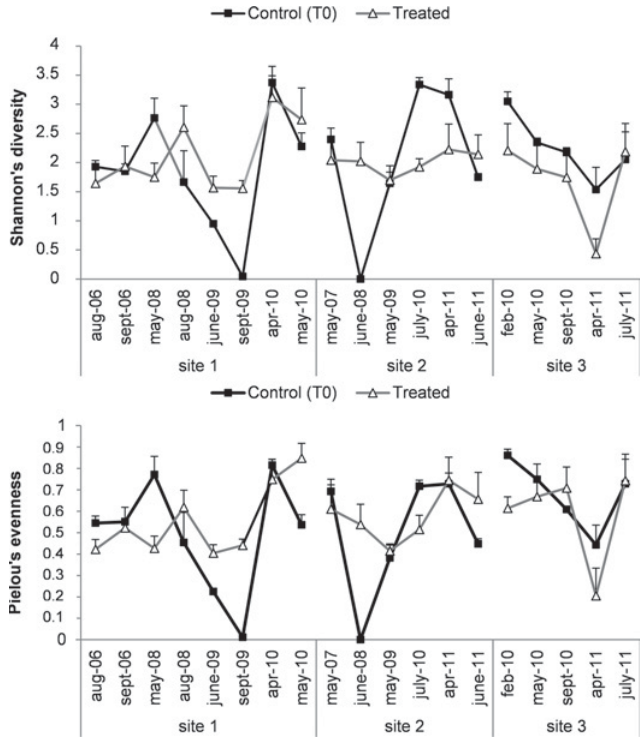

Fig. 3. Mean values ± SE of Shannon's diversity index (a) and Pielou's evenness index (b) measured on the control (date T0, $n = 3$) and post-treatment by VectoBac® dates (T2, T5 and T11; $n = 9$) at three sites and in 19 campaigns.

long-term. The differences in phytoplankton densities observed can be explained by the inter-annual variability present in coastal wetlands (Goldsborough and Robinson, 1996; Goldsborough, 2001) and can be attributed to shifts in phytoplankton community dynamics. High variability in physico-chemical parameters and hydrological conditions, including temperature, salinity, water depth, inputs of water, drought and seasonality, influenced phytoplankton colonization in these wetlands. Previous field studies failed to demonstrate short- or long-term effects of *Bti* on non-target species (Niemi *et al.*, 1999; Duchet *et al.*, 2008, 2010; Lündstrom *et al.*, 2010a, 2010b; Caquet *et al.*, 2011; Lagadic *et al.*, 2014), reaching the same conclusions. Hydrological patterns play an important role in site heterogeneity in the Camargue wetlands. Phytoplankton dynamics are influenced by the duration of the hydroperiod and by seasonality in temporary wetlands, especially in a Mediterranean climate (Nuccio *et al.*, 2003). Flooding and areal inundation were also shown to be decisive for annual heterogeneity of phytoplankton densities, with wetland landscape heterogeneity conditioned by water inflows due to areal inundation (Angeler *et al.*, 2000).

In the Camargue wetland sites treated here, no phytoplankton proliferation was observed after the disappearance of the phytophagous mosquito larvae. Previous studies revealed that algal biomass (estimated by total chlorophyll concentration) was significantly reduced under high-dosage *Bti*-treatment, approximately 2 weeks after *Bti* application (Su and Mulla, 1999; Demisse, 2013). Demisse (2013) monitored the algal biomass and showed that it ceased growing 9 days after strong *Bti* application the high-dosage *Bti*-treatment used both in this and in Su and Mulla's study was twice the concentration of *Bti* currently recommended for mosquito control in wetland wastewater treatment. It is unknown whether the reduction of algal biomass in the water column observed in this study, or in Su and Mulla (1999), was directly related to the active substances of VectoBac G, or to other proprietary components of the formulation. Another hypothesis is that application of *Bti* may have had an indirect effect, favoring the proliferation of *Bacteria* taxa with potential algicidal effects: some *Bacillus* species are known to produce algicidal toxins. For example, Reim *et al.* (1974) reported that the antibiotics produced by 128 *Bacillus bevis* (Firmicutes: *Bacillaceae*) directly inhibited the growth of Cyanobacteria, *Plectonema boryanum* and other congeneric species. Other *Bacteria* species such as *Bacillus cerus* (Firmicutes: *Bacillaceae*), *Planomicrobium* sp. (Firmicutes: *Planococcaceae*) and several species in the genera *Pseudoalteromonas* (*Gammaproteobacteria*: *Pseudoalteromonaceae*) are also reported to produce algicidal toxins (Skerratt *et al.*, 2002; and references cited therein). The elimination of some *Bacteria* taxa under strong *Bti* treatment might also be directly linked to the elimination of algae. The surfaces and surroundings of algae, also known as the phycosphere, are known to influence *Bacteria* communities as well (Eigemann *et al.*, 2013).

In our study, the dominant phytoplankton taxonomical group is inoculated naturally by water input to wetland areas, and is composed of Diatoms and Cyanobacteria. During flooding by irrigation or precipitation, this Diatom inoculum is a colonizer species (*r*-strategies) rather than a competitor species. Here, the Diatoms group tended to decrease, being replaced by species more typical of temporary wetlands such as Euglenophyta and Dinophyta, whereas the Cyanobacteria remained. Temporary pools are not a very suitable ecological niche for Diatom development and preservation, particularly in view of the hydrological fluctuations characteristic of such pools. In these temporary pools, therefore, the Diatoms group did not constitute a suitable bioindicator for monitoring the effects of *Bti*-treatment: decreasing depth and the consequent drought events contributed to their regression. Another possible explanation for the decrease in Diatoms may be low inorganic nutrient levels that were eliminated by other inorganics located very low in these wetlands, such as silicium (Dixit *et al.*, 1992). An important question is whether *Bti*-treatment may be the cause. According to Goldsborough (2001) the short generation time of algal cells means that changes in taxonomic composition of an assemblage, due to toxicant exposure for instance, can occur within a few days. Thus, changes in the proportions of phytoplankton taxonomical group, or local extinction, may be sensitive indicators of autogenic or allogenic stress. This hypothesis of a direct effect of *Bti*-treatment on the Diatoms group needs to be tested within the framework of a study in batch culture.

Laboratory bioassays should be conducted with a limited number of cultivable Diatoms species. According to Koskella and Stotzky (2002), the insecticidal toxins from *Bti* did not affect the growth of a variety of algae (primarily green and Diatoms) in pure and mixed culture. The toxins from *Bti* were also non-inhibitory in dilution tests to pure and mixed cultures of algae and Cyanobacteria. Although the results of these *in vitro* studies agree with those observed in studies in soil, none of these studies evaluated changes in the composition of mixed cultures. The authors concluded that further studies on the effects of these toxins on biodiversity are clearly needed.

These hypotheses of allogenic stress and direct effect linked to *Bti* application are not consistent with our observations. Indeed, before *Bti*-treatment (control T0), the density of phytoplankton taxonomical groups varied widely due to the coexistence of two phytoplankton communities, the exogenous community dominated by Diatoms and Cyanobacteria inoculated by inputs of water, and the endogenous community at each site represented by an algal crust (Euglenophyta, Dinophyta). The Diatoms group responds rapidly to environmental conditions, so drought events may limit development of some species. The temporal dynamics of the phytoplankton groups between T0 and T2, T5 and T11 in these temporary wetlands can be described by the following colonization dynamics. At T0: flooding (by irrigation or precipitation) leads to a phytoplankton community composed of "exogenous" algal and cyanobacteria groups represented and dominated by Diatoms (colonizers) and Cyanobacteria (competitors). Water input engenders the detachment of the algal community composed of "endogenous" algal groups, plocon and metaphyton, as described by Goldsborough (2001) in his work on phytoplankton assemblages in wetlands. The detached algae plocon and metaphyton are typical of encysted Euglenophyta, Chlorophyta and Dinophyta. Chlorophyta can be considered as competitors but this algal group is weakly represented because calcareous conditions are more marked and nutrient input lower. At dates T2 and T5, fast-growing algae enhanced by water input and the detachment of the algal crust increase, and recolonize the water column. Cyanobacteria and Euglenophyta identified in temporary wetlands possess wide ecological valency, with a tolerance for drought expressed by forming an algal crust and desiccation as well as fast recolonization (2 days). At T11, evidence of the decrease in water depth and inundation area is provided by the algal crust formed at the surface, usually composed of mucilaginous cyanobacterial trichomes, including motile Euglenophyta cysts. Euglenophyta and Cyanobacteria are common in these Mediterranean temporary wetlands, due to their ability to tolerate variations in water level and desiccation.

The changes in the density of phytoplankton taxonomical groups observed here are therefore due to natural conditions related to alternating flooding and drought events, as phytoplankton responds to hydric stress. Phytoplankton subjected to a variable moisture regime must adapt, in order to tolerate extreme conditions (Sheath and Wehr, 2003). Other filamentous forms (*Oscillatoria*, *Nostoc*) may, during the open (flooded) state, form thick mats that protect algal cells during a later dry phase (Sheath and Wher, 2003). In the Everglades wetlands, filamentous cyanobacteria, including *Scytonema*, *Schizothrix*, *Oscillatoria* and *Microcoleus*, are often abundant (Goldsborough and Robinson, 1996; Pan and Stevenson, 1996). In our temporary wetlands, the dynamics of the phytoplankton taxonomical groups changed over time, from the beginning of water input to water depletion in the pools, despite the very low nutrient input. Phosphorus limitation has been suggested as a factor structuring the taxonomic composition of phytoplankton communities in these wetlands (Beaver *et al.*, 1998). Diversity measured by the Shannon index confirmed our conclusions on phytoplankton densities, *i.e.,* there was no evidence of change for all descriptors of phytoplankton community structure after VectoBac® application. Although changes in the abundance of phytoplankton groups appeared during the monitoring of *Bti*-treatment, phytoplankton community structure was not impacted and the proportions of the different communities of the temporary wetlands remained the same after *Bti*-treatment.

Conclusion

The reduction of mosquito larva density ($>80\%$) by *Bti*-treatment did not indirectly affect phytoplankton densities and community structure in these treated temporary wetlands. Their elimination did not engender phytoplankton proliferation. It should be noted that the changes in phytoplankton taxonomical group are due to natural conditions related to alternating flooding and drought events. We can thus conclude that control of the primary consumers (top-down control) does not impact the phytoplankton community. After investigating the indirect effects of *Bti*-treatment we are able to confirm the hypothesis that this bioinsecticide does not affect phytoplankton densities and diversity and does not constitute an anthropogenic stress in phytoplankton community.

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