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Susceptibility of preparasitic stages of *Chordodes nobilii* (Gordiida, Nematomorpha) to the fungicide carbendazim

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Abstract

We evaluated the effect of carbendazim on non-target organisms using the parasite *Chordodes nobilii* as a test organism. The Gordiida act as a link between freshwater and terrestrial ecosystems; and *C. nobilii*, a neotropical representative species of this group, has been shown to be sensitive to other contaminants even at environmentally acceptable concentrations. The taxa susceptible to carbendazim, however, may not be adequately represented among the standard aquatic test species used in ecotoxicological risk assessment. Moreover, the autochthonous organisms in this area that could be used as bioindicators still need to be found. The aim of the present work was therefore to assess the susceptibility of the preparasitic stages of *C. nobilii* to noxious effects by carbendazim. The assay protocol consisted in 96- and 48-h acute exposures of early embryonic stages and larvae, respectively, to concentrations ranging from 10 to 360 μg/l. Embryonic development was not inhibited by carbendazim at any of the evaluated concentrations, but the infectivity of larvae emerging from the exposed eggs was significantly diminished. Larval survival rate was also affected at the lowest concentration assayed. Values of the mean inhibition concentration (IC_{50}) were 7 and 11 μg/l for embryos and larvae, respectively. Compared to other freshwater organisms, *C. nobilii* can be considered a species moderately to highly susceptible to carbendazim. As the expected environmental concentrations of carbendazim range from 6.25 to 41.3 μg/l, *C. nobilii* could well be a species in danger when exposed to this fungicide.

Introduction

The parasite *Chordodes nobilii* is one of the non-target organisms impacted by the pesticides usually found in water bodies near agricultural regions, mainly as a consequence of run-off. Argentina is a relevant agricultural country: the borders of its cultivated land have expanded to cover large areas of the territory, of which 22.9 million hectares are devoted to transgenic soybean plus maize and cotton (James, 2010). Moreover, 35.5 million hectares can be expected to be cultivated by 2016. In South America, harvesting losses account for 32% and 44%, respectively, of the soybean and maize

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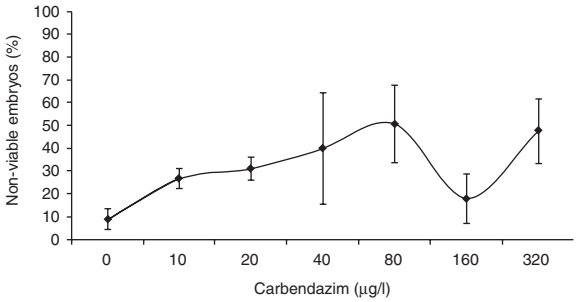

Fig. 1. The effect of carbendazim (µg/l) on the embryonic development of *Chordodes nobilii*, expressed as the mean percentage (± SEM) of non-viable embryos, i.e. those not reaching the larval stage.

produced, principally through the attack of insects, fungi and weeds, among other adverse conditions. The application of agrochemicals in general, and of phytosanitary chemicals in particular, is therefore very likely to increase proportionally in the future (Pérez Leiva & Anastasio, 2003); and accordingly pesticides will then become one of the main sources of contamination for adjacent aquatic environments.

Carbendazim (CBZ; Chemical Abstracts Service (CAS) #10 605-21-7), is one of the pesticides found in the environments near to agricultural crops. Unlike other pesticides, such as chlorpyrifos insecticide and metribuzin herbicide, carbendazim is included in the biocide group (Brock *et al.*, 2006). The compound is a broad-spectrum benzimidazole-carbamate fungicide with systemic activity. Benzimidazoles such as CBZ are widely applied in agriculture and veterinary medicine as fungicides and anthelminthic drugs. CBZ controls fungal diseases by inhibiting germ-tube development, appressoria formation, mycelial growth, nuclear division (i.e. through blocking spindle formation) and, at least temporarily, acetylcholinesterase activity (Cuppen *et al.*, 2000; Kirsch-Volders *et al.*, 2003; Álvarez *et al.*, 2006; Dang & Smit, 2008; Yenjerla *et al.*, 2009); the fungicide is also a possible endocrine disruptor (Varela bruce, 2005; Mnif *et al.*, 2011). Regulations concerning risk classifications with respect to environmental health and safety have proposed a reclassification of CBZ, to a carcinogen and a mutagen (Slijkerman, 2006; Kähkönen & Nordström, 2008; Miracle *et al.*, 2011; Palanikumar & Kumaraguru, 2014).

The criteria for risk prediction and the establishment of thresholds for permissible levels of contamination are based on species' responses to contaminants. These guidelines are derived from laboratory toxicity tests that usually use model species in single-toxicant exposures (Fleeger *et al.*, 2003). Nevertheless, little information is available specifically on the sensitivity of indigenous freshwater organisms to fungicides. The available data on CBZ in particular and its relative toxicity compared to other pesticides are still scarce, while the taxa susceptible to fungicides may also not have been adequately represented among the standard aquatic test species used in the assessment of ecotoxicological risk (Cuppen *et al.*, 2000; Van den Brink *et al.*, 2000; Warming *et al.*, 2009; Buchanan *et al.*, 2010). Consequently, a determination of the side-effects of CBZ on aquatic biota – and mainly on the resident species in Argentina – is highly relevant.

Chordodes nobilii, like the rest of the Gordiida, plays a key ecological role in the ecosystems it inhabits. This is because its life cycle consists of a series of freshwater stages – embryos, free-living larvae (i.e. the preparasites) and the postparasitic adults – all of which may be preyed upon (Cochran *et al.*, 1999; Kinziger *et al.*, 2002; Ruiz & Figueroa, 2005; Ponton *et al.*, 2006; de Villalobos *et al.*, 2008). The juveniles parasitize mainly terrestrial insects, including those of medical and agronomic relevance, causing severe injury or even death (de Villalobos, 1999; de Villalobos *et al.*, 1999, 2003, 2004; Hanelt & Janovy, 1999; Schmidt-Rhaesa & Ehrmann, 2001; Ronderos & de Villalobos, 2003; Hanelt *et al.*, 2003). An evaluation of the impact of pollutants on this class is therefore extremely appropriate and significant.

The Gordiida, in general, act as a link between the taxa of freshwater and terrestrial ecosystems, while *C. nobilii* in particular is highly vulnerable to pesticides other than CBZ, as well as to a herbicide and an insecticide, even at environmentally allowable concentrations (Achiorno *et al.*, 2008, 2009). Moreover, this species was recently validated for use in ecotoxicity assessments (Achiorno *et al.*, 2010). The aim of the present work was therefore to evaluate the effect of the fungicide CBZ on *C. nobilii* (test species) based on the hypothesis that the environmentally acceptable concentrations alter the normal development and, in turn, influence the infectivity and abundance of *C. nobilii* in aquatic habitats.

Materials and methods

Collection of nematomorphs

Adults of *C. nobilii* were collected from an unpolluted stream of the Sauce-Grande basin (38°9′0″S, 61°48′0″W; Buenos Aires, Argentina) and transported to the laboratory. There, the worms were kept at constant temperature (23 ± 1°C), at a natural photoperiod, and in containers with constant aeration; with water initially from the stream, but thereafter gradually replaced by dechlorinated tapwater. After mating, the females were transferred to individual containers and checked daily for oviposition, when they laid microscopic eggs in

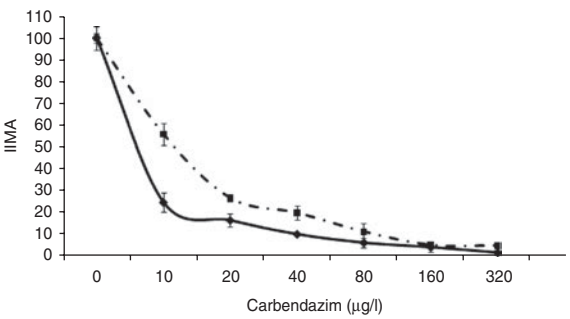

Fig. 2. The effect of carbendazim (µg/l) on the index of infection by embryos (diamonds, solid line) and by larvae (squares, dashed line) of *Chordodes nobilii* (IIMA). Values are expressed as percentages of the arithmetic means relative to the control data (± SEM).

Table 1. Tukey's comparisons of assayed carbendazim concentrations (μg/l) applied to the index of infection by embryos.

Carbendazim (μg/l)	10	20	40	80	160	320
0	*	*	*	*	*	*
10		NS	*	*	*	*
20			NS	NS	*	*
40				NS	NS	NS
80					NS	NS
160						NS

*Level of significance given as $P < 0.05$; NS, not significant.

gelatinous strings attached to the container's lower surface. The time between field collection and laying eggs was between 15 and 30 days. The bioassays were performed with embryos and larvae following the protocol described in Achiorno *et al.* (2008).

Bioassays of preparasitic stages

The test fungicide used for the bioassays (technical-grade CBZ of 98% purity) was provided by the Laboratory of the National Service of Animal Health (SENASA), Argentina. The concentrations assayed were prepared by dilution from a stock solution of 2.5 mg CBZ/litre in Milli-Q™ water (nominal value). The effective concentration of CBZ in the stock solution was determined by high-performance liquid chromatography (detection limit <0.1 mg/l), whereas the concentrations used for the embryo assay and the larval bioassay were 2.80 and 2.35 mg CBZ/litre, respectively. The CBZ concentrations assayed were 0 (control), 10, 20, 40, 80, 160 and 320 μg/l and are referred to as the nominal values. Each concentration, including that of the control, was assayed in triplicate, in a rearing chamber at 23 ± 1°C, in the dark, and under semi-static conditions involving daily medium changes (Achiorno *et al.*, 2008).

For the assays the reconstituted hard water used as the dilution solvent had the following chemical composition (mg/l): $NaHCO_3$, 192; $CaSO_4.2H_2O$, 120; $MgSO_4$, 120; KCl, 8; hardness 162 mg $CaCO_3$/litre; pH 7.99; and conductivity 1.780 μS (Weber, 1993).

The segments of egg strings selected for the embryo bioassays contained embryos mainly in the blastula stage. The bioassay consisted of three consecutive periods, as follows.

(1) Exposure: the embryos remained in the assay solution (the above water plus the appropriate concentration of CBZ) for 96 h. The solution was partially renewed every day and entirely replaced with control hard water at the end of the period.
(2) Postexposure: the containers with the embryos previously exposed to CBZ were checked periodically by light microscopy at a magnification of 40× until the appearance of free-living larvae (for more details see Achiorno *et al.*, 2008).
(3) Infection of the host insect – exposure of *Aedes aegypti* (Diptera) larvae to *C. nobilii* larvae: after hatching, larvae do not exceed 100 μm in length and are able to penetrate paratenic or definitive hosts. Since *C. nobilii* larvae exhibit little movement, we estimated their viability indirectly by evaluating the infectivity of their emerging larvae under light microscopy, as quantitated by means of the larval-infection index per mean abundance of host larvae (IIMA), i.e. the total number of *C. nobilii* larvae infecting *A. aegypti* larvae divided by the total number of *A. aegypti* larvae observed (Bush *et al.*, 1997).

The end points in the bioassay of the treated embryos were the proportions of non-viable embryos, i.e. those that produced no larvae by the end of the postexposure period, and the embryos yielding larvae whose effective viability was subsequently determined by the IIMA-E (the IIMA for the larvae hatched from those exposed eggs) at the end of the infection of the host insect.

In the bioassay of the treated larvae, the experimental protocol consisted of two consecutive periods: in the first, *C. nobilii* free-living larvae were exposed to the toxicant for 48 h, with a partial renewal of the solution at 24 h; then, in the second, *A. aegypti* larvae were exposed to *C. nobilii* free-living larvae for 72 h in control medium. In this instance, the IIMA was designated IIMA-L. Either bioassay was considered valid when the IIMA in the control group was >2.

Data analysis

The parameters calculated were the IC_{50}-IIMA-E (defined as the concentration of toxicant producing a 50% reduction in the infectivity of the larvae from the exposed eggs) and the IC_{50}-IIMA-L (defined as the toxicant concentration that resulted in a 50% survival of the exposed larvae).

Before analysis, the data were subjected to the Shapiro–Wilk and the Levene median tests to assess normality and homogeneity of variance, respectively. Statistical differences between the control and treated groups were determined by one-way analysis of variance followed by Tukey's *post-hoc* comparison test. For regression analysis, the data from control groups were omitted to prevent the wide separation from those of the experimental groups and thus disrupt an estimation of the differential effect of CBZ concentration on the dependent variable (Zar, 1999). The significance level was set at $P < 0.05$. Statistical analyses were performed with the InfoStat software (Di Rienzo *et al.*, 2008). The inhibitory concentrations (IC_{50}) were calculated from the analytical values by the linear-interpolation method recommended

Table 2. Regression analysis of the non-viable embryos (NE) of *Chordodes nobilii* and of the infection indices for embryos (IIMA-E) and for larvae (IIMA-L) upon exposure to different concentrations of carbendazim.

End Point	R^2	Regression equation	P value
NE	0.07	$0.05x + 26.67$	0.2567
IIMA-E	0.50	$-2.2^{-0.3}x + 0.72$	0.0011*
IIMA-L	0.44	$-0.01x + 2.43$	0.0025*

R^2, coefficient of determination; * level of significance with $P < 0.05$.

Table 3. Tukey's comparisons of assayed carbendazim concentrations (μg/l) applied to the index of infection by larvae.

Carbendazim (μg/l)	10	20	40	80	160	320
0	*	*	*	*	*	*
10		*	*	*	*	*
20			NS	NS	*	*
40				NS	NS	NS
80					NS	NS
160						NS

*Level of significance given as $P < 0.05$; NS, not significant.

by the United States Environmental-Protection Agency, through the use of ICp software (version 2.0; Norberg-King, 1993).

Results

Within the concentration range tested, CBZ did not significantly inhibit embryonic development. However, it is notable that we observed an elevation in the variability of response among the replicates at increased concentrations (fig. 1).

The suppression by CBZ of embryo development was reflected in the infectivity of the larvae derived from eggs that had been exposed to the fungicide (i.e. the IIMA-E values) from the lowest CBZ concentration tested (fig. 2, table 1). Furthermore, a clear concentration-dependent effect was evident from regression analysis (table 2). The median inhibitory concentration for embryos (i.e. the IC_{50}-IIMA-E) was 7 μg/l.

The responses of the larvae and embryos exposed to CBZ were qualitatively similar when assessed by the decrease in their infectivity relative to the controls; but that difference was significantly greater with the IIMA-E values than with the IIMA-L data from the lowest concentration tested (fig. 2, table 3), thus indicating a concentration-dependent differential effect (table 2). The median inhibitory concentration calculated for the larvae (i.e. the IC_{50}-IMA-L) was 11 μg/l.

Discussion

The results of this study provide useful information regarding the side-effects of CBZ on worm biology. For both the developmental stages investigated, the inhibition by CBZ of infectivity was observed at the lowest concentration tested. The embryos, though, were found to be more susceptible to the fungicide than the larvae – indicating a more drastic effect on that developmental stage (fig. 2, tables 1 and 2) – and, in turn, exhibited the lowest IC_{50}. The IIMA-E was therefore the more sensitive variable of the two measured in the toxicity tests. The reduction in the infectivity of the larvae from the treated embryos could be explained mainly by an inhibition of viable hatching by the fungicide, a result probably related to a failure at some stage during embryonic development.

Our results provide experimental evidence that low CBZ concentrations impair the viability of the preparasitic stages of the non-target organism *C. nobilii*. Only scanty information is available comparing the effect of CBZ to that of other pesticides on freshwater non-target organisms. Table 4 shows some of the results from the literature, indicating that the species currently used for ecotoxicological risk assessment – such as *Daphnia magna*, *Lepomis macrochirus* and *Onchorhynchus mykiss* – would be

Table 4. Susceptibility of selected invertebrate and fish species to concentrations of carbendazim (μg/l); end points are taken from the references, and only those values obtained under similar test conditions are included.

Species	End point	Concentration (μg/l)	Time (h)	References
Nematoda				
Caenorhabditis elegans	EC_{50}	0.38–0.68	–	Cuppen *et al.* (2000)
Annelida:				
Oligochaeta				
Dero digitata	LC_{50}	980	48	van Wijngaarden *et al.* (1998)
Platyhelminthes:				
Turbellaria				
Dugesia lugubris	EC_{50}	25	96	van Wijngaarden *et al.* (1998)
Dugesia lugubris	EC_{50}	178	48	van Wijngaarden *et al.* (1998)
Fish				
Galaxias maculatus	LC_{50}	18,900	96	Varela bruce (2005)
Ictalurus punctatus (yolk-sac fry)	LC_{50}	7	96	Palawski & Knowles (1986)
Ictalurus punctatus (fingerling, 1.2 g)	LC_{50}	19	96	Palawski & Knowles (1986)
Lepomis macrochirus (fry, 0.2 g)	LC_{50}	>3200	96	Palawski & Knowles (1986)
Oncorhynchus mykiss (yolk-sac fry)	LC_{50}	145	96	Palawski & Knowles (1986)
Oncorhynchus mykiss (fingerling, 1.2 g)	LC_{50}	870	96	Palawski & Knowles (1986)
Crustacea				
Mesocyclops sp.	LC_{50}	100.80	24	USEPA ECOTOX
Daphnia magna	NOEC	37.50	–	Ribeiro *et al.* (2011)
Daphnia magna	LC_{50}	46.62	–	Ribeiro *et al.* (2011)
Daphnia magna	LC_{50}	156	48	Ferreira *et al.* (2008)
Daphnia magna	LC_{50}	20–460	48	USEPA ECOTOX
Moina micrura	LC_{50}	118	24	Miracle *et al.* (2011)

NOEC, no observed effect concentration; EC_{50}, median effective concentration; LC_{50}, median lethal concentration.

the least sensitive ones to CBZ. Cuppen *et al.* (2000) found, in a CBZ-microcosm experiment using concentrations between 3.3 and 1000 μg/l, that the worm-like taxonomic groups exposed – such as *Oligochaeta, Turbellaria* and *Hirudinea* – along with the amphipod *Gammarus pulex* and the snail *Bithynia tentaculata* were the most sensitive taxa. Since those same authors reported that *Caenorhabditis elegans* was the most sensitive species to CBZ (table 4), and because the lowest IC_{50} recorded (7 μg/l, in the present study) was for the gordiid *C. nobilii*, we can conclude that the worm-like taxonomic groups are probably the non-target organisms most sensitive to CBZ.

We wish to emphasize that our experimental design simulated a specific period of exposure to the fungicide: in terms of embryonic development, a 96-h exposure corresponds to only about 15% of the time required to reach the free larval stage. Since the reported half-life time for CBZ in aqueous solution has been from 15 to 175 days for indoor microcosms (Cuppen *et al.*, 2000; Daam *et al.*, 2009) and 120 days for natural habitats (Varela bruce, 2005), we can assume that in the present bioassays, on both the embryo and the larvae, the CBZ retained its potency during the entirety of both exposures. The expected environmental concentration of CBZ has been found to be in the range of 6.25 μg/l for bodies of water having depths of 2 m and up to 41.3 μg/l for much shallower reaches at depths of as low as 30 cm (SETAC, 1994). Therefore, a highly relevant result from these studies is that the IC_{50} values for the embryos and larvae of *C. nobilii* fell within the range reported for the environment, and particularly those higher levels anticipated for shallow water, such as that of the streams inhabited by gordiids.

The preparasitic stages of *C. nobilii* have exhibited IC_{50}-IIMA-Es of 54 and 30 μg/l and IC_{50}-IIMA-Ls of 19 and 67 μg/l for the herbicide glyphosate and the neurotoxic insecticide malathion, respectively (Achiorno, 2011). A comparison of those values with the values obtained here for CBZ indicates an order of susceptibility for this species of CBZ > malathion > glyphosate. Thus, on the basis of these results, CBZ should be considered the most toxic pesticide of the three.

Finally, we wish to emphasize that, although no inhibition of embryonic development was observed, the maturation of the embryos was nevertheless affected since the infectivity of the larvae that were able to hatch became diminished and, as a consequence, their survival as well. From all these considerations and the literature cited, we conclude that *C. nobilii* is a species moderately to extremely susceptible to the noxious action of CBZ.

The results of the work presented here, the previous findings cited above and the ecological value of *C. nobilii* as an insect parasite all point to the usefulness of this species as a test organism for assays directed at environmental-risk assessment.

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Conflict of interest

None.

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