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Influence of environmental factors on the response of a natural population of *Daphnia magna* (Crustacea: Cladocera) to spinosad and *Bacillus thuringiensis israelensis* in Mediterranean coastal wetlands

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Significant interaction between salinity and spinosad exposure impairs the recovery of a natural population of Daphnia magna.

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ABSTRACT

The present study was undertaken to assess the impact of a candidate mosquito larvicide, spinosad (8, 17 and 33 μg L^{-1}) on a field population of *Daphnia magna* under natural variations of water temperature and salinity, using *Bti* (0.16 and 0.50 μL L^{-1}) as the reference larvicide. Microcosms (125 L) were placed in a shallow temporary marsh where *D. magna* was naturally present. The peak of salinity observed during the 21-day observation period may have been partly responsible for the decrease of daphnid population density in all the microcosms. It is also probably responsible for the absence of recovery in the microcosms treated with spinosad which caused a sharp decrease of *D. magna* abundance within the first two days following treatment whereas *Bti* had no effect. These results suggest that it may be difficult for a field population of daphnids to cope simultaneously with natural (water salinity and temperature) and anthropogenic (larvicides) stressors.

1. Introduction

Mediterranean coastal wetlands are characterized by large spatial and temporal variations of many environmental parameters (Comin and Valiela, 1993; Nuccio et al., 2003). Aquatic invertebrates living in these ecosystems may therefore be exposed to changes in water level, temperature or salinity. Under some circumstances (e.g., duration or intensity of exposure to extreme values of a given factor), the stress associated with these variations may have an impact on the physiological integrity of organisms, leading to a decrease in their overall fitness (Smolders et al., 2005). These ecosystems are also highly suitable habitats for numerous insect species, including mosquitoes (Diptera: Culicidae), which frequently exhibit mass occurrences that may become a great nuisance (Becker et al., 2003). Therefore, these ecosystems are target areas for mosquito control using chemicals that may have an impact on non-target aquatic invertebrate species. Combating the poisoning effects of toxic compounds has also a metabolic cost for these organisms, and this has implications for linking physiological stress responses observed at the level of individuals to population-level effects (Calow, 1991). Some laboratory experiments showed that environmental factors such as water salinity, hardness or temperature may interfere with the effects of toxicants on daphnids (Semsari and Haït-Amara, 2001; Heugens et al., 2006; de la Paz Gomez-Diaz and Martinez-Jeronimo, 2009). Anthropogenic and natural stressors may not simply act in additive ways; rather multiplicative interactions occur either increasing (synergistic) or dampening (antagonistic) the effects of stressors (Salbu et al., 2005; Hames et al., 2006). However, little is known on the possible interaction between toxicants and natural changes in environmental parameters in the field.

A number of natural products have been proposed as 'environment-friendly' insecticides and some of them exhibit selectivity towards certain insect taxa which promotes their use for mosquito control. Among these compounds, the bacterial larvicide *Bacillus thuringiensis* subspecies *israelensis* (*Bti*), which is well-known for its selectivity for Nematocera dipterans, in particular Culicidae (mosquitoes), Simuliidae (black flies) and Chironomidae (non-biting midges; Boisvert and Boisvert, 2000), is widely used for mosquito control all over the world (Lacoursière and Boisvert, 2004). Spinosad, a mixture of spinosyns A and D known as fermentation products of a soil bacterium (*Saccharopolyspora*

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spinosa, Actinomycetes; Crouse et al., 2001), is a neurotoxic biological insecticide that is also a potential candidate for mosquito control (Cetin et al., 2005). However, some studies indicated that it may be toxic to beneficial or non-target species (Nasreen et al., 2000; Tillman and Mulrooney, 2000; Consoli et al., 2001). The Lethal Dose which caused 50% mortality (LD_{50}) for the bee (*Apis mellifera*) was estimated at 0.057 μg/bee in the case of an oral exposure (WHO, 2007). The No Observed Effect Concentration (NOEC) in chronic toxicity was estimated at 1.6 μg L^{-1} for *Chironomus riparius* and 8 μg L^{-1} for *Daphnia magna* (WHO, 2007). Furthermore, adverse effects of spinosad have been demonstrated for the zooplankton crustacean *Daphnia pulex* (Crustacea, Cladocera) under laboratory conditions (Stark and Vargas, 2003) and in field microcosms (Duchet et al., 2008).

In a field study performed in a shallow temporary oligohaline marsh located in Western France (Duchet et al., 2008), we showed that spinosad applied at concentrations ranging from 8 to 33 μg L^{-1} had a negative impact on *D. pulex* survival and population size structure. At the lowest concentration tested, daphnid population recovered after the first week, demonstrating the recovery potential of these organisms to spinosad exposure under natural environmental conditions. However, no significant changes in water temperature or salinity were observed during the study period. Therefore, the present study was undertaken to assess the impact of spinosad on a Mediterranean coastal wetland population of *D. magna* under natural variations of water temperature and salinity, using *Bti* as a reference larvicide. Water temperature and salinity were selected because they are not supposed to vary following larvicide treatment and because they usually exhibit the highest variation in mediterranean wetlands (Waterkeyn et al., 2008). The experiment was carried out using *in situ* microcosms (enclosures). These systems give the opportunity to integrate the effects of the exposure to larvicides and to natural changes in physicochemical parameters, which is not possible with single-species laboratory toxicity tests (van den Brink et al., 2005). *D. magna* population-level effects were assessed on the basis of population density and size-structure analysis for increasing levels of larvicide exposure.

2. Materials and methods

2.1. Study site and microcosms

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) where *D. magna* populations are naturally present. The microcosms were 0.125 m^3 cube-shaped bottomless plexiglas enclosures (50 × 50 × 50 cm). They were pushed into the sediment surface (5–10 cm depth) to avoid leaking of contaminated water from the microcosms where the larvicides were applied.

2.2. Experimental design

Thirty microcosms were used to enclose fractions of the natural daphnid population. Ten microcosms were treated with *Bti* (Vectobac® 12AS; Valent Biosciences, Libertyville, IL, USA), 15 microcosms were used for the treatment with spinosad (Spinosad 120SC®; Dow AgroSciences, Indianapolis, IN, USA), and 5 microcosms remained as untreated controls. Microcosms were allowed to stabilize for 24 h before larvicide application. Treatments were randomly assigned to the microcosms using a random number table (R for Windows Version 2.7.0). Vectobac® 12AS 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 microcosms (replicates). These concentrations correspond to the minimum recommended and the maximum registered rates for terrestrial and aerial treatments, respectively (ACTA, 2008). Spinosad 120SC 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 in order to encompass the rate of 50 g ha^{-1} which would be the mean presumed recommended rate for field application. Five replicates were used for each spinosad concentration. 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 as described elsewhere (Duchet et al., 2008). Monitoring started just before the treatments (Day 0), and was carried out until 21 days after insecticide spraying. Sampling was performed on Day 0, 2, 4, 7, 14 and 21.

2.3. Water quality parameters

On each sampling date, the water temperature, dissolved oxygen concentration, salinity, and pH were measured in every microcosm at *ca.* 5 cm below the water surface, using portable apparatuses (Wissenschaftlich-Technische-Werkstätten – WTW, Champagne au Mont d'Or, France). Water level was measured to the nearest 1 mm in every microcosm using a graduated aluminium gauge. Measurements were always made between 10:00 and 12:00 AM to ensure consistency among data relative to possible circadial influence. Suspended Matter (SM) concentration was determined in 250 mL water samples filtered through pre-weighted oven-dried (2 h at 500 °C) Whatman GF/C fiberglass filters (1.2-μm mesh size; Whatman International, Maidstone, UK) that were weighted again after 48 h at 105 °C according to the AFNOR (1996) method. Chlorophyll *a* concentration in water was measured in 250 mL water samples filtered through Whatman GF/C fiberglass filters. Pigments were extracted overnight using 5 mL of an acetone/distilled water (90/10, v/v) mixture. Chlorophyll *a* was quantified spectrophotometrically (Prim Advanced, SECOMAM, Domont, France) according to Lorenzen (1967).

2.4. Sampling procedures and measurement of endpoints in daphnids

Water samples were collected using PVC tube samplers (70 cm length, 6 cm inner diameter) equipped with a 2 × 4 mm mesh screen-covered one-way valve at the bottom (Roucaute and Quemeneur, 2007). Samples were collected from twenty regularly spaced locations within each microcosm, in order to reduce the effects of plankton patchiness (Stephenson et al., 1984; SETAC, 1991), and grouped into a beaker. The resulting composite sample (mean ± SE volume = 54 ± 23 mL, depending on the water level in the microcosm) was filtered through 30-μm mesh nylon net. The retained organisms (daphnids and some other pelagic invertebrates) were transferred to a 500 mL plastic vial and preserved using neutral aqueous formaldehyde/sucrose (4%, v/v; 40 g L^{-1}) that contained 250 μg L^{-1} Bengal pink dye. All the daphnids found in the samples were identified to the species level using the key of Amoros (1984). They were counted using a stereomicroscope (Stemi SV 6, Zeiss, Thornwood, NY, USA) and their body length was measured from the eye to base of the tail spine using an ocular micrometer (Boronat and Miracle, 1997). Abundances of *D. magna* were expressed as the number of individuals per litre based on the volume of the composite samples collected in the microcosms.

2.5. Data analysis

The normality of physicochemical 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 in order to meet the requirements of parametric one-way analysis of variance (one-way ANOVA). Logarithmic ($y' = \log(y + 1)$) and square root ($y' = y^{0.5}$) transformations were tested.

For normally-distributed data (either raw or transformed data), a two-way Repeated Measures Analysis of variance (RM-ANOVA) was performed, in order to identify overall effects of the treatments. When two-way RM-ANOVA indicated that there was a significant difference between the treatments, a one-way ANOVA was performed for each sampling date. Dunnet's *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 microcosms. To evaluate the influence of larvicide treatment on the various environmental parameters, a Kruskal–Wallis test was performed for each sampling date, followed by the appropriate *post-hoc* test (*kruskalmc* function from R package *pgirmess*).

The effects of larvicides on the population density of *D. magna* were analysed for the whole study period and on each sampling date using a negative binomial Generalized Linear Model (GLM). A Dunnet's *post-hoc* test was used to test for differences between control and treated systems.

The influence of water temperature and salinity on the effects of the two larvicides on the population density of *D. magna* was checked using a three factor negative binomial GLM. Two categories, 'low' and 'high', were defined for water temperature and salinity values. Values inferior to the median values computed for temperature or salinity for the whole study period were categorized as 'low', whereas values superior to these median values were categorized as 'high'.

Preliminary investigations showed that *D. magna* length data clearly exhibited a non-normal right-skewed distribution and that neither log, nor square-root transformation was able to normalize the data or homogenize the variances. Therefore, mean length values computed for the various treatments were compared at each sampling date using a Kruskal–Wallis non-parametric test followed by a non-parametric multiple comparison *post-hoc* test. Length frequency distributions were constructed by counting the relative number of individuals in successive 0.5 mm width classes. For each sampling date, values of the relative abundance of a given length class were compared using a proportion comparison test. When the test indicated a significant between-treatment difference, the values obtained for