Ecotoxicology and Environmental Safety 115 (2015) 272–278

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Effects of *Bacillus thuringiensis israelensis* and spinosad on adult emergence of the non-biting midges *Polypedilum nubifer* (Skuse) and *Tanytarsus curticornis* Kieffer (Diptera: Chironomidae) in coastal wetlands

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ARTICLE INFO

Article history:
Received 28 September 2014
Received in revised form
10 February 2015
Accepted 18 February 2015

Keywords:
Biological insecticide
Mosquito control
Field enclosure
Non-biting midge
Non-target invertebrate

ABSTRACT

To optimize their efficacy, some insecticides used for mosquito control are introduced into aquatic ecosystems where mosquito larvae develop (marshes, ponds, sanitation devices) and cannot escape from the treated water. However, this raises the question of possible effects of mosquito larvicides on non-target aquatic species. *Bacillus thuringiensis* var. *israelensis* (*Bti*), which is well-known for its selectivity for *Nematocera dipterans*, is widely used for mosquito control all over the world. Spinosad, a mixture of spinosyns A and D known as fermentation products of a soil actinomycete (*Saccharopolyspora spinosa*), is a biological neurotoxic insecticide with a broader action spectrum. It is a candidate larvicide for mosquito control, but some studies showed that it may be toxic to beneficial or non-target species, including non-biting midges. The present study was therefore undertaken to assess the impact of *Bti* and spinosad on natural populations of *Polypedilum nubifer* (Skuse) and *Tanytarsus curticornis* Kieffer (Diptera: Chironomidae) in field enclosures implemented in Mediterranean coastal wetlands. Unlike *Bti*, spinosad had a strong lethal effect on *P. nubifer* and seems to affect *T. curticornis* at presumed recommended rates for field application. Differences in the sensitivity of these two species to spinosad confirm that population dynamics need to be known for a proper assessment of the risk encountered by chironomids in wetlands where larvicide-based mosquito control occurs.

1. Introduction

Larvicides used for mosquito control are introduced into aquatic ecosystems where mosquito larvae develop (marshes, ponds, sanitation devices). This optimizes the efficiency of chemical control because mosquito larvae cannot escape from the treated water. However, treatment of aquatic ecosystems raises the question of possible effects of mosquito larvicides on non-target aquatic species. The use of insecticides is accompanied by risks to biodiversity and to the function and services of ecosystems (FAO, 2010). The primary concern is direct toxicity, which may be seen as mortality of non-target species, or as effects which, while not directly lethal, increase the susceptibility of the affected organisms to other causes of morbidity or mortality. Precautions must be taken when using insecticides to maximise effectiveness while minimising undesirable side effects such as mortality of non-target species.

A number of natural products have been proposed as 'environment-friendly' insecticides, and some of them exhibit high selectivity towards certain insect taxa which promotes their use for mosquito control. Among these compounds, the bacterial larvicide *Bacillus thuringiensis* var. *israelensis* (*Bti*), which is well-known for its selectivity for nematoceran Diptera (Boisvert and Lacoursière, 2004), is widely used for mosquito control (Després et al., 2011). Spinosad, a mixture of spinosyns A and D known as

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http://dx.doi.org/10.1016/j.ecoenv.2015.02.029
0147-6513/

fermentation products of a soil actinomycete (*Saccharopolyspora spinosa*; (Crouse et al., 2001), is a biological neurotoxic insecticide that was approved and registered by the USEPA as a larvicide for mosquito control in October 2007 (Hertlein et al., 2010). Spinosad has been evaluated and accepted for listing by the WHOPES working group, the official WHO body in charge of the assessment of pesticides for their effectiveness and safety (WHO, 2007). Suitability of spinosad for larval mosquito control has been progressively highlighted in a series of scientific publications dating from 2003. However, to our knowledge, most of the field studies were conducted in small outdoor containers, plastic jars or catch basins (Bond et al., 2004, Perez et al., 2007, Jiang and Mulla, 2009, Thavara et al., 2009, Darriet et al., 2010, Anderson et al., 2011), but very few in open field (oasis: Müller et al., 2008; agricultural area: Schlein and Müller, 2008; mosquito breeding sites surrounding houses and cultivated lands: Bahgat et al., 2007). These studies mainly concerned *Culex pipiens*, *Cx. quinquefasciatus*, *Aedes aegypti* and *Ae. albopictus* (Marina et al., 2011, Marina et al., 2012) which are efficient vectors of human pathogenic viruses (Rezza et al., 2007, Fontenille et al., 2009, Butler, 2012, Vega-Rua et al., 2013).

Spinosad is efficient against a broad array of target pests: lepidopteran pests of cotton and other crops, Diptera (including fruit flies and mosquitoes), Thysanoptera, Isoptera and Coleoptera (Thompson et al., 2000, Legocki et al., 2010). Some studies showed that it is toxic to non-target parasitoids which play prominent a role in biological control of pests (Nasreen et al., 2000, Tillman and Mulrooney, 2000, Consoli et al., 2001). Previous studies also demonstrated side effects of spinosad on cohort dynamics of the zooplanktonic crustacean *Daphnia pulex* (Crustacea, Cladocera) in laboratory conditions (Stark and Vargas, 2003) and in the field (Duchet et al., 2008, Duchet et al., 2010a).

There is a large amount of data available on the effects of *Bti* on non-target species (Boisvert and Lacoursière, 2004), but only a few studies dealt with effects in the field. In both continental and coastal wetlands, most studies, including long-term monitoring (6 years or more), did not show any significant effects of Bti on aquatic invertebrate communities living in habitats where mosquito larvae develop (Russell et al., 2009, Vinnersten et al., 2009, Lundström et al., 2010, Vinnersten et al., 2010, Caquet et al., 2011, Lagadic et al., 2014). Nevertheless, transient effects of *Bti* on nematoceran Diptera larvae, including the family Chironomidae, have been observed in Minnesota freshwater wetlands aerially sprayed for three years (1991–1993) with VectoBac® G (Hershey et al., 1998, Niemi et al., 1999). However, after intensive and continuous use of VectoBac® G, study performed in those wetlands in 1997 and 1998 showed that the only difference between treated and untreated sites was lower population levels of some groups within the Chironomidae in treated sites, but other chironomid groups were more abundant, resulting in no difference in chironomid numbers or biomass as a whole (Balcer et al., 1999). Moreover, field studies did not show any effects of *Bti* on secondary consumers, including birds (Panel, 1996, Hanowski et al., 1997, US EPA, 1998, MMCD, 1999, Niemi et al., 1999, Boisvert and Boisvert, 2000, Boisvert and Lacoursière, 2004). However, Poulin et al. (2010) have described changes in the reproductive success of bird populations (the house martin *Delichon urbicum*) in Camargue (Southern France), and raised possible indirect effects of *Bti* treatments, *via* effects on the abundance of prey such as adult midges (Poulin, 2012). Such side effects of mosquito control are not welcome because midges, and more specifically chironomids, may have a major role in the aquatic ecosystems (Armitage et al., 1995). Due to their high abundance, and their habit of forming large swarms, chironomids are commonly used as food items by many bird and bat species (Laursen, 1978, Jong and Ahlén, 1991, Vaughan, 1997, Cox et al., 1998, Buchanan et al., 2006, Encarnação and Dietz, 2006). Therefore, besides studying the effects of larvicides on larval stages, it is essential to investigate the emergence phase, which is a critical step in chironomid life-cycles. Indeed, reduced densities of emerging midges may ultimately result in a decrease in food resources for predatory vertebrates.

Therefore, the present study was undertaken to assess the impact of *Bti* and spinosad on the emergence of populations of two chironomid species, *Polypedilum nubifer* (Skuse) and *Tanytarsus curticornis* Kieffer (Diptera: Chironomidae). The experiment was conducted in field enclosures implemented in Mediterranean coastal wetlands controlled for mosquito outbreaks. The larvicides were used as for operational larval mosquito control.

2. Materials and methods

2.1. Study site and field enclosures

The study was performed in a shallow temporary oligohaline marsh located in Les Saintes-Maries-de-la-Mer (Bouches-du-Rhône, Camargue, France; 43° 29′36.98″N–4° 23′31.83″ E; (Duchet et al., 2010a). The study site is a red bed marshpool artificially flooded mid-July by water pumped in the Petit-Rhône River to provide suitable habitats for ducks, the wetland being used as a hunting site in autumn.

The field enclosures were 0.125 m^3 cube-shaped bottomless plexiglas enclosures ($50 \times 50 \times 50$ cm^3). They were pushed into the sediment surface (5–10 cm depth) to avoid leaking of contaminated water from the enclosures where the larvicides were applied, spaced from another by 20 cm. These enclosures prevented aquatic organisms to escape but, because they were not covered, they allowed aerial colonisation and egg deposits by insects.

2.2. Experimental design

Thirty field enclosures were allowed to stabilize for 24 h before larvicide application. Treatments were randomly assigned to the field enclosures using a random number table (R for Windows Version 2.7.0). As the marsh was homogenous in term of sun, depth, and vegetation, no block design was used. Vectobac® 12AS (*Bti*, 1200 International Toxic Units—ITU per mg; Valent BioSciences, Libertyville, IL, USA) was applied at 0.8 and 2.5 L ha^{-1} (nominal concentration for 30 cm water depth: 0.16 and 0.50 μL L^{-1}, respectively), each concentration being applied to 5 enclosures (replicates). These concentrations correspond to the recommended rates for terrestrial and aerial treatments for mosquito control, respectively (ACTA, 2008). Conserve® 120SC (spinosad; Dow AgroSciences, Indianapolis, IN, USA) was applied as a suspension concentrate formulation containing 120 g active substance per litre at 25, 50 and 100 g ha^{-1} (nominal concentration for 30 cm water depth: 8, 17 and 33 μg L^{-1}, respectively). The treatment rates were chosen to encompass the rate for field application in mosquito control as LC_{50} ranged from 5 μg L^{-1} to 39 μg L^{-1} (Romi et al., 2006, Perez et al., 2007, Legocki et al., 2010). Five replicates were used for each spinosad concentration. Five enclosures remained as the untreated controls. The treatments were performed on August 10, 2005. Each larvicide was diluted into tap water before spraying at the water surface using a portable spraying apparatus. To prevent cross-contamination, the treated enclosures were covered with a PVC plate with a hole in its center to allow the spray to enter. Additionally, adjacent enclosures were covered with PVC plates while spraying.

2.3. Sampling procedure

Monitoring of chironomid emergence started just before the treatments (Day 0), and was carried out every two days until 21

days after insecticide spraying. Sampling was performed on Day 0, 2, 4, 7, 9, 11, 14, 16, 19 and 21. Population-level effects in *P. nubifer* and *T. curticornis* were assessed through exuvia enumeration, as described by (Pont et al., 1999) in similar conditions. Chironomid pupae exuviae, were collected at the water surface using a 250 μm mesh-size net (10 cm diameter) every other day (Wilson and Bright, 1973, Franquet, 1999). The retained exuviae were transferred to 125 mL plastic vials and preserved in 70% ethanol. All the exuviae found in the samples were identified to the genus or the species level when possible, using the taxonomic keys of (Wiederholm, 1986, Wilson and McGill, 1982, Langton and Cranston, 1991). They were then counted using a stereomicroscope (Stemi SV 6, Zeiss, Thornwood, NY, USA). Results were expressed as the number of pupae exuviae per m^2.

2.4. Water quality parameters

On each sampling date, the temperature, dissolved oxygen concentration, salinity, pH, depth, suspended matter (SM) and chlorophyll *a* concentration in water were measured in every enclosure. Water temperature, dissolved oxygen concentration, salinity and pH were measured at *ca.* 5 cm below the water surface, using a portable apparatus (Wissenschaftlich-Technische-Werkstätten— WTW, Champagne au Mont d'Or, France). Water level was measured to the nearest 1 mm in every enclosure using a graduated aluminium gauge. Measurements were always made between 10:00 and 12:00 AM (Duchet et al., 2010a). However, only three of these parameters, namely salinity, temperature and depth, are really crucial for the development of chironomid larvae (Armitage et al., 1995). Therefore, only the results obtained for these three physico-chemical parameters are considered thereafter.

2.5. Data analysis

The normality of physico-chemical and exuviae density data was tested using Shapiro–Wilks test, and the homogeneity of variances between treatments was tested using Bartlett's test. When one of these tests failed, data were transformed to meet the requirements of parametric tests. Logarithmic ($y'=\log(y+1)$) and square root ($y'=y^{0.5}$) transformations were used. Two-way Repeated Measures Analysis of Variance (RM-ANOVA) was performed over the whole study period for determining overall trends among treatments. When RM-ANOVA indicated a treatment effect, a Dunnet *post-hoc* test was used to identify which treatments were different from the control.

When data transformation failed, non-parametric Friedman's test was used to check for heterogeneity in the temporal dynamics of the different parameters between the enclosures. To evaluate influence of the larvicide treatment on the various environmental parameters, a Kruskal–Wallis test was performed for each sampling date, followed by the appropriate *post-hoc* test.

RM-ANOVA was performed using Statistica® for Windows Version 6.0 (Statsoft, Tulsa, OK, USA). Significance was set at $\alpha=0.05$ for all tests. Kruskal–Wallis test was performed using R (*kruskalmc* function from R package *pgirmess*; R Development Core Team, 2008).

3. Results

3.1. Environmental parameters

Only raw water temperature data met the requirements of parametric methods of statistical analysis. Two-way RM-ANOVA showed that water temperature varied during the study in all the enclosures ($p<0.001$), and no significant between-treatment differences were observed ($p=0.27$; average over the study period: 21.8 ± 0.10 °C). Water depth gradually decreased in all the enclosures during the study (23.4 ± 0.30 cm on Day 0; 12.0 ± 0.50 cm on Day 21). However, no significant difference between treatments was identified with the date-by-date analysis (Kruskal–Wallis test; $p>0.05$). Water salinity increased from Day 0 to Day 7 (3.2 ± 0.01 g L^{-1} and 5.3 ± 0.06 g L^{-1}, respectively), and showed further decline until the end of the experiment (3.7 ± 0.01 g L^{-1}). Again, no significant between-treatment differences were shown by the date-by-date analysis (Kruskal–Wallis test; $p>0.05$).

3.2. Chironomid emergence

Five chironomid species were identified: *Polypedilum nubifer*, *Tanytarsus curticornis*, *Cryptochironomus rostratus*, *Paratanytarsus* sp. and *Cricotopus sylvestris*. Only two species, namely *P. nubifer* and *T. curticornis*, were considered thereafter as they were the most abundant. *P. nubifer* largely dominated the chironomid assemblage (83.8% and 15.8% for *P. nubifer* and *T. curticornis*, respectively).

3.2.1. Cumulative emergence

Cumulative emergence of *P. nubifer* calculated for each sampling date (Table 1) showed that exuvia densities were not significantly different between the enclosures before introduction of the larvicides (Day 0; $p=0.739$). The highest exuvia density was observed in the control enclosures from Day 2 to Day 21 (2331 exuviae m^{-2} at Day 21). Dunnet's *post-hoc* test showed a negative effect ($p<0.001$) of the 2 highest spinosad concentrations from Day 4 to the end of the study, and on Day 4 and Day 9 only, for the lowest concentration of spinosad. The densities of exuviae collected in the enclosures treated with *Bti* at 0.16 and 0.50 μL L^{-1} remained very close during all the experiment. No effect of *Bti* was detected (RM-ANOVA, $p>0.05$).

Table 1
Cumulative emergence (mean ± standard-error; $n=5$) of *Polypedilum nubifer* (expressed as the number of pupae exuviae collected/ m²) in the control enclosures, the enclosures treated with *Bti* at 0.16 and 0.50 μL L^{-1}, and the enclosures treated with spinosad at 8, 17 and 33 μg L^{-1}. (Significantly different from control, Dunnet's *post-hoc* test: *: $0.05<p<0.01$; ***: $p<0.001$).

Dates	Control	Bti 0.16 μL L^{-1}	Bti 0.50 μL L^{-1}	Spi 8 μg L^{-1}	Spi 17 μg L^{-1}	Spi 33 μg L^{-1}
Day 0	1 ± 0.5	1 ± 0.4	1 ± 0.3	1 ± 0.6	1 ± 0.4	1 ± 0.2
Day 2	155 ± 37.2	120 ± 32.9	121 ± 36.0	99 ± 22.5	59 ± 17.0	50 ± 10.6
Day 4	463 ± 40.5	353 ± 32.6	394 ± 34.9	174 ± 6.3*	120 ± 5.2*	84 ± 2.9*
Day 7	828 ± 45.1	568 ± 24.9	577 ± 22.7	223 ± 11.1	138 ± 1.2	96 ± 1.7
Day 9	1158 ± 44.1	834 ± 50.7	861 ± 43.2	243 ± 0.0***	143 ± 0.6***	107 ± 2.9***
Day 12	1348 ± 14.8	1049 ± 6.7	1038 ± 10.9	243 ± 0.0	145 ± 0.2***	115 ± 0.0***
Day 14	1511 ± 26.2	1184 ± 23.5	1198 ± 29.3	245 ± 1.2	149 ± 2.8***	118 ± 1.9***
Day 16	1736 ± 38.8	1396 ± 36.8	1452 ± 42.3	254 ± 2.8	167 ± 3.4***	128 ± 1.5***
Day 19	2019 ± 41.4	1709 ± 53.5	1739 ± 41.3	265 ± 0.7	180 ± 1.2***	137 ± 2.1***
Day 21	2331 ± 43.5	2002 ± 32.4	2035 ± 38.7	267 ± 0.4	197 ± 6.7***	145 ± 1.4***

Table 2
Cumulative emergence (mean ± standard-error; $n=5$) of *Tanytarsus curticornis* (expressed as the number of pupae exuviae collected/ m²) in the control microcosms, the microcosms treated with *Bti* at 0.16 and 0.50 μL L^{-1}, and the microcosms treated with spinosad at 8, 17 and 33 μg L^{-1}. (Significantly different from control, Dunnet's *post-hoc* test: *: $0.05 < p < 0.01$; **: $p < 0.01$).

Dates	Control	Bti 0.16 μL L^{-1}	Bti 0.50 μL L^{-1}	Spi 8 μg L^{-1}	Spi 17 μg L^{-1}	Spi 33 μg L^{-1}
Day 0	1 ± 0.5	1 ± 0.4	1 ± 0.3	1 ± 0.6	1 ± 0.4	1 ± 0.2
Day 2	40 ± 6.1	31 ± 12.5	32 ± 9.7	39 ± 9.2	28 ± 4.1	11 ± 3.2
Day 4	69 ± 4.8	83 ± 4.5	70 ± 3.0	90 ± 7.2	38 ± 0.8	31 ± 1.0
Day 7	110 ± 1.6	114 ± 8.1	93 ± 7.0	165 ± 16.3	53 ± 4.4	33 ± 0.0
Day 9	146 ± 13.9	160 ± 11.5	151 ± 11.3	300 ± 34.2	71 ± 1.6	33 ± 0.0
Day 12	271 ± 10.9	222 ± 10.8	254 ± 16.2	550 ± 45.7*	84 ± 0.8*	55 ± 4.7*
Day 14	382 ± 12.3	334 ± 26.0	385 ± 37.9	808 ± 49.5*	99 ± 6.4*	79 ± 4.8*
Day 16	474 ± 20.2	493 ± 34.8	577 ± 24.2	1294 ± 88.1*	137 ± 5.4*	118 ± 4.8*
Day 19	692 ± 26.2	695 ± 22.1	753 ± 26.4	1638 ± 41.5	177 ± 6.4**	129 ± 1.3**
Day 21	1045 ± 55.0	1012 ± 58.9	1046 ± 45.4	1861 ± 30.4	265 ± 18.2**	164 ± 7.7**

Cumulative emergence of *T. curticornis* for each sampling date is presented in Table 2. No significant difference was observed at Day 0. The highest exuvia density was observed in the enclosures treated with spinosad at 8 μg L^{-1} from Day 4 to Day 21 (1968 exuviae m^{-2} at Day 21; Dunnet's *post-hoc* test, $p < 0.05$ on Day 12, Day 14 and Day 16). The densities of exuviae collected in control enclosures and in the enclosures treated with *Bti* at 0.16 and 0.50 μ L L^{-1} remained very close during all the experiment (1224, 1136, 1148 exuviae m^{-2} at Day 21, respectively; Dunnet's *post-hoc* test, $p > 0.05$). The densities of exuviae collected in the enclosures treated with spinosad at 17 and 33 μg L^{-1} were significantly lower than control from Day 12 to Day 21 (Dunnet's *post-hoc* test, $p < 0.05$; 200 and 145 exuviae m^{-2} at Day 21, respectively).

3.2.2. Emergence dynamics

Fig. 1 shows the changes of mean (± SE) *P. nubifer* exuvia density during the experiment for the different types of field enclosures. The mean (± SE) density varied between 83 ± 18 and 300 ± 44 exuvia m^{-2} in the control enclosures. In the *Bti*-treated enclosures, the mean density varied between 63 ± 6 and 286 ± 37 exuviae m^{-2}. In the enclosures treated with spinosad, emergence density ranged between 0 and 126 ± 19 exuviae m^{-2}, with a sharp decrease from Day 7 after sprayed until the end of the experiment. In the control enclosures, two emergence peaks could be identified: the first one at Day 7 and the second one at Day 21.

RM-ANOVA showed significant differences between the treatments ($p < 0.0001$). Dunnet's *post-hoc* test did not show any

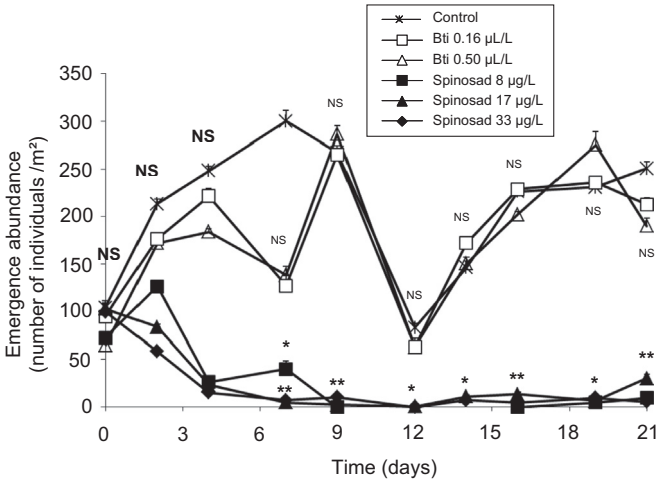

Fig. 1. Change in mean values (+ SE; $n=5$) of *Polypedilum nubifer* emergence abundance (expressed as the number of pupae exuviae collected) in the control enclosures, the enclosures treated with *Bti* at 0.16 and 0.50 μL L^{-1}, and the enclosures treated with spinosad at 8, 17 and 33 μg L^{-1}. (Significantly different from control, Dunnet's *post-hoc* test: *: $0.05 < p < 0.01$; **: $p < 0.01$).

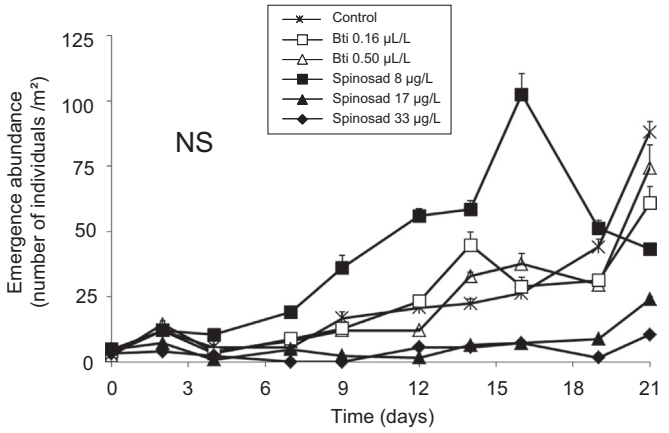

Fig. 2. Change in mean values (+ SE; $n=5$) of *Tanytarsus curticornis* emergence abundance (expressed as the number of pupae exuviae collected) in the control enclosures, the enclosures treated with *Bti* at 0.16 and 0.50 μL L^{-1}, and the enclosures treated with spinosad at 8, 17 and 33 μg L^{-1}. (Significantly different from control, Dunnet's *post-hoc* test: *: $0.05 < p < 0.01$; **: $p < 0.01$).

significant difference between *Bti*-treated and untreated enclosures. In contrast, all spinosad concentrations (8, 17 and 33 μ g L^{-1}) caused a significant reduction in the emergence of *P. nubifer* as compared to the controls, from Day 7 to the end of the study (Dunnet's *post hoc* test, $p < 0.05$).

Fig. 2 shows the changes of mean (± SE) exuvia density for *T. curticornis* for the different types of field enclosures. Emergence density increased with time, and the peak of emergence seems to appear after Day 21. At Day 21, mean (± SE) emergence density reached 88 ± 16 exuviae m^{-2} in the control enclosures, 74 ± 35 and 61 ± 26 exuviae m^{-2} in the enclosures treated with *Bti* at 0.16 and 0.50 μL L^{-1}, respectively, and 43 ± 9 exuviae m^{-2} in the enclosures treated with spinosad at 8 μg L^{-1} (but 102 ± 33 exuviae m^{-2} were found in these enclosures at Day 16). In the enclosures treated with spinosad at 17 and 33 μg L^{-1}, mean (± SE) emergence densities reached only 24 ± 5 and 10 ± 2 exuviae m^{-2}, respectively, at day 21.

RM-ANOVA did not show any significant differences between the treatments ($p > 0.475$). However, emergence densities in the field enclosures treated with spinosad at 17 and 33 μg L^{-1} remained lower than those observed in the control enclosures, in the *Bti*-treated enclosures and in the enclosures treated with spinosad at 8 μg L^{-1}.

4. Discussion

The chironomid species *P. nubifer* and *T. curticornis* are well represented in the temporary wetlands where this study was

conducted. *P. nubifer* is one of the dominant species in Camargue, where it is able to survive in various aquatic habitats and in water salinities between 0 and 40 mg L^{-1} (Tourenq, 1976; Pont et al., 1999). Both *P. nubifer* and *T. curticornis* colonize habitats with low current and large amounts of particulate organic matter (Tourenq, 1976; Syrovátka et al., 2009). This explains their dominance in temporary marshes of Camargue where they can be seen as relevant sentinel species for monitoring the unwanted effects of larvicides used against mosquito larvae in these biotopes. Because of its capacity to tolerate drought periods, *P. nubifer* larvae develop rapidly after flooding and emerge before *T. curticornis* whose larvae develop from eggs laid by the females in flooded areas. The succession of two emergence peaks observed for *P. nubifer* in our control field enclosures (Fig. 1) is consistent with the results reported by Trayler et al. (1994).

In the present study, effects of three spinosad concentrations (8, 17 and 33 μg L^{-1}) on *P. nubifer* and *T. curticornis* were compared to those of *Bti*, applied at the recommended rates of 0.16 and 0.50 μL L^{-1} for respective terrestrial and aerial operational control of mosquito larvae. *Bti* had no effect on the emergence of both species. This bacterial larvicide is recognized as highly selective for Nematocera, the taxonomic group of dipterans to which belong Culicidae, Simuliidae and Chironomidae. All the field studies showing effects of *Bti* on chironomids were carried out in controlled conditions (*in situ* enclosures or outdoor artificial ponds; Miura et al., 1980, Ali, 1981, Ali et al., 1981, Charbonneau et al., 1994, Liber et al., 1998, Pont et al., 1999, Russell et al., 2009), or in rivers where black fly larvae were controlled (Car and de Moor, 1984, Back et al., 1985, Merritt et al., 1989, Molloy, 1992, Palmer, 1993; Jackson et al., 1994; MacCracken and Matthews, 1997, Jackson et al., 2002), with dosages of *Bti* much higher than those used for mosquito control. Stevens et al. (2004) compared toxicity of a *Bti* commercial formulation (Vectobac WDG; 3000 UTI/mg) and a spore/crystal mixture against *Chironomus tepperi* larvae, considering chironomids as a pest for rice cultures. They estimated LC_{50} values ranging from 0.20 to 1 mg L^{-1} depending on larvae age, density, temperature and substrate tested. These values are much higher than the concentrations tested in our study (0.16 and 0.50 μg L^{-1}). This is consistent with the observations made in laboratory, semi-field and field conditions, as reviewed by Ali et al., (2002a). *Bti* application rates required to kill chironomids are significantly higher than those required for mosquito control, since *Bti* is generally formulated for mosquito control, where it is advantageous for the spores and toxins to remain suspended in the water column. As an example, Craggs et al. (2005) observed a slight reduction in midge larvae numbers compared to controls and had little effect on adult emergence after a *Bti* treatment at 1000 μg/L. To impact chironomids, formulations should deliver the component of *Bti* at the mud-water interface where chironomid larvae feed (Ali et al., 2002a). Consequently, although *Bti* has been commercially available in the USA for the past 30 years, its high application rates (and hence high costs) needed to control nuisance midges resulted in very little use for such a purpose, compared to mosquito and blackfly control (Ali et al., 2002b).

Monitoring adult emergence allows taking into account the whole aquatic phase of chironomid life cycle. This is important to evaluate success for the midges to cope with every stress (e.g., environmental stress factors, pollution, competition, predation) the larvae have been subjected to during their development. Although the stock of chironomid larvae in our field enclosures remained unknown and in spite of the absence of control of egg laying during the experiment, using emergence as an endpoint for assessing the impacts of larvicides offered the advantage to be non-destructive (*i.e.*, no consequence for studying population dynamics) as the living organisms were not sampled.

Adult emergence has been considered only in some studies on the effects of *Bti* on chironomids (Liber et al., 1998, Craggs et al., 2005, Ali et al., 2008, Vinnersten et al., 2010). Our results showed that *Bti* had no significant effect on the emergence of both *P. nubifer* and *T. curticornis*.

Following spinosad treatments, emergence of *P. nubifer* dramatically decreased from Day 4 up to the end of the observation period. Adult emergence never recovered in spite of the rapid degradation of the insecticide (half-life of 1–2 d for the sum of spinosyns A and D; (Saunders and Bret, 1997, Cleveland et al., 2002, Duchet et al., 2008). Concerning *T. curticornis*, adult emergence remained low in the enclosures treated with spinosad at 17 and 33 μg L^{-1}, though not statically different from control. The emergence was higher in the enclosures treated with spinosad at 8 μg L^{-1} (Fig. 2). The short half-life of spinosad resulted in the fast dissipation of such a low concentration of the insecticide from the water, so that *T. curticornis* could rapidly recolonize the enclosures from eggs laid by females. In addition, it cannot be excluded that even at low concentration, spinosad may have reduced the abundance of chironomid larvae competitors and/or predators. This may explain why adult emergence rate was higher in the enclosures treated with 8 μg L^{-1} spinosad as compared to the control enclosures and the enclosures treated with *Bti* which is known to preserve non-target invertebrate communities in wetlands (Barnes and Chapman, 1998, Russell et al., 2009, Lagadic et al., 2014).

Most of the published studies on spinosad side effects were carried out in controlled conditions (laboratory and outdoor artificial ponds), and only a few papers concern the effects of spinosad on chironomids. For *Chironomus circumadatus*, laboratory 48-h LC_{90} of spinosad was estimated at 63 and 177 μg L^{-1} for the 1st and 4th larval instars, respectively (Kumar et al., 2011). Spinosad also showed a notable activity against *C. tepperi* with laboratory 24- h LC_{50} and LC_{90} estimated at 28.9 and 61.8 μg L^{-1}, respectively (Stevens et al., 2005). In outdoor containers treated with spinosad at 5 ppm (equivalent to 5 mg L^{-1}), chironomid larvae were not able to develop (Perez et al., 2007). Also in outdoor containers, the same spinosad concentration (5 ppm equivalent to 5 mg L^{-1}) resulted in a complete inhibition of chironomid reproduction during 7 weeks, and the number of chironomid larvae was reduced by 72% (Bond et al., 2004).

As a consequence, Bond et al. (2004) pointed to possible adverse effects of a spinosad-based larvicide on non-target aquatic invertebrates. This was confirmed in experiments conducted with Conserve® 120SC in field enclosures implemented in Atlantic and Mediterranean wetlands (Duchet et al., 2008, Duchet et al., 2010a). Natural populations of *Daphnia pulex* and *Daphnia magna* were dramatically reduced by the exposure to 8, 17 and 33 μg L^{-1} spinosad. Although recovery was observed in *D. pulex* in the Atlantic wetlands (Duchet et al., 2008), population model forecasted quasi extinction after 43.9 weeks (Duchet et al., 2010b).

In all the references quoted above, spinosad concentrations which induced an effect on chironomids were higher than the concentrations tested in the present study. Nevertheless, these low concentrations significantly impacted the emergence of *P. nubifer*, and no population recovery was observed in spinosad-treated field enclosures. This may be due to the different test conditions between our study and the others. Laboratory toxicity tests are usually conducted under stable conditions, while exposures in the field occur under variable environmental conditions. Environmental contamination is often associated with a combination of stress factors of various sources (biological, physical and chemical), and non-target organisms are exposed to multiple natural and anthropogenic stressors at the same time (Duchet et al., 2010a, Owojori and Reinecke, 2010). Thus, variability in response of chironomid communities to spinosad treatments was attributed to both environmental factors and variations in the susceptibility of

individual chironomid taxa (Stevens et al., 2004). Indeed, various environmental variables, including temperature, larval instar, water depth, water surface area coverage by macrophytes, etc., influence the toxicity of larvicides to non-target species (Charbonneau et al., 1994), and this may be of particular importance for toxins which act through ingestion (Duchet et al., 2010a). Nevertheless, previous studies also ran in field enclosures with both *Bti* and spinosad, in different climate regions and at different seasons, showed similarly strong lethal effects of spinosad and no observed effects of *Bti* on two different *Daphnia* species (Duchet et al., 2008, 2010). This strongly suggests that the response pattern observed in the present study is truly the result of the treatments and not a function of a time- and/or location-by-treatment interaction.

Finally, several studies showed that although pesticide contaminations alter species abundance, community context can determine the response to pesticide effects (Relyea, 2005, Rohr and Crumrine, 2005). By changing the community composition, a pesticide can have a stronger effect on a population either by altering the traits of a prey species or by decreasing its physiological tolerance and to make it more sensitive to stressors (Relyea and Mills, 2001, Schulz and Dabrowski, 2001, Relyea, 2003). This points out the importance of considering population dynamics, for a proper risk assessment of larvicides.

5. Conclusions

Unlike *Bti*, spinosad had a strong lethal effect on *P. nubifer* population and it affected *T. curticornis* at presumed recommended rates for field use. Differences in sensitivity between *P. nubifer* and *T. curticornis* populations toward spinosad were observed, showing that population dynamics needs to be considered for a proper risk assessment of larvicides (Pont et al., 1999). Our study confirms that field application of *Bti*-based larvicides, at recommended dosages, does not cause any major direct negative effects on chironomids, and therefore remains the most acceptable mode of chemical control of mosquito populations nowadays.

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