Ecotoxicology and Environmental Safety 74 (2011) 1122–1130

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Effects of repeated field applications of two formulations of *Bacillus thuringiensis* var. *israelensis* on non-target saltmarsh invertebrates in Atlantic coastal wetlands

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ARTICLE INFO

Article history:
Received 21 December 2010
Received in revised form
16 April 2011
Accepted 26 April 2011
Available online 17 May 2011

Keywords:
Larvicide
Mosquito control
Non-target invertebrates
Field biomonitoring
Brackishwater pools

ABSTRACT

Bacillus thuringiensis var. *israelensis* (*Bti*) is commonly used for selective control of larval populations of mosquitoes in coastal wetlands. A two year-study was implemented to investigate whether repeated treatments with *Bti* applied either as a liquid (VectoBac® 12AS) or a water-dispersible granule (VectoBac® WG) formulation may affect the abundance and diversity of non-target aquatic invertebrates in saltmarsh pools. Taxonomic composition of the invertebrate communities was typical of brackishwater intermittent ecosystems, with a dominance of annelids, crustaceans and nematocerans. Conditions were contrasted between the two years of the survey, both in terms of annual cumulative rainfall and rainfall distribution throughout the year. As a consequence, the hydroperiod and some other environmental characteristics associated with pool drying played a major role in the dynamics of the invertebrate community. In summer 2006, pool drying reduced the abundance of the polychaete worm *Nereis diversicolor*, of the amphipod crustacean *Corophium volutator* and of chironomid larvae. These taxa were able to recolonize rapidly the pools after flooding in September 2006. In 2007, rainfall was more regularly distributed across the year, and the pools did not get dry. Hydrozoans, Chironomini and Orthocladiinae larvae, and oligochaetes were more abundant in treated than in control pools, especially in VectoBac® WG-treated pools. No adverse effects of the treatments were shown on the abundance of *N. diversicolor*, *C. volutator* and midge larvae, suggesting that the availability of these food sources for birds was not negatively affected by *Bti* applications. It is concluded that, as currently performed in Western France coastal wetlands, land-based treatments of saltmarsh pools for larval mosquito control with *Bti*, used either as VectoBac® 12AS or VectoBac® WG, did not adversely impact non-target aquatic invertebrate communities.

1. Introduction

Saltmarshes develop on wave-sheltered shores, usually between mean high water neap tide level and mean high water spring tide level, where moderate erosion enhances vegetation development (Hughes, 2004). They export organic matter that is a major resource for marsh and coastal food webs (Valiela et al., 2002), and they are especially important as feeding, growing and reproduction areas for many fish and bird species (Swanson, 1985; Hughes, 2004). In addition to marine invertebrates (e.g., annelids, crustaceans, molluscs), these areas are colonized by aquatic insects, especially Diptera of the sub-order Nematocera, mainly Chironomidae, Ceratopogonidae and Culicidae (Giberson et al., 2001). These temporarily flooded systems are highly suitable habitats for mosquito species that deposit their eggs on the edge of receding tidal creeks and pools. The eggs hatch when high tides or rain flood the marsh, and massive emergences of adult mosquitoes frequently become a great nuisance. These areas may therefore be subjected to mosquito control for protection of public comfort or health (Becker et al., 2003).

In the recent years, commercial formulations of *Bacillus thuringiensis* var. *israelensis* (*Bti*) have been increasingly used for selective control of larval populations of mosquitoes in many types of continental aquatic ecosystems (e.g., Upper Rhine; Becker, 1997; Becker et al., 2003). Coastal wetlands may also be subjected to such treatments (Russell et al., 2009). Currently, *Bti* is the only larvicide used in Europe as the result of the implementation of the EU Biocidal Products Directive 98/8/EC. For mosquito control, *Bti* may be applied as liquid (AS), granule (G) or water-dispersible granule (WG) formulations. The main inconvenience of G formulations is their application rate (several kg ha^{-1}), which makes them unsuitable for ground-based application by

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doi:10.1016/j.ecoenv.2011.04.028

portable apparatus. The WG formulation of the commercial product VectoBac® proved to be as efficient or even more efficient than the AS formulation, and it has additional advantages of economical transport, storage and longer shelf life (Russell et al., 2003). This is why this formulation currently tends to replace the liquid formulation (VectoBac® 12AS) for use in mosquito control in many countries worldwide. However, data on the comparative potential impacts of the AS and WG formulations of *Bti* on non-target invertebrates are scarce.

With respect to environmental impact, controversial results have been obtained for *Bti*-containing larvicides. Laboratory tests and field studies showed that *Bti* may be considered as 'safe' to the environment due to its selectivity (Mulla et al., 1982; Barnes and Chapman, 1998; Boisvert and Lacoursière, 2004). However, larvae of some taxa of non-target Nematocera such as Chironomidae have been shown to be susceptible to *Bti* (Kondo et al., 1992; Rey et al., 1998), and there are indications that non-target organisms may be impacted by *Bti*-containing larvicides in wetlands (Hershey et al., 1998; Niemi et al., 1999; Boisvert and Boisvert, 2000). In contrast, results from experiments performed in temporary freshwater ponds showed that *Bti*, applied at two week-intervals as VectoBac® G at the rate of 7.85 kg ha^{-1}, did not adversely affect the density or biomass of various invertebrate groups, nor the taxonomic richness of benthic invertebrate communities (Hershey et al., 1995). In an *in situ* experiment in the River Dalälven floodplain (Central Sweden) designed to assess indirect effects of reduction of the abundance of mosquito larvae by *Bti* (applied as VectoBac® G at 13–15 kg ha^{-1}) on predatory beetles (Dytiscidae), Vinnersten et al. (2009) found that, although the abundance of mosquito larvae was strongly reduced (close to 100% reduction), there was no general effect of treatments on the abundance of diving beetles.

Implementation of repeated *Bti* treatments during several years raises the question of possible long term effects of the larvicide. A long-term (6 years) field study in Minnesota freshwater wetlands showed that *Bti* (applied as VectoBac® G at rates ranging from 5.9 to 17.2 kg ha^{-1}) induced a significant decrease in the abundance of non-target invertebrates, especially Nematocera, associated with a reduction in insect genera richness and an increase in dominance indices (Hershey et al., 1998; Niemi et al., 1999). In a six-year survey performed in the River Dalälven floodplain, no significant effect of flood-water mosquito control using aerial application of VectoBac® G (13–15 kg ha^{-1}) was shown on the production of emerging insects (Persson Vinnersten et al., 2010), nor on chironomid species richness (Lundström et al., 2010a) and production (Lundström et al., 2010b). Most of the available information about long term effects of repeated *Bti* applications concerns continental wetlands, and is not applicable to saltwater coastal wetlands due to differences in terms of hydrology, aquatic invertebrate community composition and food web structure (Russell et al., 2009). Furthermore, there is no data on the comparative effects of various *Bti* formulations on non-target aquatic communities living in these areas. The present two year-study was therefore implemented to investigate whether repeated treatments with *Bti* applied either as a liquid (VectoBac® 12AS) or a water-dispersible granule (VectoBac® WG) formulation may have long term effects on the abundance and diversity of non-target aquatic invertebrates in saltmarsh pools located in southern Brittany. This region benefits from particular protection status within European or global networks of protected sites created under the European Natura 2000 or international Ramsar Conventions. It is also well-known as a feeding and breeding ground for birds. Therefore, a particular attention has been devoted therein to non-target invertebrate species that represent a food source for birds. Correlations between fluctuations of environmental parameters and responses of the aquatic invertebrate community to *Bti* exposure have been explored for data interpretation.

2. Materials and methods

2.1. Study site

The study was performed from March 2006 to September 2007 in a 5 ha saltmarsh close to a tidal estuary (Ria d'Etel, Locoal-Mendon, Morbihan, France; 47°42′30″N–03°07′48″W). This is a former saltern where dozens of small pools are regularly flooded by incoming estuary water at high tide (tide coefficient > 90) and by rainfall. A part of this wetland (*ca.* 1 ha) was delimited as a reference area before the beginning of the mosquito control program in 1998, and it has never been exposed to larvicides (Fourcy et al., 2002). The rest of the site has been subjected to larvicide treatments with VectoBac® 12AS since 1998 in order to control *Ochlerotatus* (*Aedes*) *caspius* and *Ochlerotatus (Aedes)* larvae. Based upon the information available for the 1998–2007 period, the total number of treatments per year varied from 5 to 8, with an average number of 6.2. For the purpose of the present study, this part of the site has been divided into two treated zones of equivalent area (*ca.* 2 ha each) where VectoBac® 12AS and VectoBac® WG were applied, respectively. The three zones were adjacent but care was taken to have a 50 to 100 m wide buffer zone between the different zones in order to prevent cross contamination between the treated zones or contamination of the control zone. Within each zone 5 pools (from 10 to 30 m^2 surface) were randomly chosen and numbered before the beginning of treatments in 2006. They were then repeatedly sampled during the study.

2.2. Treatments

VectoBac® 12AS (11.6% *Bti* at 1200 International Toxic Units—ITU per mg, i.e., 923.96 g a.i. L^{-1}; Valent Biosciences, Libertyville, IL, USA) was applied at 0.5 L ha^{-1} whereas VectoBac® WG (37.4% *Bti* at 3000 ITU mg^{-1}; Valent Biosciences, Libertyville, IL, USA) was applied at 0.4 kg ha^{-1}. Actual applied doses were 5- and 2.5-fold lower than the currently recommended application rates in France for VectoBac® 12AS (2.5 L ha^{-1}) and VectoBac® WG (1 kg ha^{-1}), respectively, but they resulted in equivalent applied concentrations of *Bti* (*ca.* 500×10^6 ITU ha^{-1}). Each larvicide was diluted into tap water (0.5 L VectoBac® 12AS in 10 L tap water, 400 g VectoBac® WG in 12 L tap water) before spraying at the water surface, using a portable spraying apparatus. Treatments of the pools were performed by personnel from the Entente Interdépartementale pour la Démoustication du littoral Atlantique (EID Atlantique, Rochefort-sur-Mer, France) on the same dates as those of mosquito control operation in this area. According to the procedure adopted for the mosquito control program in this region, treatments are not performed according to a pre-determined basis but only if the abundance of mosquito larvae exceeds a given threshold (defined by field survey of the abundance of larvae; Carron et al., 2003). Hatching of mosquito larvae occurs after flooding of the saltmarshes, especially by high coefficient tides that are more frequent in February–March and from September to November on the Atlantic Coast. The interval between two treatments may therefore vary from one week to more than one month depending on tide and rainfall intensity. In the context of the present study, *Bti* applications occurred on 6 dates in 2006 (April 07, May 03, June 01, July 15, August 16 and September 17) and on 5 dates in 2007 (March 28, April 25, May 23, July 19 and August 03).

2.3. Invertebrate community sampling and analysis

Invertebrate sampling was scheduled in order to encompass the whole treatment period. The first and the last samples were collected a few weeks before and after the first and the last treatment dates, respectively. Samples were collected on 6 dates in 2006 (March 21, May 04, June 14, July 25, September 05 and October 17) and 4 dates in 2007 (February 21, April 04, June 25 and September 11). Invertebrates were sampled using a stainless steel hand-driven cylindrical corer (20 cm inner diameter, 50 cm height). A sampling point was randomly chosen in each pool, and the corer was rapidly pushed into the sediment. Once the corer was in place, water depth was measured in order to calculate the enclosed volume and the organisms present in the water column were collected using a 250-μm mesh size net, which content was transferred to a 100 mL polystyrene vial. The volume of the water column was used to correct abundances for differences in volume between samples. The sediment core (*ca.* 2.2 L volume) was then retrieved and placed into a 4 L polyethylene box. Both types of samples were then preserved using neutral aqueous formaldehyde (10%, v/v). Back to the laboratory, the sediment cores were manually broken up and placed for 30 min in an aqueous solution of sodium hexametaphosphate (1 g L^{-1}) in order to enhance clay dispersion. Water column and sediment samples were then passed through a series of sieves of decreasing mesh size (8, 4, 2, 1, 0.5 mm for both types of samples, and an additional 0.25 mm mesh size sieve for water column samples). In order to speed up the analysis, subsamples of the sieve content were obtained using

a Motoda splitting box (Motoda, 1959). The collected organisms were enumerated and identified at the lowest practical taxonomic level under a dissecting microscope (maximum magnification 1:75).

Abundance data for the various groups were used to compute several common quantitative descriptors of community structure (Clarke and Warwick, 2001): taxonomic richness (S), total abundance (N), Shannon's diversity index (H') and Pielou's evenness (J). These indices provide complementary information on the number of different taxa (S), on the total abundance of individuals within the samples (N) and on the distribution of the individuals between the different taxa (H', J). Since the study area is known to be an important feeding and breeding area for many bird species, the abundance of four invertebrate taxa known to be of great importance for waterfowl feeding, namely *N. diversicolor*, *C. volutator* and larvae of midges from the Chironomini tribe and Orthocladiinae subfamily (Mackenzie, 2005) were subjected to a more detailed analysis.

2.4. Water quality parameters

In the pools where invertebrates were sampled, water depth was measured to the nearest 1 mm using a graduated aluminum gauge at the same point every sampling date. In parallel, water temperature, dissolved oxygen concentration, salinity and pH were measured at *ca.* 5 cm below the water surface, using portable apparatuses (Wissenschaftlich-Technische-Werkstätten—WTW, Champagne au Mont d'Or, France). Measurements were always made between 10:00 AM and noon to ensure consistency among data relative to possible circadial influence. In each pool, one 1.5 L water sample was collected and stored in opaque polyethylene bottles. Subsamples (500–750 mL) were filtered through Whatman GF/C fiberglass filters (1.2-µm mesh size; Whatman International, Maidstone, UK), which were immersed overnight in 5 mL of an acetone/distilled water (90/10, v/v) mixture at 4 °C for pigment extraction. Chlorophyll *a* was quantified spectrophotometrically (Specord 205, AnalytikJena AG, Jena, Germany) according to Lorenzen (1967). Suspended solids concentration was determined in 250 mL water subsamples filtered through pre-weighted oven-dried (2 h at 500 °C) Whatman GF/C fiberglass filters that were weighted again after 48 h at 105 °C according to the AFNOR (1996) method. Data on daily rainfall were provided by Météo France (weather station of Theix, 47°37′59″N–02°40′01″W).

2.5. Data analysis

Since the same pools were repeatedly sampled, all the data were analyzed using mixed models. Environmental parameters, H' and J values were analyzed using linear mixed models (*lmer* function of R package *lme4*), with time and zone as fixed factors and pool identity as a random factor. In addition, on each sampling date, mean values of H' and J were compared using one-way ANOVA followed by Tukey's *post-hoc* test (*glht* function from R package *multcomp*) to detect point differences between the three zones. Normality of H' and J data was verified using Shapiro–Wilk normality test ($p=0.121$ and 0.500 for H' and J, respectively). The effects of treatments on S, N and on the abundance of *N. diversicolor*, *C. volutator*, Chironomini and Orthocladiinae larvae were analyzed using generalized linear mixed models because the data did not follow a normal distribution and usually exhibited a significant covariation between mean and variance (O'Hara and Kotze, 2010). Analysis were performed using the *glmer* function of R package *lme4*, with time and zone as fixed factors and pool identity as a random factor, and using a Poisson distribution for S (no overdispersion of data) and a quasi-Poisson distribution for the other variables (high overdispersion of data). In addition, on each sampling date, mean values of the different parameters were compared using a quasi-Poisson generalized linear model followed by Tukey's *post-hoc* test (*glht* function from R package *multcomp*) to detect point differences between the three zones.

Changes in the structure of the invertebrate community due to *Bti* exposure were analyzed by the principal response curve (PRC) method (van den Brink and ter Braak, 1999). The PRC method is a multivariate technique based on the redundancy analysis ordination technique. PRC results in a diagram showing the sampling weeks on the x-axis and the first principal component of the treatment (C_{dt}) effects on the y-axis. This results in a diagram showing the deviations, in time, of treatments compared to controls. The species weights (b_k) shown on the right side of the diagram can be interpreted as the weight of each species for the response given in the diagram. For each pool and date, abundance data from water column and sediment core were grouped and the resulting values were $\ln(20x+1)$ transformed before analysis. A Monte Carlo permutation test was used to identify the dates for which a significant difference was apparent among treatments. Correlation between environmental parameters and between environmental parameters and community response descriptors was tested using Spearman's correlation coefficient.

PRC analysis was performed with CANOCO for Windows software package, Version 4.5 (Center for Biometry–Wageningen, Wageningen, The Netherlands). The other tests were performed using R for Windows Version 2.8.0 (R foundation for Statistical Computing). Statistical threshold was $\alpha=0.05$ for all tests.

3. Results

3.1. Environmental parameters

The results obtained for the various environmental parameters measured in 2006 and 2007 are summarized in Table 1. For all environmental parameters, time had a highly significant effect (χ^2 values ranging from 44.6 to 469.3, df=5 to 7 and $p<0.001$). A similar temporal pattern was observed in the three zones for the various environmental parameters. Inter-zone differences were detected for water temperature ($\chi^2=25.0$, df=2 and $p<0.001$) and salinity ($\chi^2=8.9$, df=2 and $p=0.011$). For both parameters, there was a significant time × zone interaction ($\chi^2=77.3$ and 55.6 for temperature and salinity, respectively, df=14, $p<0.001$). Differences were within the natural range of variation of these parameters within the different zones (on average less than 0.8 °C for water temperature and than 1.7 g L^{-1} for salinity). It seems rather unlikely that they might have significant effects on the various biological parameters that were monitored during this study.

Many environmental parameters were significantly correlated (Table 2). Water depth was negatively correlated with salinity, and with chlorophyll *a* and suspended solid concentrations, and positively correlated with water pH. An opposite pattern was observed for water temperature. Correlation between water temperature and depth was not significant. Salinity was positively correlated with suspended solids and chlorophyll *a* concentrations, whereas it was negatively correlated with pH and dissolved oxygen concentration. Suspended solids and chlorophyll *a* concentrations were positively correlated. Finally, a significant negative correlation was found between pH and chlorophyll *a* concentration, whereas pH and dissolved oxygen concentration were positively correlated.

3.2. Invertebrates

A total of 38 different invertebrate taxa were identified in the samples collected during the study period. In the three zones, the aquatic invertebrate community mainly comprised annelids (6 groups among which oligochaetes, *N. diversicolor* and polychaetes of the family Capitellidae were the most abundant ones),

Table 1
Mean ± standard error and range of variation (minimum to maximum) of environmental parameters measured in water for the pools of the three zones in 2006 and 2007.

Zone	Control		VectoBac® WG		VectoBac® 12AS	
Year	2006	2007	2006	2007	2006	2007
Depth (cm)	9.9 ± 1.3 (0–24.5)	15.1 ± 1.8 (4.5–29)	5.7 ± 0.8 (0–16.0)	10.7 ± 1.2 (2.5–19.0)	7.6 ± 1.0 (0–17.0)	12.1 ± 1.5 (3.0–21.0)
Temperature (°C)	14.5 ± 0.7 (8.4–20.8)	12.8 ± 1.0 (6.6–18.4)	14.0 ± 0.7 (8.4–21.8)	13.3 ± 1.0 (7.4–18.9)	14.3 ± 0.8 (8.1–23.0)	14.0 ± 0.9 (8.1–18.7)
Salinity (g L^{-1})	34.0 ± 1.7 (24.2–43.1)	29.5 ± 1.4 (23.8–39.0)	33.4 ± 2.8 (20.4–53.2)	30.6 ± 1.7 (24.2–45.9)	32.2 ± 2.4 (21.7–46.8)	29.6 ± 1.6 (23.2–41.9)
pH	7.2 ± 0.1 (5.2–8.0)	7.4 ± 0.1 (6.7–7.9)	7.2 ± 0.1 (4.9–8.0)	7.4 ± 0.1 (7.0–7.9)	7.1 ± 0.1 (5.3–7.9)	7.3 ± 0.1 (6.7–7.8)
Dissolved oxygen (mg L^{-1})	7.0 ± 0.4 (2.6–13.4)	5.8 ± 0.4 (2.4–9.1)	6.4 ± 0.4 (3.1–10.5)	6.2 ± 0.7 (2.8–9.2)	7.2 ± 0.4 (3.7–13.0)	6.7 ± 0.4 (4.5–11.0)
Suspended solids (mg L^{-1})	17.8 ± 2.2 (5.1–48.0)	10.9 ± 0.8 (5.5–18.9)	19.1 ± 2.0 (5.5–41.9)	11.7 ± 1.1 (5.1–23.8)	17.1 ± 1.4 (7.9–33.5)	13.3 ± 1.3 (6.3–29.9)
Chlorophyll *a* (µg L^{-1})	53.7 ± 23.5 (1.2–648.0)	6.9 ± 1.4 (2.6–30.0)	37.5 ± 12.0 (1.1–267.5)	9.4 ± 3.0 (1.9–53.3)	42.0 ± 13.6 (1.0–247.4)	18.6 ± 7.0 (1.8–127.7)S

Table 2
Values of Spearman correlation coefficient ($n=91$) for water parameters and descriptors of the invertebrate community of the pools in the three areas in 2006 and 2007. *S*: taxonomic richness, *N*: total abundance, *H′*: Shannon's diversity index, *J*: Pielou's evenness. Significant values ($p<0.05$) are indicated in bold characters.

	Depth	Temperature	pH	Dissolved oxygen	Salinity	Chlorophyll *a*	Suspended solids
Temperature	−0.18						
pH	**0.41**	**−0.49**					
Dissolved oxygen	0.07	−0.11	0.13	0.13			
Salinity	**−0.63**	**0.67**	**−0.42**	**−0.24**			
Chlorophyll *a*	**−0.38**	**0.57**	**−0.47**	−0.01	**0.60**		
Suspended solids	**−0.44**	**0.19**	**−0.18**	−0.10	**0.48**	**0.42**	
S	0.02	**−0.26**	**0.23**	**−0.24**	**−0.26**	**−0.20**	−0.01
N	0.02	**−0.25**	0.16	**−0.47**	−0.13	**−0.39**	−0.05
H′	**−0.25**	−0.06	0.08	**−0.21**	0.17	−0.01	0.12
J	**−0.33**	0.10	−0.07	−0.10	**0.37**	0.12	0.17

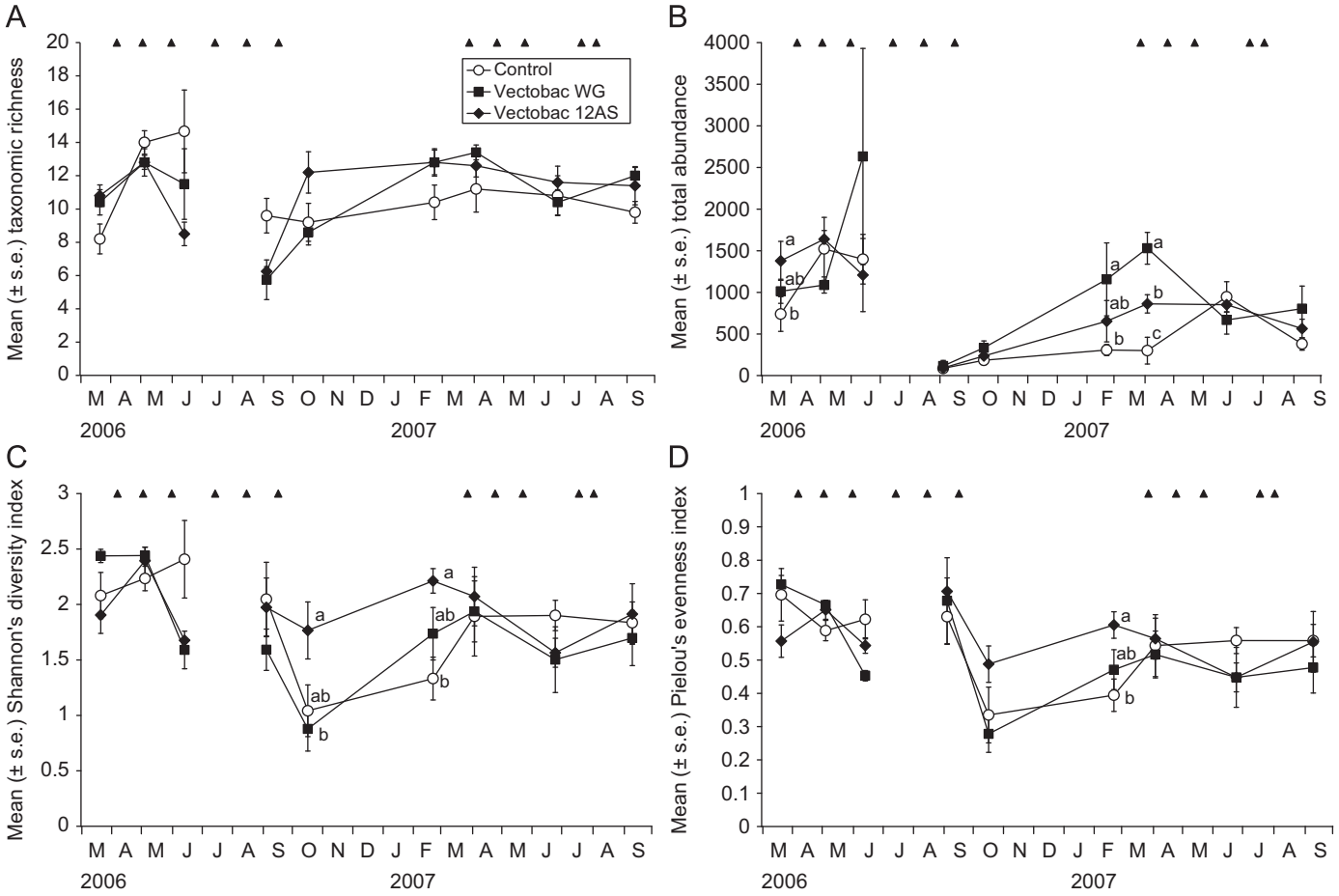

Fig. 1. Changes in mean ± s.e. (standard error; $n=5$) values of the various descriptors of invertebrate communities in control, VectoBac® 12AS- and VectoBac® WG-treated pools. The triangles indicate the dates of larvicide applications in the study area. (A) Taxonomic richness; (B) Total abundance; (C) Shannon's diversity index; (D) Pielou's evenness index. Different letters indicate significant differences according to Tukey's *post-hoc* test following GLM analysis or ANOVA ($p<0.05$). The interruption of the lines corresponds to drying of the pools which prevented sampling.

crustaceans (12 groups among which *C. volutator*, Copepods and Ostracods were the most abundant ones) and insects (11 groups among which larvae of Chironomini from the *Chironomus salinarius* group and of Orthocladiinae were the most abundant ones). Taxa found in the water column included various crustaceans (Copepods, Ostracods, *Palaemonetes varians* adult and larvae, *Lekanesphaera* sp.) and water mites. *Ochlerotatus* sp. larvae and pupae were sometimes found in the samples but their abundance was always very low in all the pools. Important temporal variations were observed for all the descriptors and patterns were almost identical in the three zones (Fig. 1 and Table. 3). Time had a highly significant effect (χ^2 values ranging from 30.6 to 34,312, df=from 6 to 8, $p<0.001$) and drying of the pools in summer 2006 had a very strong negative effect on taxonomic richness and an even stronger effect on total abundance. Significant differences between zones were shown for total abundance ($\chi^2=12.1$, df=2 and $p=0.002$) and for the abundance of the four invertebrate groups identified as food resource for birds ($\chi^2=6.1$, 12.4, 11.9 and 9.5, df=2, $p=0.048$, 0.002, 0.0025 and 0.008 for *N. diversicolor*, *C. volutator*, Chironomini and Orthocladiinae larvae, respectively). For all these variables, there was a significant time × zone interaction ($p<0.001$). Detailed results of the statistical analysis are

provided as Supplementary material. No significant differences were observed between control and treated zones for taxonomic richness (Fig. 1A). Significantly higher total invertebrate abundances in treated than in control pools were observed at the beginning of the study and on two dates in spring 2007 (Fig. 1B). Significantly higher Shannon's diversity index mean values were observed in VectoBac® 12 AS-treated pools on two dates (Fig. 1C). For Pielou's evenness, the only difference between control and treated pools was observed on February 21, 2007 with higher values for VectoBac® 12AS-treated pools than for control pools (Fig. 1D). When significant differences were shown, values of the various parameters were often higher for VectoBac® 12AS-treated pools than for the other pools.

Correlation analysis (Table 2) showed that taxonomic richness and total abundance of invertebrates were negatively correlated with water temperature, dissolved oxygen and chlorophyll *a* concentration. Taxonomic richness was positively correlated with pH and negatively correlated with salinity. Both *N* and *S* were not significantly correlated with water depth or suspended solid concentration. A negative correlation was shown between Shannon's diversity index and dissolved oxygen concentration and water depth. Pielou's evenness was positively correlated with salinity and negatively correlated with depth.

PRC analysis of invertebrate abundance data (Fig. 2) showed significant differences between control and treated pools ($p=0.005$). Monte Carlo permutation tests for the whole data set indicated that a significant amount of the variance explained by the treatments is displayed in the diagram of the first PRC ($p=0.005$). Of all the variance in the abundance data, 49.7% could be attributed to the sampling date, and 15.5% could be attributed to the treatments (31.6% of this variance is displayed on the vertical axis of the first PRC). Monte Carlo permutation tests for each sampling date indicated that the dynamics of invertebrate communities were identical in VectoBac® 12AS- and VectoBac® WG-treated pools. In particular, very large differences between control and treated pools were observed just before and after the drying episode in 2006, and also in spring 2007. The analysis of the distribution of species weights (b_k) indicates that several invertebrate groups were more abundant in treated than in control pools in 2007. Hydrozoans, chironomid larvae (Chironomini tribe and Orthocladiinae subfamily), oligochaetes and chironomid pupae were the most positively impacted groups. On the other hand, although some groups exhibited a tendency to be less abundant in treated than in control pools (Brachycera pupae and Hydrobiidae), the low values of b_k for these groups (close to -0.5) suggest that differences were moderate.

Table 3
Mean ± standard error and range of variation of macroinvertebrate community descriptors for the pools of the three zones in 2006 and 2007.

	Control		VectoBac® WG		VectoBac® 12AS	
	2006	2007	2006	2007	2006	2007
Total abundance (*N*)	733.0 ± 144.0[a] (19–2539)[b]	484.0 ± 81.9 (57–1331)	907.2 ± 142.6 (38–1962)	732.8 ± 69.5 (170–1525)	852.1 ± 158.6 (5–3550)	1039.2 ± 140.8 (252–2717)
Taxonomic richness (*S*)	10.9 ± 0.6 (6–18)	10.7 ± 0.5 (8–16)	10.6 ± 0.6 (6–15)	12.2 ± 0.4 (9–15)	10.0 ± 0.6 (6–15)	12.2 ± 0.4 (9–15)
Shannon's diversity index (*H'*)	1.93 ± 0.13 (0.44–3.33)	1.74 ± 0.11 (0.86–2.67)	1.96 ± 0.08 (1.13–2.77)	1.94 ± 0.10 (1.03–2.67)	1.83 ± 0.14 (0.38–2.66)	1.72 ± 0.11 (0.73–2.53)
Pielou's evenness (*J*)	0.57 ± 0.04 (0.15–0.93)	0.51 ± 0.03 (0.26–0.77)	0.59 ± 0.02 (0.38–0.80)	0.48 ± 0.03 (0.22–0.68)	0.57 ± 0.04 (0.13–0.95)	0.54 ± 0.03 (0.29–0.76)

[a] Mean ± standard error of the mean.
[b] Range (minimum to maximum) for the period under consideration.

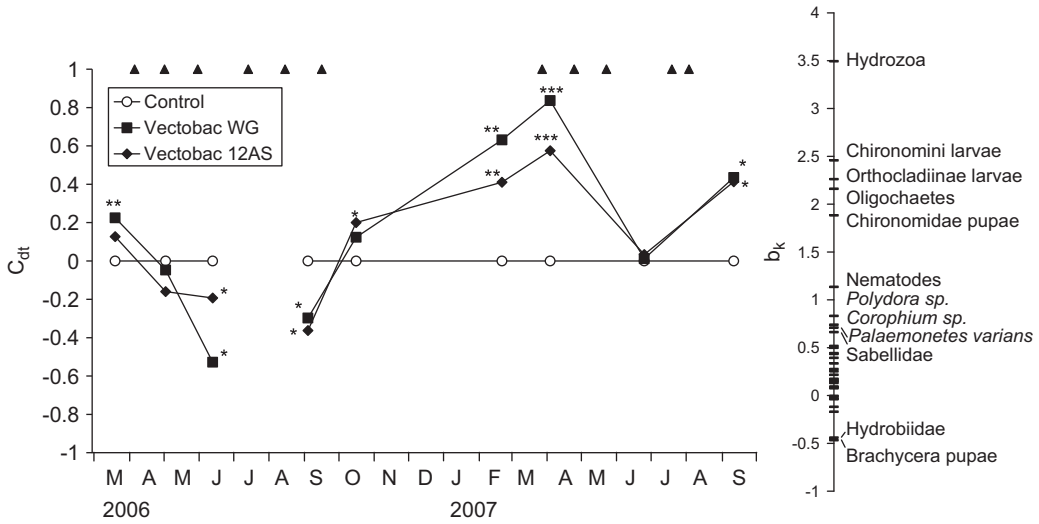

Fig. 2. Principal response curves (PRC) resulting from the analysis of invertebrate data set. The lines represent the course of the treatments in time. The vertical axis represents the difference in community structure between treated and control zones expressed as regression coefficients (C_{dt}) of the PRC model. The species weights (b_k) can be interpreted as the affinity of the taxon to the principal response. Only species with a weight superior to 0.5 or inferior to -0.5 are shown. Results for Monte Carlo permutation test for each sampling date: $^{***}p<0.001$; $^{**}0.001<p<0.01$; $^{*}0.01<p<0.05$. The interruption of the lines corresponds to drying of the pools which prevented sampling.

The drying period in 2006 had a very strong effect on the abundance of *N. diversicolor* and *C. volutator* (Fig. 3). Only *C. volutator* recovered on the following spring. Although chironomid populations were also deeply affected by drying, they recovered rapidly in autumn, after flooding of the pools. Detailed results of the statistical analysis are provided as Supplementary material. There was a huge difference in *N. diversicolor* mean abundance between 2006 and 2007, with much lower abundance values recorded during the second year of the survey (Fig. 3A). Differences between control and treated pools were shown on five dates but with no clear relationship with the treatments. For both years, there was a clear seasonal pattern in the abundance of *C. volutator*, with maximum values in summer and lower values in autumn–winter (Fig. 3B). In spring 2006 and 2007, *C. volutator* abundance was higher in *Bti*-treated than in control pools, with similar patterns for VectoBac® 12AS- and VectoBac® WG-treated pools. Significantly higher abundance values of Chironomini larvae in VectoBac® WG-treated pools than in control and VectoBac® 12AS-treated pools were repeatedly observed. Abundance of these organisms was significantly higher in control than in treated pools only on one date. Higher abundance values of Orthocladiinae larvae were shown in VectoBac® WG-treated pools than in control and VectoBac® 12AS-treated pools in March 2006 and spring 2007.

4. Discussion

During the present two-year study, repeated applications of either VectoBac® 12AS or VectoBac® WG in brackishwater pools had no significant negative impact of invertebrate communities. In particular, no adverse effects of the treatments were shown on the abundance of *N. diversicolor*, *C. volutator* and midge larvae, suggesting that the availability of invertebrate food sources for birds was not negatively affected by *Bti* applications. In addition, the results of PRC analysis showed that hydrozoans, Chironomini and Orthocladiinae larvae and oligochaetes were more abundant in treated than in control pools, especially in VectoBac® WG-treated pools in 2007. There are no available data on the toxicity of *Bti* on brackishwater hydrozoans, but according to Becker and Margalit (1993), freshwater cnidaria of the genus *Hydra* were not affected by *Bti* in laboratory tests at a concentration of 100 mg L^{-1}. Oligochaetes and chironomid larvae differ in their sensitivity to *Bti.* According to Becker and Margalit (1993), oligochaetes of the genus *Tubifex* were not affected by *Bti* in laboratory tests at a concentration of 180 mg L^{-1}. In their 6-year survey on the effects of *Bti* on non-target invertebrates in Minnesota wetlands, Hershey et al. (1998) did not find any significant difference in the abundance of annelids (including oligochaetes) between control and VectoBac® G-treated areas, which is in accordance with the very low sensitivity of these

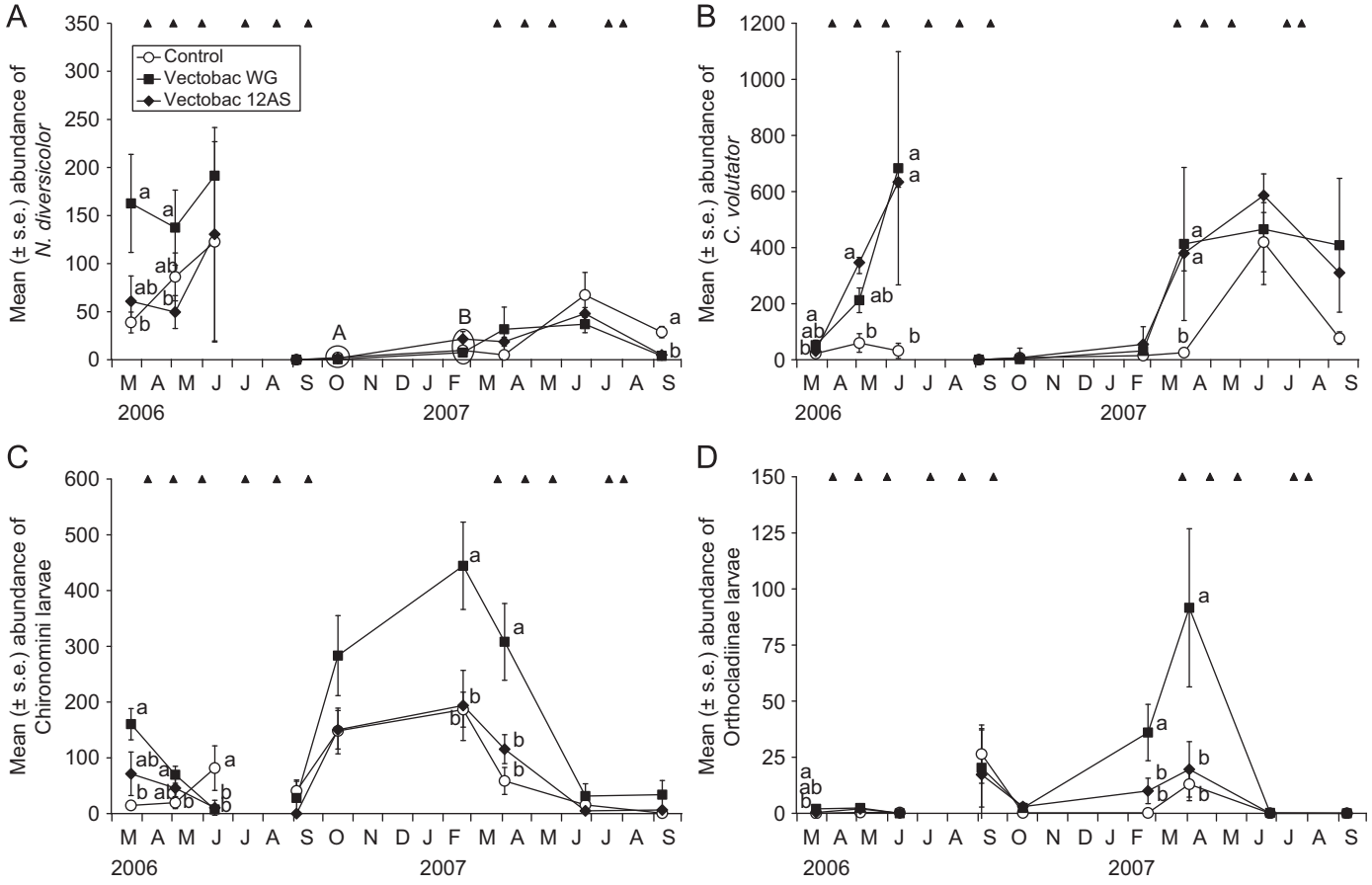

Fig. 3. Changes in mean ± s.e. (standard error; $n=5$) abundance of the four groups of invertebrates considered as major food resources for waterfowl in control, VectoBac® 12AS- and VectoBac® WG-treated pools. The triangles indicate the dates of larvicide applications in the study area. (A) *Nereis diversicolor*; (B) *Corophium volutator*; (C) Chironomini larvae; (D) Orthocladiinae larvae. Different letters indicate significant differences according to Tukey's *post-hoc* test following GLM analysis or ANOVA ($p<0.05$). Ellipses indicate that significant between zones differences were shown but cannot be indicated on the figure due to non sufficient place. A: Control ≥ (VectoBac® 12AS = VectoBac® WG), B: VectoBac® 12AS ≥ (Control = VectoBac® WG). The interruption of the lines corresponds to drying of the pools, which prevented sampling.

organisms to *Bti*. Similarly, Barnes and Chapman (1998) found no effect of VectoBac® 12AS on the abundance of insect larvae (chironomids), crustaceans or molluscs collected in sediment samples from temperate New South Wales saltmarshes. In a pond mesocosm study, Liber et al. (1998) showed that *Bti*, applied as VectoBac® G twice at a 21-day interval, had a negative effect on the abundance of chironomid larvae only for application rates 1.5 to 2 times superior to the operational rate used for mosquito control (9 kg ha^{-1}). They also showed that the various subfamilies of Chironomidae exhibited different responses to *Bti* exposure, Orthocladiinae being more susceptible than Chironominae, whereas Tanypodinae were not affected even at application rate 10-fold higher than the operational rate. Among Chironominae, Chironomini appeared to be less susceptible than Tanytarsini although the reason for this difference is unclear (Liber et al., 1998). Other *in situ* studies found no significant *Bti* toxicity to chironomids when the larvicide was applied as VectoBac® G at rate ranging from 5.6 to 28.1 kg ha^{-1} (Charbonneau et al., 1994; Vinnersten et al., 2009; Lundström et al., 2010a, b) or as VectoBac® 12AS at the rate of 1 L ha^{-1} (Lagadic et al., 2002). In temperate New South Wales saltmarshes, Barnes and Chapman (1998) did not find any effect of VectoBac® 12AS on the abundance of insect larvae (chironomids), crustaceans or molluscs collected in sediment samples.

One of the objectives of this study was to compare the long-term effects of repeated treatments with AS and WG formulations of VectoBac® on non-target aquatic invertebrates. The results of the PRC analysis strongly suggest that there was no difference between the overall effects of the two formulations on the dynamics of aquatic invertebrate community structure. Higher Shannon's diversity and Pielou's evenness values were sometimes observed for VectoBac® 12 AS- than VectoBac® WG-treated pools. This is the consequence of the higher increase in the abundance of midge larvae in VectoBac® WG-treated pools on the same dates. The reasons for the increase in the abundance of some non-target groups, especially midge larvae, observed in *Bti*-treated zones remain unclear. The fact that this positive effect was more pronounced in the pools treated with VectoBac® WG than in those treated with VectoBac® 12AS also raises unanswered questions. Increase in abundance of non-target taxa following exposure to insecticides is frequently interpreted as an indirect effect of the reduction in the abundance of susceptible groups (Caquet et al., 1992, 2007), including the targeted taxa (i.e., mosquito larvae in this case). In natural wetlands where VectoBac® G was aerially applied at 15 kg ha^{-1}, Östman et al. (2008) observed a reduction in the abundance of mosquito larvae in treated sites, followed by an increase in the taxonomic richness and abundance of protozoans which are prey for mosquito larvae but also for other invertebrates such as rotifers or hydrozoans. However in this study, the abundance of mosquito larvae in samples from the three zones was always very low, suggesting that such an indirect effect was probably not responsible for the observed changes in the abundance of non-target groups or that its contribution to this phenomenon was very limited. Russell et al. (2009) attributed the absence of detectable effects of VectoBac® 12AS on the composition of the arthropod community in ephemeral pools in Australian saltmarshes to the fact that the systems used for their study had been exposed to previous larvicide application for mosquito control. According to this hypothesis, repeated applications of larvicides might have resulted in only the more resilient or more tolerant species remaining in the treated areas. In our study, treated pools are located in an area which has been subjected to *Bti* treatments for nearly ten years (Fourcy et al., 2002). Therefore, it could be argued that the absence of negative effects of both *Bti* formulations would be due to a kind of 'selection' of taxa by previous treatments. However, this hypothesis is challenged by the fact that identical sets of taxa were found in pools from the three zones and because frequent drying of the pools would act as a 'reset' phase for the invertebrate communities that develop in these systems. Since recovery of most of the species when pools are flooded again is caused by propagules coming from the open ocean or other flooded areas, it is unlikely that such a selection would have occurred at our study site.

The results presented in this paper confirm the major influence of hydrology on the dynamics of communities in this type of ecosystem. The taxonomic composition of the invertebrate communities was typical of brackishwater intermittent ecosystems (Ward and FitzGerald, 1983; Giberson et al., 2001; Gascón et al., 2008). Short hydroperiods are associated with species with fast developmental time, abundant food resources, and reduced impact of predators (Wellborn et al., 1996; Williams, 1996). Inter-annual differences in hydrological regime had a very strong impact on invertebrate dynamics. Annual cumulative rainfall was higher in 2006 than in 2007 (871.3 and 801.2 mm, respectively) but rainfall was more regularly distributed across the year in 2007, which explains why all the pools were filled with water on the different sampling dates in 2007. Spring 2006 was characterized by low rainfalls, which led to drying of the pools in the following summer. Intense rainfalls in August and September 2006 enhanced refilling of the pools and chironomid larvae were among the first organisms to recolonize the pools after flooding. These insects are known to rapidly (re)colonize newly flooded habitat or formerly disturbed aquatic ecosystems due to the presence of several cohorts of dispersing mature flying adults per year (i.e., multivoltine life cycle; Caquet et al., 2007). *C. volutator* recovery was probably associated with immigration of young individuals with tide. Some species are theoretically able to survive unfavorable conditions through deep burrowing in the sediment, although this strategy is not always successful as exemplified by the data on the abundance of *N. diversicolor*. This species gradually recovered in the following year through the recruitment of young individuals, but abundance values were generally lower in 2007 than in 2006 in both control and treated pools. Strong environmental fluctuations, especially those associated with hydrological regime, may also have a major influence on the effects of *Bti* treatments in the field. Effects of *Bti* on target and non-target species are known to be influenced by various environmental parameters such as sunlight, water depth, temperature, salinity, organisms density in water and concentration of suspended solids (especially organic particles; Becker and Margalit, 1993; Charbonneau et al., 1994; Russell et al., 2003; Osborn et al., 2007). Russell et al. (2009) observed inconsistent short-term differences in the composition of the arthropod community in ephemeral pools treated with VectoBac® 12AS as compared to control pools. In their study, drying of the pools had a very strong effect on aquatic communities, and temporal fluctuations in numbers of arthropod taxa was generally much greater in magnitude than any difference between control and treated pools. In Minnesota freshwater wetlands, Hershey et al. (1995) showed that drought was more important than *Bti* (as VectoBac® G) treatment in determining insect abundance, suggesting that temporal changes in environmental factors might have an influence at least as important as the larvicide on non-target species. Similarly, Persson Vinnersten et al. (2010) showed that natural environmental factors had a more important influence on structuring of the insect fauna of temporary wetlands than *Bti* treatments.

5. Conclusion

The results presented in this paper suggest that, for invertebrates living in temporary brackish pools, natural factors have a stronger effect on invertebrate community structure than exposure

to *Bti*. Environmental conditions during the two years of the survey were very different, especially when considering rainfall. As a consequence, the hydroperiod and some other environmental characteristics of the pools were much contrasted between 2006 and 2007. As shown by Hershey et al. (1995) and Russell et al. (2009), drying of the pools had a very strong effect on aquatic communities and temporal fluctuations in numbers of arthropod taxa within a given zone was generally much greater in magnitude than any difference between control and treated pools. Our results also clearly show that pool drying played a major role in the dynamics of the invertebrate community. As currently performed in Western France coastal wetlands, larval mosquito control with *Bti*, especially when it is used as the VectoBac® WG formulation, does not adversely affect non-target invertebrates. Treatments are performed using portable spray apparatus by qualified personnel who select the treated zones on the basis of the presence and abundance of mosquito larvae (Carron et al., 2003). Furthermore, there is a tendency to reduce the application dosages. This strategy drastically reduces the amount of dispersed larvicide and ensures targeted applications in places inhabited by mosquito larvae, thus minimizing the risk of non-intentional impacts. Indirect positive effects on some non-target species should be confirmed by additional observations, over the long term. Such long-term surveys should encompass climatically contrasted years in order to take into account the influence of natural fluctuations of environmental parameters on the dynamics of non-target communities.

Acknowledgments

Financial support for this work was provided by the Conseil Général du Morbihan. The authors wish to thank the personnel from the EID-Atlantique, and more specifically Benoît Le Hunsec and Sébastien Chouin, for their assistance.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2011.04.028.

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