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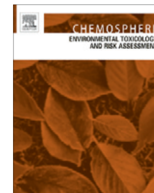

Validation test with embryonic and larval stages of *Chordodes nobilii* (Gordiida, Nematomorpha): Sensitivity to three reference toxicants

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ABSTRACT

Chordodes nobilii is a parasite whose pre- and postparasitic stages are found in different types of freshwater bodies. Due to the peculiarities of its life cycle, it acts as a link between freshwater bodies and terrestrial ecosystems. There is little toxicological information on the group Gordiida. It is only known that embryos and larvae of *C. nobilii* are sensitive to glyphosate and malathion at relevant concentrations in the environment. On this basis, the aims of this study were to characterize the sensitivity of the pre-parasitic stages of *C. nobilii* to three reference toxicants: sodium dodecyl sulfate (SDS), cadmium chloride and potassium dichromate (Cr^{6+}), and to validate a previous experimental protocol for ecotoxicological risk assessment. The protocol involved acute exposure of early embryonic stages and larvae to the three toxicants for 96 h and 48 h, respectively. Embryo development was inhibited only by Cr^{6+} which presented a IC_{50} of 0.71 mg Cr^{6+} L^{-1}. The development of the eggs exposed to SDS and those exposed to cadmium chloride showed no differences as compared to that of controls. However, the infective capacity of larvae derived from the eggs exposed to the three toxicants was lower than that of controls. Larval survival was affected even at the lowest concentration of the three toxicants assayed. In relation to other freshwater organisms, *C. nobilii* can be characterized as an organism medium to highly sensitive to the toxicants tested.

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1. Introduction

Invertebrates represent most of the existing species and are usually placed at the lower levels of the trophic chain. The many bioassays carried out with aquatic invertebrates have shown that they generally have greater sensitivity to toxicants than aquatic vertebrates (Natale, 2006). Thus, alterations in their development and reproduction are of great importance for the biomass and community structure of aquatic environments. In this sense, the bioassays to assess ecotoxicological effects have focused mainly on producer and consumer organisms, and not on the importance of other interspecific relationships, such as parasitism. Although many results have been reported in the last 15 years regarding the effects of different toxicants on some parasites (Donkin and Williams, 1995; Lafferty, 1997; Zuck et al., 1997; Mackenzie, 1999; Sures et al., 1999; Bergey et al., 2002; Sures, 2004, 2008; Gheorghiou et al., 2007; Achiorno et al., 2008a; Achiorno et al., 2009), the information is still scare.

The class Gordiida (Phylum Nematomorpha), commonly known as horsehair worms, is composed of parasitic species whose life cycle includes a larval parasitic stage and freshwater free-living adults. After mating, females move to hard substrates, where they attach the laid eggs in gelatinous strings; recently hatched larvae do not exceed 100 μm in length and bear hooks and stylets on the anterior end, with which they are able to penetrate paratenic or definitive hosts. Insects, including those of medical and agronomical importance, are regarded as the most frequent definitive hosts; gordiid infection in insects may lead to severe damage or even death (de Villalobos and Miralles, 1997; Hanelt and Janovy, 1999; Schmidt-Rhaesa, 2001; Schmidt-Rhaesa and Ehrmann, 2001). We may consider that the characteristics of these parasites and the peculiarities of its life cycle allow a connection between freshwater bodies and terrestrial ecosystems.

Abbreviations: NE, non-viable embryos; LC, ready-to-hatch larvae within egg capsules; FL, free-living larvae; IIMA, infection index mean abundance; IIMA-E, IIMA for embryo bioassays; IIMA-L, IIMA for larval bioassays.

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In particular, gordiids play an important ecological role in the ecosystems they inhabit, because this group include in their life cycle freshwater stages, i.e. embryos, free-living larvae and adults, which may play a role of prey (de Villalobos et al., 2008; Cochran et al., 1999; Kinziger et al., 2002; Ponton et al., 2006; Ruiz and Figueroa, 2005) and parasitic stage, juveniles, which parasitize insects. It is therefore extremely important to know the impact of pollutants on these organisms. However, this group has been scarcely studied from the ecological and toxicological points of view (de Villalobos et al., 2003; de Villalobos and Ronderos, 2003; Achiorno et al., 2008b). The effects of glyphosate and malathion have been evaluated only in early pre-parasitic stages of *Chordodes nobilii*, which have been found to be very sensitive to these pesticides at concentrations expected in the environment (Achiorno et al., 2008a, 2009). This clearly shows the importance of characterizing the responses of this species in order to investigate its potential as a test organism for ecotoxicological assessment.

The development of scientifically consistent protocols to evaluate the sensitivity of these organisms to different toxicants is an extensive and complex process for non-standardized groups. This requires not only to determine the sensitivity of the group, but also to determine whether the techniques used are suitable and reproducible to test different toxicants.

The objective evaluation of the suitability of candidate organisms for toxicity testing requires the comparison of biologically measurable responses (end-points) with a range of concentrations of a common reference toxicant. In this manner, the sensitivity of the various organisms can be ranked, and the most suitable species identified (Gopalakrishnan et al., 2008).

Reference toxicants are organic and inorganic compounds used in standard procedures, which provide information for the interpretation of the results from the toxicity tests; they are also used for intra- and interlaboratory calibration (McNulty et al., 1999; Jorge and Moreira, 2005).

US EPA (1993) recommends the following substances as reference toxicants: the detergent sodium dodecyl sulfate (SDS), cadmium – as cadmium chloride, and chromium – as potassium dichromate.

SDS ($C_{12}H_{25}NaO_4S$) is one of the most frequently used anionic synthetic surfactants worldwide, with applications in household products, industrial mixtures, cleansing products, cosmetics, liquid soaps, shampoos, bubble baths, and tooth pastes. It has been used as an emulsifier, wetting agent and adjuvant in insecticides, as well as an emulsifier and penetrant in varnish and paint remover. Furthermore, it has been used as a tool in biological research. This has been possible because of its ability to reduce the surface tension of water solutions, to form microemulsions, and to solubilize fats and oils, thus leading to solubilization of lipid membranes. Due to its wide applications, SDS is commonly found in domestic sewage.

Since the main routes of environmental exposure to SDS in animals are contaminated water, sediments and soils, SDS is used as a reference substance in toxicity tests evaluating sediment quality (Singer and Tjeerdema, 1993; Cserháti et al., 2002; Jorge and Moreira, 2005).

Concentrations of cadmium and chromium in freshwater environments have increased substantially as a consequence of anthropogenic activities.

Cadmium is more mobile in aquatic environments than most other metals and is regarded as one of the most toxic elements for aquatic organisms; it has a relatively long residence time in aquatic systems; for example, the mean residence time calculated in Lake Michigan is of 4–10 years (Bouche et al., 2000; Lienesch et al., 2000; US EPA, 2007).

Chromium is naturally found in rocks, animals, plants, soil, volcanic dust and gases. In the environment it is often found as Cr (III), a naturally produced, essential oligoelement for life, and as hexavalent Cr (VI), the second most stable state, resulting from industrial activity. The solubility of chromium compounds in water varies depending on the oxidation state, with Cr^{6+} being the most soluble form (Segura-Muñoz et al., 2003; Téllez et al., 2004).

Therefore, the aims of this study were to characterize the sensitivity of embryos and larvae of *C. nobilii* to three reference toxicants (SDS, $CdCl_2$ and $K_2Cr_2O_7$), and to validate previously designed protocols of ecotoxicological assays.

2. Materials and methods

2.1. Test organisms

Adults of *C. nobilii* were collected from an unpolluted stream of the Sauce Grande basin, in an area of small urban settlements in Sierra de la Ventana (38°9′0″S, 61°48′0″W), Buenos Aires Province, Argentina. Once in the laboratory, worms were kept in containers with water from the stream, which was gradually replaced by dechlorinated tap water. The worms were kept under constant temperature (23 ± 1 °C), natural photoperiod and constant aeration. Immediately after mating, females were transferred to individual containers and checked daily for oviposition. The time between field collection and egg laying was about 15–30 d. Bioassays were performed with embryos (mainly in blastula stage) and larvae, following the protocol described in Achiorno et al. (2008a).

2.2. Test substances

The test substances were cadmium (Cd^{2+}: $Cl_2Cd{\cdot}2H_2O$, J.T. Baker), chromium (Cr^{6+}: $K_2Cr_2O_7$, Sigma), and sodium dodecyl sulfate (SDS, Fluka).

The following stock solutions were prepared in distilled water for each toxicant: 1000 mg Cd^{2+} L^{-1}; 1000 mg Cr^{6+} L^{-1} and 100 g SDS L^{-1}. The test solutions were prepared by dilution from their respective stock solutions.

The dilution medium used in exposure assays was reconstituted hard water (pH 7.99, hardness 162 mg CO_3Ca L^{-1}), with the following chemical composition (mg L^{-1}): $NaHCO_3$ 192, $CaSO_4{\cdot}2H_2O$ 120, $MgSO_4$ 120, KCl 8 (US EPA, 1993). The dilution medium was chosen because it provides values of water hardness similar to those found at natural collection sites.

Each concentration including that of controls was tested in triplicate, in a rearing chamber at 23 ± 1 °C, under semi-static conditions and in the dark. The effective concentrations of Cd^{2+} and Cr^{6+} were determined by atomic absorption spectroscopy using direct aspiration into an air–acetylene flame (Method 3111B, APHA, 1999). The analytical concentration of SDS was the methylene blue active substance (MBAS) assay. The detection limits were 0.05, 0.1 and 0.05 mg $L^{-1}$for Cd^{2+}, Cr^{6+} and SDS respectively. The nominal and analytical concentrations of the solutions are shown in Table 1.

2.3. Embryo bioassay

The pieces of egg strings selected for the bioassays contained embryos mainly in the blastula stage. The egg strings were cut into segments of about 3 mm in length, each containing approximately 4000 eggs (see Hanelt and Janovy, 2002, 2004). Each egg segment was cut to increase the exposed surface area and randomly placed in a 1.5-mL container, resulting in an egg density of approximately 2500–3000 eggs mL^{-1}.

The bioassay consisted of the following three consecutive periods:

1. *Exposure*: Embryos remained in the assay medium for 96 h; the medium was partially renewed every day and entirely replaced by control medium at the end of the period.

Table 1
Nominal and analytical concentrations tested for each bioassay.

Toxic substance	Assay	Values	Assayed concentrations (mg L^{-1})						
Cadmium	Embryos	Nominal	0	0.5	1	2	4	8	
		Analytical	ND	0.4	0.92	1.9	3.6	7.1	
	Larval	Nominal	0	0.5	1	2	4	8	–
		Analytical	ND	0.4	0.9	1.9	2.2	7.8	–
Chromium	Embryos	Nominal	0	0.5	1	2	4	8	–
		Analytical	ND	0.4	0.9	1.8	3.8	7.8	–
	Larval	Nominal	0	0.5	1	2	4	8	–
		Analytical	ND	0.16	0.33	0.5	3.2	6.7	–
Sodium dodecyl sulfate	Embryos	Nominal	0	0.1	0.5	1	2	4	8
		Analytical							
	Larval	Nominal	0	0.1	0.5	1	2	4	8
		Analytical	0	0.12	0.46	1.01	1.88	3.94	8.55

2. *Post-exposure*: The segments with embryos previously exposed to the test chemicals were periodically observed. At each observation, samples were taken from each container to check the progress of embryonic development, until the appearance of free-living larvae (FL), using light microscopy. Then, in the subsamples containing between 300 and 600 individuals, 100 individuals were randomly selected as follows: the sample was located using light microscope, with a magnification of 3×, then a sector of the sample was randomly selected for observation with a magnification of 10×, and finally, 25 eggs were classified with a magnification of 40×. The procedure was repeated up to 100 individuals, and the proportion of free-living larvae (FL), ready-to-hatch larvae within egg capsules (LC) and non-viable embryos (NE) with collapsed envelopes and patches of amorphous material were determined. The third experimental period (infection of host insects exposed to FL) was started when the proportion of FL in the sample was >50%, (between 28 and 38 d after the bioassay was started) and the infective capacity was assured in control conditions. This methodology was chosen because Gordiida embryos of the same egg string may develop at dissimilar rates, and thus the proportion of different stages in the egg string, as well as the microscopic size of the embryos, are variable (Montgomery, 1904; Dorier, 1930; Zanca et al., 2007).
3. *Infection of host insect*: This period was started when the proportion of FL in each sample from the post-exposure period (period 2) was nearly 50% for each replicate. Then, under control conditions, 30 *Aedes aegypti* larvae were exposed to samples containing *C. nobilii* larvae for 72 h. Because of the difficulty to determine activity of *C. nobilii* larvae, viability was determined by evaluation of their infective capacity with the infection index mean abundance, IIMA, as the total number of *C. nobilii* larvae that could infect *A. aegypti* larvae, divided by the total number of *A. aegypti* larvae observed (Bush et al., 1997) under light microscopy.

The embryo bioassay was considered valid either when the proportion of non-viable embryos (NE) in the control groups was ⩽10% or when the proportion of viable embryos (NE) in the control groups was <10%.

In the embryo bioassay, the endpoints were either the proportion of NE at the end of the second period, and the IIMA at the end of the third period.

For the first endpoint, the inhibitory concentration (IC_{50}) was defined as the toxic concentration that resulted in 50% abnormal development of viable embryos. For the second endpoint (IIMA-E), the inhibitory concentration (IC_{50}) was defined as the toxic concentration that resulted in 50% decreased infective capacity of larvae hatched from exposed eggs.

2.4. Larval bioassay

Pieces of egg strings were placed in 50-mL plastic containers until the FL stage was reached. The experimental protocol consisted of two consecutive periods: in the first one, egg strings with high proportion (>50%) of fry larvae were cut into segments about

Table 2
Summary of test conditions used in conducting sensibility toxicity tests with pre-parasitic *Chordodes nobilii* satges.

Test parameter	Conditions	
	Embryo bioassays	Larval bioassays
Test type	Static-renewal	Static-renewal
Test duration	±30 d	5 d
Exposition duration	4 d	2 d
Temperature	23 ± 1 °C	23 ± 1 °C
Photoperiod	Dark	Dark
Test chamber size	3 mL	3 mL
Test solution volume	1.5 mL	1.5 mL
Partially renewal of test solution	Daily	Daily
Stage of test organisms in the start of bioassay	Blastula stage (in higher proportion)	Larval stage (in higher proportion)
Density organisms/test chamber	±2500 mL^{-1}	±2500 mL^{-1}
Replicate test chambers	3	3
Dilution water	Hard reconstituted water	Hard reconstituted water
Feeding regime	None	None
Endpoint	% NE IIMA-E	IIMA-L
Test acceptability	% NE ⩽ 10 IIMA-E > 2	IIMA-L > 2

Table 3
Inhibition concentration values and results of the regression analysis for embryos and larvae of *Chordodes nobilii* exposed to sodium dodecyl sulfate, cadmium and chromium.

Chemical	End point	IC_{50} (mg L^{-1})	CLe (mg L^{-1})	Regression equation	*p*-valor	R^2
SDS	IIMA-E	0.064	(0.053–0.083)	$0.76 + (-0.09)x$	0.0001*	0.45
	IIMA-L	0.061	(0.051–0.089)	$0.41 + (-0.05)x$	0.0034*	0.42
Cd	IIMA-E	0.63	(0.29–0.93)	$1.80 + (-0.28)x$	0.0001*	0.71
	IIMA-L	0.36	(0.18–1.75)	$0.59 + (-0.07)x$	0.0810 ns	0.22
Cr	NE	0.71	(0.37–1.26)	$89.9 + (-51.73)x$	0.0026*	0.75
	IIMA-E	0.29	(0.22–0.44)	$1.3 + (-1.23)x$	0.0130*	0.90
	IIMA-L	0.12	(0.093–0.16)	$2.33 + (-0.33)x$	0.0038*	0.49

IC_{50}: Inhibition concentration; CLe: expanded confidence limits; NE: non-viable embryos survival. R^2: coefficient of determination, ns: not significant.
* Differ statistically significant ($p < 0.05$).

3 mm in length, each containing approximately 4000 individuals and segments with high proportion of FL were exposed to the toxicant for 48 h, with partial renewal of the solution every 24 h. In the second period, 30 *A. aegypti* larvae were exposed to the *C. nobilii* larvae for 72 h in control medium.

The IIMA-L, used as a parameter of the infective capacity of the *C. nobilii* larvae to the host (*A. aegypti*), was chosen as endpoint. This bioassay was considered valid when the IIMA in the control group was >2. The test acceptability criteria for control worms were determined based on the method development tests conducted (Achiorno et al., 2008a,b; 2009) and the literature data available (Lazorchak et al., 2009).

For the endpoint (IIMA-L), the inhibitory concentration (IC_{50}) was defined as the toxic concentration that resulted in 50% survival of exposed larvae, obtained from the effectiveness of infection capacity.

A summary of the conditions of the embryo and larval tests is presented in Table 2.

2.5. Statistical analysis

Significant differences between the control and exposed groups were determined using one-way ANOVA followed by Tukey's post

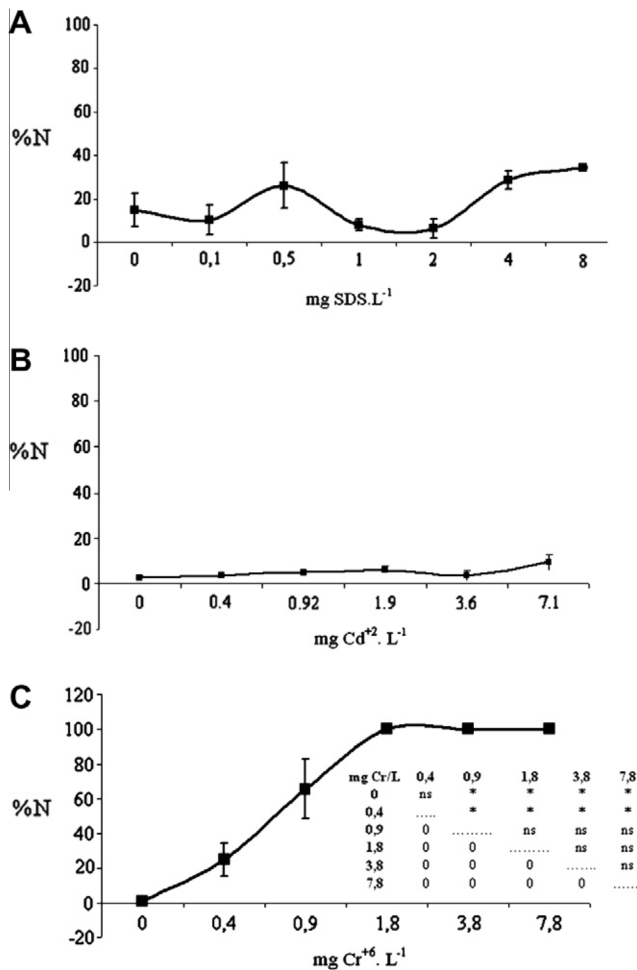

Fig. 1. Non-viable embryos (NE) exposed to SDS (A), Cd^{2+} (B) and Cr^{6+} (C). Value expressed as arithmetic mean by treatment ± SEM. For C, the text box of Tukey's comparisons is shown. * Differ statistically significant ($p < 0.05$), ns: not significant.

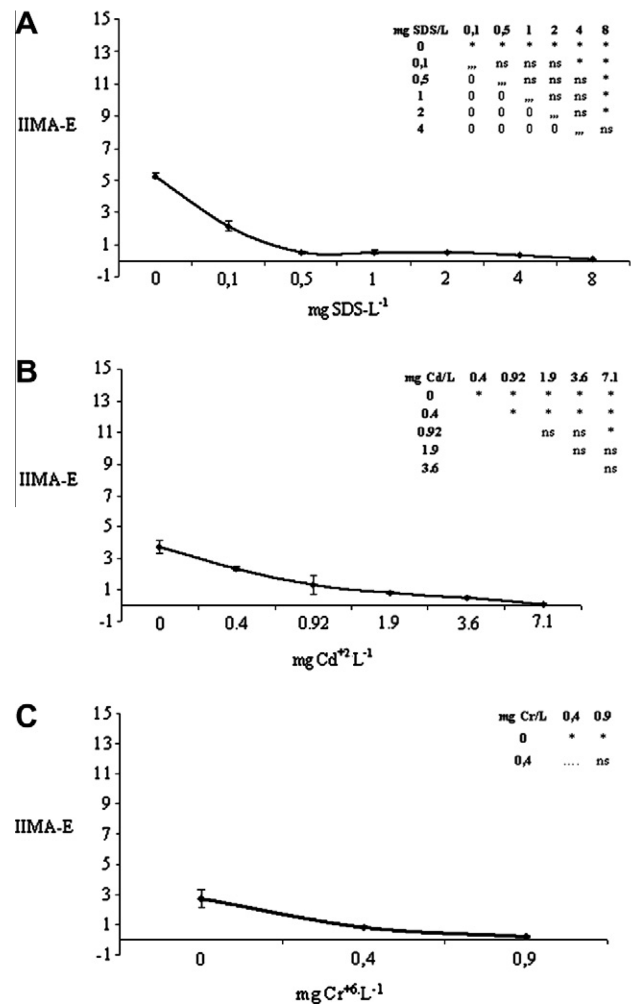

Fig. 2. Infection index mean abundance observed for the control and the assay concentrations of SDS (A), Cd^{2+} (B) and Cr^{6+} (C), in the embryonic development's assays (IIMA-E). Value expressed as arithmetic mean ± SEM by treatment. The text box of Tukey's comparisons is shown. * Differ statistically significant ($p < 0.05$), ns: not significant.

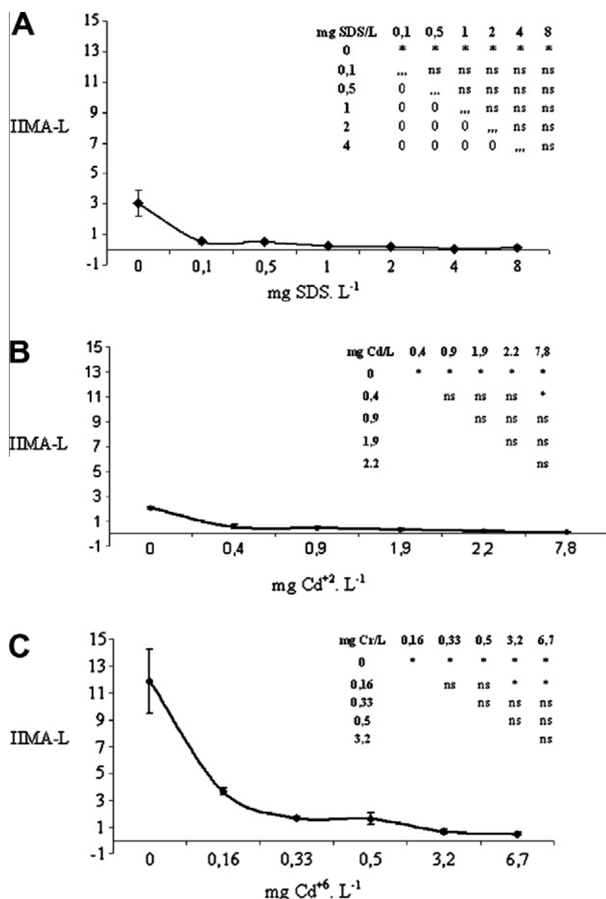

Fig. 3. Infection index mean abundance (IIMA-L) observed for the control and the assay concentrations of SDS (A), Cd^{2+} (B) and Cr^{6+} (C), in larvae's assay. Value expressed as arithmetic mean ± SEM by treatment. The text box of Tukey's comparisons is shown. * Differ statistically significant ($p < 0.05$), ns: not significant.

hoc comparison test. The Shapiro–Wilk test and the Levene median test were used to assess normality and homogeneity of variance of data, respectively. When significant differences were found, regression analysis was carried out in order to determine whether the concentration-depend effect exist. Regression analysis was carried out only for exposed groups to prevent that the significant gap with the control disrupts the effect of concentrations on the dependent variable. An arcsin transformation was applied to the IIMA (Zar, 1999) when was necessary, before statistical analysis, but the IIMA is presented in the figures as non-transformed percentages. The significance level was set at $p < 0.05$. Statistical analyses were performed using Infostat software.

The inhibitory concentrations (IC_{50}) were calculated by the linear interpolation method, recommended by US EPA, using the ICp software (Version 2.0) (Norberg-King, 1993).

3. Results

Table 3 shows the IC_{50} values obtained for each toxicant and each assay and the results of the regression analysis.

3.1. Embryo bioassays

Under our experimental conditions neither SDS nor Cd, even at the maximum concentration tested, inhibited the embryonic development of *C. nobilii* (Fig. 1A and B), but the proportion of normal larvae was significantly decreased ($p < 0.05$). This significant decrease was reflected in the infective capacity of larvae derived from eggs that had been exposed to these chemicals even at the lowest concentrations, i.e. 0.1 mg SDS L^{-1} and 0.4 mg Cd^{2+} L^{-1}. According to the IC_{50} values obtained, the embryos resulted more sensitive to SDS than cadmium in one order of magnitude (Table 3).

In contrast, chromium completely inhibited embryo development at concentrations equal to or higher than 1.8 mg Cr^{6+} L^{-1}, and larvae that hatched from eggs exposed to the lowest concentrations (0.4 and 0.9 mg Cr^{6+} L^{-1}) showed a significantly lower infective capacity than that of the control group (Figs. 1C and 2C, Table 3).

The three toxicants have a very important concentration-dependent effect at a significance level of 0.05 (see Table 3).

The results thus indicate that *C. nobilii* is more sensitive to Cr than to the other toxicants tested, since for the same range of concentrations, Cr was the only one that inhibited embryonic development. However, we should consider that the sublethal effect (IIMA-E) observed was higher for SDS than for the other toxicants tested, since the IC_{50}–IIMA-E, which determines that the effectiveness of the hatched larvae of the eggs exposed would decrease by 50%, is much lower than that of the detergent (Table 3).

This fact implies that the infectiousness of the larvae hatched from eggs exposed to the toxicants is affected by SDS > Cr > Cd.

3.2. Larval bioassays

SDS, Cd and Cr had not only severe effects on *C. nobilii* development, but also adverse affects on larval survival when the larvae were exposed to these toxicants for 48 h.

The IIMA values of larvae exposed to all the tested concentrations of the three toxicants were significantly lower than those of the control, even at the lowest concentration tested at a significance level of 0.05 (Fig. 3). Only Cr and SDS showed a significant concentration-dependent effect at a significance level of 0.05 (Table 3).

When the IC_{50} for embryos and larvae were compared, we observed that the larvae were as sensitive as or even more sensitive than the embryos for a given toxicant, mainly for Cd, since the concentration-dependent effect was not observed at the larval stage.

Considering the IC_{50} obtained for the three toxicants in this work, the toxicity ranking considered was SDS > Cr > Cd (Table 3).

4. Discussion and conclusions

In order to develop and validate the assay protocols it is crucial to prove whether the endpoints considered are viable indicators of lethal or sublethal effects (Lazorchak et al., 2009). Based on our results, the endpoints used in our assays seem to be appropriate to indicate both lethal (IIMA-L and NE) and sublethal effects (IIMA-E).

We thus considered these to be reliable endpoints. Therefore, the results obtained in this work for the three reference toxicants validate these endpoints to be used in ecotoxicological assays in pre-parasitic stages of Gordiida species.

The results of this study validate an experimental protocol using early developmental stages of *C. nobilii* for ecotoxicological assessment, designed to simulate a situation of acute exposure. To evaluate the sensitivity of embryos, fertilized eggs of *C. nobilii* were exposed during a period representing just about 15% of the time elapsed from the blastula stage to the free-living larval stage; embryos underwent complete development in clean medium. Likewise, the protocol with larvae included a shorter exposure period because larvae must find a host within 7 d of hatching to survive. These two protocols allow evaluating the sensitivity of this

Table 4
Sensitivity of different species to sodium dodecyl sulfate, cadmium and chromium. Endpoints values under similar test conditions.

Test organisms	Endpoint	Concentration (mg L^{-1})	Exposure time (h)	Reference
Sodium dodecyl sulfate				
Ceriodaphnia dubia	LC_{50}	1.26	48	US EPA ECOTOX
Daphnia magna	LC_{50}	1.80	48	US EPA ECOTOX
Daphnia ambigua	LC_{50}	2.43	48	US EPA ECOTOX
Diaphanosoma brachyurum	LC_{50}	6.14	48	US EPA ECOTOX
Daphnia magna	LC_{50}	9.60	48	Toussaint et al. (1995)
Daphnia pulex	LC_{50}	10.20	48	Lewis and Horning II (1991)
Daphnia magna	LC_{50}	10.80	48	Lewis and Horning II (1991)
Daphnia pulex	LC_{50}	12.60	48	Lewis and Horning II (1991)
Daphnia magna	LC_{50}	13.50	48	Lewis and Horning II (1991)
Ceriodaphnia rigaudi	LC_{50}	20.87	48	US EPA ECOTOX
Moinodaphnia macleayi	LC_{50}	27.71	48	US EPA ECOTOX
Ceriodaphnia dubia	LC_{50}	48.40	48	Toussaint et al. (1995)
Mysidopsis bahia	LC_{50}	6.60	96	Toussaint et al. (1995)
Pimephales promelas	LC_{50}	8.00	96	Toussaint et al. (1995)
Lampsilis teres	LC_{50}	17.00	96	US EPA ECOTOX
Utterbackia imbecillis	LC_{50}	22.82	96	US EPA ECOTOX
Villosa vibex	LC_{50}	24.00	96	US EPA ECOTOX
Utterbackia imbecillis	LC_{50}	30.78	96	US EPA ECOTOX
Actinonaias pectorosa	LC_{50}	33.56	96	US EPA ECOTOX
Actinonaias pectorosa	LC_{50}	34.38	96	US EPA ECOTOX
Utterbackia ibecillis	LC_{50}	40.36	96	US EPA ECOTOX
Cadmium				
Hydra vulgaris	LC_{50}	0.31	48	US EPA ECOTOX
Tubifex tubifex	EC_{50}	61.47	48	US EPA ECOTOX
Tubifex tubifex	LC_{50}	0.03	96	Bouche et al. (2000)
Tubifex tubifex	LC_{50}	0.06	48	Bouche et al. (2000)
Tubifex tubifex	LC_{50}	1.03	96	US EPA ECOTOX
Tubifex tubifex	LC_{50}	1.46	48	US EPA ECOTOX
Daphnia magna	EC_{50}	0.02	48	Barata et al. (2006)
Daphnia ambigua	LC_{50}	0.00009	48	US EPA ECOTOX
Daphnia pulex	LC_{50}	0.0004	48	US EPA ECOTOX
H. curvispina	LC_{50}	0.00171	96	García et al. (2010)
Daphnia magna	LC_{50}	0.003	48	US EPA ECOTOX
Hyalella azteca	LC_{50}	0.008	96	Nebeker et al. (1986)
Daphnia magna	LC_{50}	0.017	48	US EPA ECOTOX
H. curvispina	LC_{50}	0.02999	96	García et al. (2010)
Daphnia magna	LC_{50}	0.033	48	Nebeker et al. (1986)
Daphnia magna	LC_{50}	0.038	48	Lewis and Horning II (1991)
Daphnia pulex	LC_{50}	0.042	48	Lewis and Horning II (1991)
Daphnia magna	LC_{50}	0.069	48	US EPA ECOTOX
Ceriodaphnia dubia	LC_{50}	0.100	48	US EPA ECOTOX
Ceriodaphnia rigaudi	LC_{50}	0.160	48	US EPA ECOTOX
Ceriodaphnia rigaudi	LC_{50}	0.200	48	US EPA ECOTOX
Daphnia magna	LC_{50}	0.200	48	US EPA ECOTOX
Daphnia magna	LC_{50}	0.36	48	Fargasová (1994)
Daphnia magna	LC_{50}	0.520	48	US EPA ECOTOX
Cherax destructor	LC_{50}	0.913	48	US EPA ECOTOX
Diaphanosoma brachyurum	LC_{50}	1.06	48	US EPA ECOTOX
Chironomus riparius	EC_{50}	0.0000145	96	US EPA ECOTOX
Chironomus ripartus	LC_{50}	0.013	48	Iannacone Oliver et al. (2003)
Chironomus calligraphus	LC_{50}	0.132	48	Iannacone Oliver et al. (2003)
Chironomus calligraphus	LC_{50}	0.28	48	Iannacone Oliver et al. (2003)
Ruditapes decussatus	EC_{50}	0.424	48	Beiras and Albentosa (2004)
Mytilus galloprovincialis	EC_{50}	1.925	48	Beiras and Albentosa (2004)
Lampsilis teres	LC_{50}	0.011	96	US EPA ECOTOX
Villosa vibex	LC_{50}	0.030	96	US EPA ECOTOX
Lampsilis teres	LC_{50}	0.038	96	US EPA ECOTOX
Actinonaias pectorosa	LC_{50}	0.057	96	US EPA ECOTOX
Chromium				
Cypris subglobosa	EC_{50}	8.75	48	Khangarot and Das (2009)
Ceriodaphnia rigaudi	LC_{50}	0.002	48	US EPA ECOTOX
Bosmina longirostris	LC_{50}	0.05	96	Wu et al. (2007)
Daphnia carinata	LC_{50}	0.05	96	Wu et al. (2007)
Daphnia pulex	LC_{50}	0.06	96	Wu et al. (2007)
Ceriodaphnia quadrangular	LC_{50}	0.07	96	Wu et al. (2007)
Simocephalus vetulus	LC_{50}	0.08	96	Wu et al., 2007
Daphnia carinata	LC_{50}	0.12	48	Wu et al. (2007)
B. longirostris	LC_{50}	0.12	48	Wu et al. (2007)
C. quadrangular	LC_{50}	0.14	48	Wu et al. (2007)
D. pulex	LC_{50}	0.15	48	Wu et al. (2007)
S. vetulus	LC_{50}	0.16	48	Wu et al. (2007)
Daphnia magna	LC_{50}	0.25	96	Wu et al. (2007)

Table 4 (*continued*)

Test organisms	Endpoint	Concentration (mg L^{-1})	Exposure time (h)	Reference
D. magna	LC_{50}	0.78	48	Wu et al. (2007)
Bryocamptus echinatus	LC_{50}	1.26	96	US EPA ECOTOX
Bryocamptus zschokkei	LC_{50}	1.85	96	US EPA ECOTOX
Bryocamptus pygmaeus	LC_{50}	3.48	96	US EPA ECOTOX
Bryocamptus minutus	LC_{50}	3.56	96	US EPA ECOTOX
Attheyella crassa	LC_{50}	3.82	96	US EPA ECOTOX
Diplodon chilensis	LC_{50}	20.40	96	Silva et al. (2007)

taxonomic group in particular, and the protocol for embryonic development is able to discern sublethal effects (IIMA-E) that might otherwise go unnoticed.

According to Gopalakrishnan et al. (2008), to be of practical use in ecotoxicological assessment bioassays, a candidate species, or at least one stage of its life history, should be not only sensitive to potential contaminants, but also relatively easy to collect from the field (i.e. abundant) as well as amenable to routine maintenance, culture and rearing in the laboratory. If early developmental stages are to be used, spawning should be readily inducible and gametes should be freely available from the natural habitat. Then the pre-parasitic stages of *C. nobilii* seem to be suitable test organisms for ecotoxicological assessment, although further research has to be carried out in order to optimize larval rearing in the laboratory.

Another point to that should be taken into account to consider *C. nobilii* as a test organism for ecotoxicological assessment is its relative sensitivity as compared to that of other organisms. Although the endpoints IIMA-E and IIMA-L obtained in the present study are not reported elsewhere, which makes the comparisons with those shown below difficult, it is possible to establish the relative sensitivity of *C. nobilii* to the assayed toxicants with respect to that of other aquatic organisms.

There are few reports concerning the effect of the tested reference substances on parasites. Pietrock et al. (2002), who studied the effect of cadmium on the life span of the trematode *Diplostomum* sp., found significantly higher mortality in cercariae exposed to concentrations higher than 20 mg Cd L^{-1} than in control cercariae. Many studies have been performed in Nematoda, a group closely related to Nematomorpha, to evaluate the effect of different pollutants on free-living species. We can also mention Donkin and Williams (1995), who reported a LOEC of 11.2 mg Cd^{2+} L^{-1} in larvae of *Caenorhabditis elegans* after a 96-h exposure. In *C. elegans*, Chu and Chow (2002) determined 96-h LC50 values of 12.3 (12.5–12.2) mg Cr^{6+} L^{-1} and 21.1 mg Cd^{2+} L^{-1} after exposure. Murillo Zabala and Diaz Baez (1997) exposed larvae of *Panagrellus redivivus* to different concentrations of $K_2Cr_2O_7$ as a reference toxicant, and obtained a 96-h LC_{50} of 44.3 mg Cr^{6+} L^{-1}, and 96-h EC_{50} values for survival, growth and sexual maturity of 38, 39 and 13.2 mg Cr^{6+} L^{-1}, respectively.

The toxicity of SDS, Cd^{2+}, and Cr^{6+} to different aquatic organisms has been widely investigated. Table 4 summarizes some toxicity endpoint values reported for these toxicants.

The freshwater crustacean *Ceriodaphnia dubia* is among the organisms with higher sensitivity to SDS (Table 4), with values of at least two orders of magnitude higher than those required to induce a toxic response in pre-parasitic stages of *C. nobilii*.

There is great variability in the toxic effects of cadmium (Table 4), depending on the organisms, the dilution media and assay conditions tested. This accounts for the EC_{50} and LC_{50} values, even within the same species and development stage reported. In general terms, toxicity is lower in marine organisms, while in freshwater environments it is lower in vertebrates than in invertebrates, even at early development stages. If the LC_{50} is considered, the most sensitive freshwater invertebrates seem to be *Hyalella curvispina* at the neonate stage, with 96-h LC_{50} of 0.00171 mg L^{-1}, while the most resistant species seems to be *Artemia salina*, with 96-h LC_{50} of 160 mg L^{-1}. If the EC_{50} is considered, *Daphnia magna* is among the most sensitive freshwater invertebrates, with 48-h EC_{50} of 0.024 mg L^{-1}, and *Tubifex tubifex* is among the most resistant species, with 48-h EC_{50} of 61.47 mg L^{-1}. Based on the results obtained in this study, the pre-parasitic stages of *C. nobilii* are likely to have a medium to low sensitivity to cadmium (see Figs. 1–3, and Table 4).

Ceriodaphnia rigaudi and *Bosmina longirostris* are among the freshwater invertebrates most sensitive to Cr (48-h LC_{50} of 0.002 mg L^{-1} and 96-h LC_{50} of 0.05 mg L^{-1}; Table 4), while larvae of *Panagrellus redivivus* seem to be the most resistant, with a 96-h LC_{50} of 44.3 mg Cr^{6+} L^{-1} (Murillo Zabala and Diaz Baez, 1997). In comparison with these values, *C. nobilii*, with 96-h IC_{50} of 0.71 mg Cr^{6+} L^{-1} for embryo development and 0.12 mg Cr^{6+} L^{-1} for larval development, seems to have a medium to high sensitivity.

The results obtained in these bioassays indicate that these protocols give consistent lethal (NE, and IIMA-L) and sublethal endpoints (IIMA-E) with *C. nobilii*, which, based on the responses to SDS, chromium and cadmium as reference toxicants, allow it to be characterized as having medium to high sensitivity in relation to other freshwater organisms.

As regards the protocols, the length of the realization is one point to discuss. In the protocols used in this work, the embryonic assay has a extended duration, whereas the larval assay does not. Another point to discuss is the special equipment needed: in our work, neither assay requires special equipment. To finish, we can say that these protocols are reproducible.

Finally, another interesting point to be taken into account is that from the scientific point of view, these protocols lead us to wonder how it is possible that a brief exposure during embryonic development may determine that hatched larvae lose their infective capacity and how pollutants act in the parasitic metabolism. Therefore, further research must be carried out in order to respond to these new concerns.

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