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Chitobiase activity as an indicator of altered survival, growth and reproduction in *Daphnia pulex* and *Daphnia magna* (Crustacea: Cladocera) exposed to spinosad and diflubenzuron

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ABSTRACT

Chitobiase is involved in exoskeleton degradation and recycling during the moulting process in arthropods. In aquatic species, the moulting fluid is released into the aqueous environment, and chitobiase activity present therein can be used to follow the dynamics of arthropod populations. Here, chitobiase activity was used for monitoring the impact of mosquito candidate larvicides on *Daphnia pulex* and *Daphnia magna* under laboratory conditions. Both species were exposed to spinosad (2, 4, 8 μg L^{-1}) and diflubenzuron (0.2, 0.4, 0.8 μg L^{-1}) for 14 days. *Bacillus thuringiensis* var. *israelensis* (*Bti*; 0.25, 0.5, 1 μL L^{-1}) was used as the reference larvicide. Chitobiase activity, adult survival, individual growth and fecundity, expressed as the number of neonates produced, were measured every 2 days. Average Exposure Concentrations of spinosad were ten-fold lower than the nominal concentrations, whereas only a slight deviation was observed for diflubenzuron. In contrast to *Bti*, spinosad and diflubenzuron significantly affected both species in terms of adult survival, and production of neonates. As compared to *D. pulex*, *D. magna* was more severely affected by diflubenzuron, at low and medium concentrations, with reduced adult growth and much lower chitobiase activity. Chitobiase activity was positively correlated with the individual body length, number of neonates produced between two consecutive observation dates, and number of females and neonates. In addition, the significant positive correlations between chitobiase activity measured on the last sampling date before the first emission of neonates and the cumulative number of neonates produced during the whole observation period strongly support the potential of the activity of this chitinolytic enzyme as a proxy for assessing the dynamics of arthropod populations exposed to larvicides used for mosquito control.

1. Introduction

Chitobiase is one of the two chitinolytic enzymes involved in exoskeleton degradation and recycling during ecdysis in arthropods. A first enzyme, chitinase, hydrolyses chitin, a polymer of β-(1–4)-linked *N*-acetyl-β-glucosamine (NAG), to oligomers and trimers of NAG, while chitobiase subsequently hydrolyses oligomers and trimers of NAG to NAG monomers (Muzzarelli, 1977; Merzendorfer and Zimoch, 2003). Chitobiase activity is located in both the epidermis and hepatopancreas of arthropods (Spindler-Barth et al., 1990; Zou and Fingerman, 1999a). During ecdysis, the moulting fluid, containing chitobiase, is released into the aqueous environment. Thus, chitobiase theoretically offers the possibility to perform non-destructive measurements of moulting activity of arthropods under field or laboratory conditions. Indeed, analysis of chitobiase activity in water showed promising results as an assessment tool for estimating arthropod biomass, population dynamics and secondary production both in laboratory (Espie and Roff, 1995; Oosterhuis et al., 2000; Sastri and Roff, 2000; Vrba et al., 2004; Sastri and Dower, 2006) and field studies (Hanson and Lagadic, 2005; Sastri and Dower, 2006; Conley et al., 2009). Exposure to toxicants can influence moulting of arthropods with measurable changes in chitobiase activity. Such changes have been observed in crabs exposed to endocrine disruptors (Zou and Fingerman, 1999b, c; Zou, 2005), in crabs and shrimps exposed to metals (Zhang et al., 2006; Xie et al., 2009), and in *Daphnia magna*

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exposed to pharmaceuticals (Richards et al., 2008). For the vast majority of the chemicals that have been tested, inhibition of chitobiase activity was recorded, except with pharmaceuticals for which a more complex pattern of induction and inhibition has been observed (Richards et al., 2008). It is worth noting that pesticides have rarely been studied with respect to their action on chitinolytic enzyme activity and the moulting process in arthropods, the only reported data concerning the inhibition of chitobiase by endosulfan in *Uca pugilator* (Zou and Fingerman, 1999b).

For mosquito control programmes, insecticides are mostly applied as larvicides. The compounds are directly introduced into aquatic systems inhabited by mosquito larvae (e.g., marshes, ponds and sanitation devices). Cladocerans and other zooplankton groups are water column-dwelling organisms that share the habitat and, at least in part, the food resources of mosquito larvae (Blaustein and Chase, 2007). They may therefore be exposed to larvicides in treated areas, and there is a need for methods that can be used for monitoring the impacts of mosquito control programmes on these non-target organisms.

The present study was undertaken to assess the potential of chitobiase activity for monitoring the impact of the candidate larvicides spinosad and diflubenzuron on survival, growth and fecundity of *Daphnia pulex* and *D. magna* (Crustacea: Cladocera) under laboratory conditions. These two *Daphnia* species were chosen because they are frequently found in biotopes where mosquito larvae develop (Metge, 1986). The larvicides were chosen based upon their different modes of action, and possible use for mosquito control. Currently, *Bacillus thuringiensis* var. *israelensis* (*Bti*) is the only larvicide used in Europe as the result of the implementation of the EU Biocidal Products Directive 98/8/EC. It is also widely used for mosquito control all over the world (Boisvert and Lacoursière, 2004). Therefore, the risk of resistance to *Bti* in target species should not be neglected, and there is an urgent need to develop new larvicides that could be used alternately. In the present study, *Bti* was considered as a reference compound since it has the most favourable ecotoxicological profile among all mosquito larvicides. Lebrun and Vlayen (1981) estimated the $LC50_{24\,h}$ for *D. magna* at 2700 mL L^{-1}, and assigned the mortality of daphnids to gill clogging, instead of toxic effects peculiar to *Bti* at this concentration. Spinosad (DowElanco, Indianapolis, IN, USA) is a new biological insecticide that seems promising for mosquito control (DowElanco, 1996). It is a mixture of spinosyns A and D known as fermentation products of a soil actinomycete (*Saccharopolyspora spinosa*; Crouse et al., 2001). Spinosad acts as a contact and stomach poison (DowElanco, 1996; Salgado, 1998), and persistently stimulates the insect central nervous system by interacting with nicotinic acetylcholine receptors (Watson, 2001). It is considered as a selective insecticide for insect pest species (Miles and Dutton, 2000), but it may also be toxic to non-target species (Nasreen et al., 2000; Tillman and Mulrooney, 2000; Consoli et al., 2001). In particular, spinosad reduced survival and altered life history traits of *D. pulex* at 10 μg L^{-1} in laboratory experiments (Stark and Vargas, 2003). Diflubenzuron is a halogenated benzoylphenylurea. It is an effective stomach and contact insecticide acting by inhibition of chitin synthesis and so interfering with the formation of arthropod cuticle (WHO, 1996; Zhang and Zhu, 2006), leading to death. In crab larvae, diflubenzuron markedly affected the incorporation of glucose and NAG in the premoult stage during secretion of exocuticle (Christiansen et al., 1984). Aquatic arthropods, especially cladocerans (e.g., $LC50_{48\,h}$ for *D. magna* = 3.7 μg L^{-1}; EPA, 1997), are considered as highly susceptible to diflubenzuron (Pest Management Regulatory Agency, 2001).

In this study, biological parameters related to survival, growth and fecundity of groups of control and larvicide-exposed daphnids were measured in laboratory conditions at various dates following the beginning of the exposure. Chitobiase activity in the exposure medium was measured in parallel. Correlations between chitobiase activity and survival, growth and fecundity were analysed. Special attention was given to the correlation between chitobiase activity measured on the last sampling date before the first emission of neonates and the cumulative number of neonates produced during the whole experiment, in order to assess the capacity of chitobiase activity to predict the reproduction dynamics of daphnid population. Results are discussed with regard to the possible use of chitobiase activity as an endpoint to be included into the framework of ecological risk assessment of substances used for mosquito control.

2. Materials and methods

2.1. Chemicals

B. thuringiensis var. *israelensis* (*Bti*) was applied as VectoBac® 12AS (1.2% AI, i.e., 1200 ITU mg^{-1}), a flowable formulation produced by Valent Biosciences (Libertyville, IL, USA). Spinosad was applied as Conserve® 120SC (11.6% AI; DowElanco, Indianapolis, IN, USA). Diflubenzuron was applied as wettable powder (Dimilin® WP 25, 25% AI, Uniroyal Chemical SARL, Switzerland). Substrate, standard, and buffer components for chitobiase activity measurements were obtained from Sigma–Aldrich Co., Lyon, France. All the products and solvents used for residue analysis were of analytical grade and were purchased from Carlo Erba Réactifs (Val de Reuil, France).

2.2. Test organisms

Experiments were carried out using the 4–6th brood offspring (< 24 h old) of *D. pulex* and *D. magna* reared at the INRA Experimental Unit of Aquatic Ecology and Ecotoxicology (Rennes, France). Daphnid strains were originally from the field and maintained under laboratory conditions for 1 year prior to testing. They were reared in 20 L glass aquaria filled with dechlorinated, charcoal-filtered tap water at 20 ± 1 °C in a light–dark regimen of 16:8 with light intensity of ~15 μE m^{-2} s^{-1} (OECD, 1998). They were fed three times a week with a suspension of green microalgae (*Desmodesmus subspicatus* for *D. pulex* and *Chlorella vulgaris* for *D. magna*), batch-cultured according to AFNOR T90-304 (AFNOR, 1993).

2.3. Exposure conditions

Tests were performed in 125 mL polystyrene beakers containing 100 mL of exposure medium (dechlorinated, charcoal-filtered tap water and green microalgae suspension). Three nominal concentrations were tested for each compound: 2, 4 and 8 μg L^{-1} for spinosad, 0.2, 0.4 and 0.8 μg L^{-1} for diflubenzuron, and 0.25, 0.5 and 1 μL L^{-1} for *Bti*. *Bti* concentrations encompassed the maximum registered rates for aerial treatments (0.50 μL L^{-1}; ACTA, 2008), whereas 8 μg L^{-1} spinosad was the lowest concentration that affected *D. pulex* and *D. magna* populations in field microcosms (Duchet et al., 2008, 2010a), and 0.8 μg L^{-1} diflubenzuron corresponds to the minimum $EC50_{48\,h}$ measured under static laboratory conditions on *D. magna* neonates (Majori et al., 1984). Concentrations were hence chosen to obtain sufficient survival in the beakers to measure chitobiase activities. Test solutions were obtained by diluting the commercial preparations of the three insecticides in dechlorinated water. Exposure to each concentration was performed in 5 replicates, and 15 beakers remained as untreated controls. Neonates (< 24 h) of *D. pulex* (20 per beaker) or *D. magna* (15 per beaker) were introduced into each beaker at the beginning of the test (Sanchez et al., 2000). Duration of the test was 14 days for both species (time needed to observe at least 3 broods in our conditions). The exposure medium was renewed on each observation day using stock solutions prepared at the beginning of the experiment in order to avoid a sudden and unrealistic change in chemical composition and larvicide concentration of exposure water.

2.4. Spinosad and diflubenzuron residue analysis

To determine spinosad and diflubenzuron concentrations in the treated beakers, exposure medium was collected on renewal days d2, d4, d7 and d10. Samples were stored at −20 °C in 125 mL amber glass bottles until analysis.

For spinosad analysis, 100 mL of each sample were acidified with 6 mL HCl (pH 2). Spinosad was extracted 3 times with 50 mL dichloromethane. The pooled extracts were evaporated to dryness at 30 °C under a nitrogen flow, and residues were resuspended in 1 mL acetonitrile. Volumes of 50 μL were injected into an HPLC device (Thermoquest P4000) equipped with a UV detector set at 243 nm, and an Eclipse XDB C8 column (150 × 4.6 mm, 5 μm, Agilent Technologies, Santa Clara CA, USA). The mobile phase consisting of acetonitrile and ammonium formate buffer at