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Sublethal Effects of Trace Metals (Cd, Cr, Cu, Hg) on Embryogenesis and Larval Settlement of the Ascidian *Ciona intestinalis*

J. Bellas, R. Beiras, E. Vázquez

Facultade de Ciencias, Departamento de Ecoloxía e Bioloxía Animal, Universidade de Vigo E-36200, Vigo, Galicia, Spain

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Abstract. Toxicity of Cadmium (Cd), Chromium (Cr), Copper (Cu), and Mercury (Hg) on the early developmental stages of *Ciona intestinalis* was investigated. Developmental defects of larvae after exposure of gametes throughout their development to the larval stage were assessed. Gamete exposure to increasing metal concentrations resulted in a significant decrease of the percentage of normally hatched larvae, showing median effective concentrations (EC_{50}) of 721 μg/L (6.42 μM) for Cd, 12772 μg/L (226 μM) for Cr, 36.6 μg/L (0.576 μM) for Cu, and 44.7 μg/L (0.223 μM) for Hg. Larval attachment was significantly affected when gametes were exposed to the metals throughout development. The EC_{50} reducing larval attachment by 50% were 752 μg/L (6.7 μM) for Cd, 15026 μg/L (289 μM) for Cr, 67.8 μg/L (1.607 μM) for Cu, and 78.1 μg/L (0.389 μM) for Hg. Therefore, on a molar basis Hg is three times more toxic than Cu, 20–30 times more than Cd, and 700–1000 times more toxic than Cr, for both responses.

Industrialization and development of the coastal zone have caused a continuous increase in trace metal pollution of estuarine and marine waters, and high concentrations of metals have been recorded in seawater, sediments, and organisms in coastal areas (Kennish 1992; Clark 2001; OSPAR Commission 2000). Trace metals have long been recognized as major pollutants of the marine environment, constituting a hazard for the associated organisms (Weis and Weis 1991; Kennish 1992; Depledge *et al.* 1994). Many studies have been done that demonstrated the high toxicity of trace metals on early developmental stages of marine invertebrates, such as bivalves (Calabrese *et al.* 1973, 1977; His and Robert 1981, 1982; Beiras and His 1994, 1995), echinoderms (Waterman 1937; Kobayashi 1981; Dinnel *et al.* 1989; Warnau *et al.* 1996), or crustaceans (Corner and Sparrow 1956; Ramachandran *et al.* 1997; Itow *et al.* 1998a, 1998b), and the use of these invertebrate embryo and larval bioassays, in particular bivalves and sea urchins, have been proposed as sensitive, simple, and reliable methods for assessing and monitoring marine pollution (Woelke 1972; Kobayashi 1981; His *et al.* 1997). In contrast, little work has been carried on the effects of trace metals on early stages of ascidians. *Ciona intestinalis* (Chordata, Ascidiacea) is the most cosmopolitan and the most studied species of ascidians (Berrill 1947; Millar 1971). It shows a long spawning season from March to November, and occurs in dense aggregations as the dominant occupier of space (Dybern 1965), and playing an important ecological role as a filter-feeder (Kayser 1982). Besides assessing the effects of those metals on the response of embryonic development, we investigated a sublethal response relatively unstudied previously, the larval attachment (*e.g.*, Grave and Nicoll 1939; Wisely 1963).

The present work aimed to determine the toxicity of four trace metals of environmental concern on the early development of *Ciona intestinalis*. We studied the toxic effects of trace metals cadmium (Cd), chromium (Cr), copper (Cu), and mercury (Hg), on the rates of embryonic development and larval attachment of *Ciona intestinalis* in order to establish the dose-response relationships for each tested metal, and give biological criteria for the implementation of marine water quality standards to protect these organisms.

Materials and Methods

Biological Material

Adults of *Ciona intestinalis* collected from a natural population in the Ría de Vigo (Galicia, northwestern Spain) were transported in a portable icebox to the laboratory, transferred into aquaria, and fed on the microalgae *Isochrysis galbana* and *Chaetoceros calcitrans*. Handling conditions of adult stock were 14–17°C temperature, 34.5–36.0 ppt salinity, 6.2–6.6 mg/L O_2, and 7.5–8.4 pH.

Gametes were obtained from the gonoducts with a Pasteur pipette by dissection of 12 adults. Since this species is hermaphroditic and self-fertilizing, unfertilized mature oocytes were taken from the oviduct of eight specimens and sperm from the spermduct of four different ones. Eggs were transferred into glass beakers containing artificial seawater (ASW) at 20°C and sperm was stored undiluted at 4°C until use. ASW was prepared following Zaroogian *et al.* (1969) but salinity was adjusted to 33 ppt by adding double-distilled water.

Correspondence to: J. Bellas, Kristineberg Marine Research Station, Fiskebäckskil SE-450 34, Sweden; *email:* juan.bellas@navegalia.com

Metal Solutions

Experiments were performed using the following analytical grade salts: mercuric chloride ($HgCl_2$), cupric chloride ($CuCl_2 \cdot 2H_2O$), cadmium chloride ($CdCl_2 \cdot H_2O$), and chromium VI oxide (CrO_3) (Merck, Darmstadt, Germany). We chose these salts because of their high solubility in seawater (Calabrese *et al.* 1973) and the nontoxicity of the anion. Stock solutions were prepared in deionized water (Milli-Q) within four hours before the beginning of the experiments, at concentrations high enough to prevent weighing errors and salinity change ≤10%. Mercury solutions were 100-fold more concentrated because of the low amount of salt. Toxicity tests included five metal concentrations, prepared by dilution of the stock in ASW.

The range of experimental concentrations was based on literature data (*e.g.*, Calabrese *et al.* 1973, 1977; Beiras and His 1994, 1995; Hoare *et al.* 1995a, 1995b, with bivalves; Corner and Sparrow 1956, with crustaceans; Waterman 1937; Dinnel *et al.* 1989; Kobayashi 1995, with echinoderms; Franchet *et al.* 1997, with tunicates), and our own previous work (Bellas *et al.* 2001). The metal concentrations used in the toxicity tests were: 512–2048 μg/L for Cd, 4096–16384 μg/L for Cr, 8–256 μg/L for Cu, and 8–128 μg/L for Hg. Concentrations are reported in μg/L of metal ion added to the ASW.

The control solution in each experiment was ASW. For calculations we incorporated the 10% dilution of metal concentrations caused by adding 2 mL of egg suspension in 20 mL of experimental solution; however nominal concentrations are retained for presentation. All glassware was acid-washed (HNO_3 10% vol) and rinsed in deionized water before the experiments.

Experiments with Cd, Cr, and Cu were done by adding 20 mL of each metal concentration to 25 mL polypropylene vials. Experiments with Hg were run in Teflon vials to avoid loss of Hg by binding onto the walls of the vessels. Since polypropylene vials did not offer a suitable substrate for larval attachment, these experiments were carried out in 14-mL glass Petri dishes that had previously been used and washed thoroughly. Five replicates for each metal concentration were tested.

Physicochemical conditions of the experiments were: 20 ± 1°C temperature, 33.3 ± 0.27 ppt salinity, 7.47 ± 0.76 mg/L O_2, and 8.06 ± 0.14 pH (mean ± sd).

Larval Hatching Experiments

Unfertilized eggs (2 mL, approximately 150 eggs/mL) and 100 μL of diluted sperm (approximately 10^8 sperm/mL) were delivered into experimental vials containing metal solutions and were incubated for 20 h at 20°C. After incubation, a few drops of 40% buffered formalin were added and the percentage of normal hatched larvae was recorded.

Larval Attachment Experiments

To study the effects of metals from gametes to larval attachment, 1.3 mL of unfertilized eggs (approximately 100 eggs/mL) and 100 μL of sperm suspension (approximately 10^8 sperm/mL) were added to 14-mL glass Petri dishes containing 13 mL of metal solutions, to obtain the same dilution as in the other experiments. Thus, Petri dishes were filled up to the rim, so that when they were capped, the experimental solution was touching the upper dish and larvae could attach to it, offering a larger attachment surface. Vials were incubated for 70 h. After incubation and without adding formalin, the number of attached larvae was recorded for each treatment.

Statistical Analyses

Differences among treatments were tested for significance using the nonparametric Kruskal-Wallis test, since some sets of data did not meet the assumptions of normality and homoscedasticity, even after angular transformation. When differences among groups were significant, the Games-Howell test was employed to compare the control group and each of the experimental groups for calculation of Lowest Observed Effect Concentrations (LOEC) and No Observed Effect Concentrations (NOEC). The EC_{50} values and their 95% confidence limits were determined according to the Probit method for normal larvae (%) data and, since it was not possible to fit a sigmoidal curve for larval attachment experiments, we used the graphic interpolation method of Litchfield-Wilcoxon for those data. The experimental data were normalized to the control mean percentage of larval abnormality, previously to EC_{50} calculations using Abbot's formula (Emmens 1948):

$$P = (P_e - P_c/100 - P_c) \times 100$$

where P_c and P_e are control and experimental percentages of response, respectively. Statistical analyses were performed according to Newman (1995) and Sokal and Rohlf (1995).

Results

Effects on Larval Hatching

When *Ciona intestinalis* embryos, were exposed for 20 h to increasing Cd, Cr, Cu, and Hg concentrations, the percentage of normal larvae showed a significant decrease (Table 1, Figure 1). The metals showing the highest toxicity were Cu and Hg, causing significant effects at 32 μg/L (LOEC), reducing larval hatching by 20% for Hg and by 20–40% for Cu, compared to controls (Table 2). No larval hatching was observed at 128 μg/L for either Cu or Hg. The lowest Cd concentration with significant effects on larval hatching was 512 μg/L, and for Cr 11385 μg/L.

Table 3 shows the EC_{50} values and their 95% confidence intervals (95 CI) in μg/L and after conversion into molar units for comparison. On a molar basis, Hg was almost three times more toxic than Cu, 30 times more than Cd, and 1000 times more than Cr.

Effects on Larval Attachment

Exposure of early developmental stages of *Ciona intestinalis* from gametes to larval attachment to increasing metal concentrations caused a significant reduction in the number of larvae attached to the Petri dishes (Table 1, Figure 2). Again, Hg and Cu showed the highest toxic effects. Cu significantly reduced the larval attachment by 60–70% at 64 μg/L, and at 32 μg/L of Hg there was a significant increase in the number of attached larvae. Settlement was completely inhibited at 128 μg/L. Cd and Cr showed lower toxicity. Cd caused a 70% decrease in larval attachment at 2048 μg/L. In contrast, Cr caused a significant increase in larval attachment at 8192 μg/L, while higher concentrations (16384 μg/L) inhibited larval attachment.

Table 3 presents EC_{50} values and their 95% confidence intervals. On a molar basis, Hg was three times more toxic than Cu, 20 more than Cd, and 700 more than Cr.

Table 1. Results of Kruskal-Wallis test for *Ciona intestinalis* normal larvae and attached larvae data incubated at different concentrations (μg/L) of Cd, Cr, Cu, and Hg

	Experiment[1]	χ^2	d.f.	p	Experimental treatments
Embryonic development	Cd 1	28.250	5	0.000***	0^a, 512^b, 724^c, 1024^d, 1448^e, 2048^e
	Cd 2	27.484	5	0.000***	0^a, 512^a, 724^b, 1024^c, 1448^d, 2048^d
	Cr 1	23.433	5	0.000***	0^a, 4096^a, 5793^a, 8192^a, 11385^b, 16384^c
	Cr 2	15.044	5	0.010*	0^a, 1024^a, 2048^a, 4096^a, 8192^a, 16384^b
	Cu 1	27.294	5	0.000***	0^a, 16^a, 32^b, 64^c, 128^d, 256^d
	Cu 2	27.360	5	0.000***	0^a, 16^a, 32^b, 64^c, 128^d, 256^d
	Cu 3	23.265	5	0.000***	0^a, 8^a, 16^a, 32^a, 64^b, 128^c
	Hg 1	26.591	5	0.000***	0^a, 8^a, 16^a, 32^b, 64^c, 128^d
	Hg 2	24.332	5	0.001**	0^a, 8^a, 16^{ab}, 32^b, 64^c, 128^d
	Hg 3	26.490	5	0.000***	0^{ab}, 16^a, 23^{bc}, 32^{cd}, 45^d, 64^e
Larval attachment	Cd 1	21.699	5	0.001**	0^{ab}, 128^{ab}, 256^a, 512^a, 1024^b, 2048^c
	Cr 1	14.895	5	0.011**	0^a, 1024^a, 2048^a, 4096^a, 8192^a, 16384^a
	Cr 2	18.675	5	0.003**	0^a, 1024^{ab}, 2048^{abc}, 4096^{ab}, 8192^b, 16384^c
	Cu 1	17.917	5	0.002**	0^{ab}, 8^{ab}, 16^a, 32^a, 64^a, 128^b
	Cu 2	20.782	5	0.008**	0^a, 8^a, 16^{ab}, 32^{ab}, 64^b, 128^c
	Cu 3	19.687	5	0.001**	0^{ab}, 8^{ac}, 16^{ab}, 32^{ab}, 64^b, 128^c
	Cu 4	15.772	5	0.008**	0^a, 8^{ab}, 16^a, 32^a, 64^{ab}, 128^b
	Hg 1	20.123	5	0.001**	0^{ab}, 8^a, 16^a, 32^a, 64^a, 128^b
	Hg 2	12.458	5	0.029**	0^{ab}, 8^{ab}, 16^a, 32^a, 64^a, 128^b
	Hg 3	24.276	5	0.000***	0^a, 8^a, 16^a, 32^b, 64^a, 128^c
	Hg 4	24.215	5	0.000***	0^a, 8^a, 16^a, 32^b, 64^{ab}, 128^c

[1] Numbers indicate different experiments.
*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.
[a, b, c, d]: designate homogeneous groups obtained with the Games-Howell method.

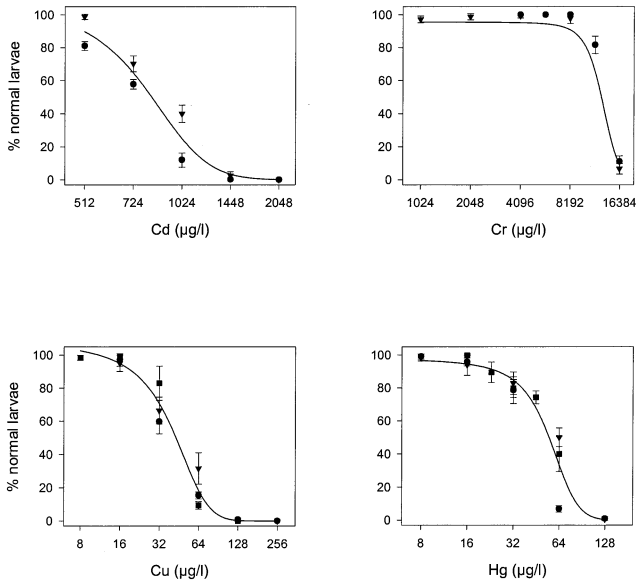

Fig. 1. Percentage of normal hatched larvae of *Ciona intestinalis* after 20 h exposure of gametes throughout their development to the larval stage to different concentrations (μg/L) of dissolved Cd, Cr, Cu, and Hg. Symbols indicate different experiments. Error bars represent the standard deviation for each treatment ($n = 5$).

Discussion

In a previous study (Bellas *et al.* 2001) we exposed gametes of *Ciona intestinalis* to metals for 1 h, embryos at two-cell stage for 20 h, and nearly hatched larvae for 48 h. We reported that

Table 2. NOEC and LOEC of Cd, Cr, Cu, and Hg (μg/L) for toxicity tests with early developmental stages of *Ciona intestinalis*

	Cd	Cr	Cu	Hg
Embryonic development				
NOEC	<512	8192	16	23
LOEC	512	11385	32	32
Larval attachment				
NOEC	1024	4096	32	64
LOEC	2048	16384	64	128

fertilization rate was an insensitive endpoint for exposures to Cd, Cr, Cu, and Hg compared to embryonic development and larval attachment of *Ciona intestinalis*, which showed a sensitivity to metals similar to bivalve and sea urchin embryos and larvae. Later experiments confirmed those results, but revealed some toxicity of Cu on gametes of *Ciona intestinalis*, reducing the fertilization rate by 30% at 128 μg/L (Bellas 2002). After studying the effects of trace metals on different developmental stages of *Ciona intestinalis* separately (sperm viability, fertilization, embryonic development, and larval attachment), in the present work we ran toxicity tests to study the toxicity on the whole early life cycle, exposing gametes before fertilization through larval hatching and larval attachment. Thus, we can observe if metals caused damage to the gametes that, although not inhibiting fertilization, could become apparent on the offspring. Kobayashi (1981, 1995) reported inducible damage of trace metals and organic compounds to sea urchin gametes that can affect the embryonic and larval stages, and proposed the use of "aged gametes" (pretreated eggs and sperm) for marine pollution bioassays. Also, it has been found that metals induced

Table 3. Median effective concentrations (EC_{50}, μg/L and μM) and 95% confidence intervals (in brackets) of tested metals for the biological responses tested

	Cd	Cr	Cu	Hg
Embryonic development				
μg/L	721 (692–751)	12772 (11131–12465)	36.6 (34.1–39.3)	44.7 (40.7–49.5)
μM	6.42 (6.16–6.68)	226 (214–238)	0.58 (0.54–0.62)	0.22 (0.20–0.25)
Larval attachment				
μg/L	752 (699–806)	15026 (14411–15671)	67.8 (65.6–70.2)	78.1 (76.3–80.1)
μM	6.76 (6.22–7.17)	289 (277–301)	1.07 (1.03–1.11)	0.39 (0.38–0.40)

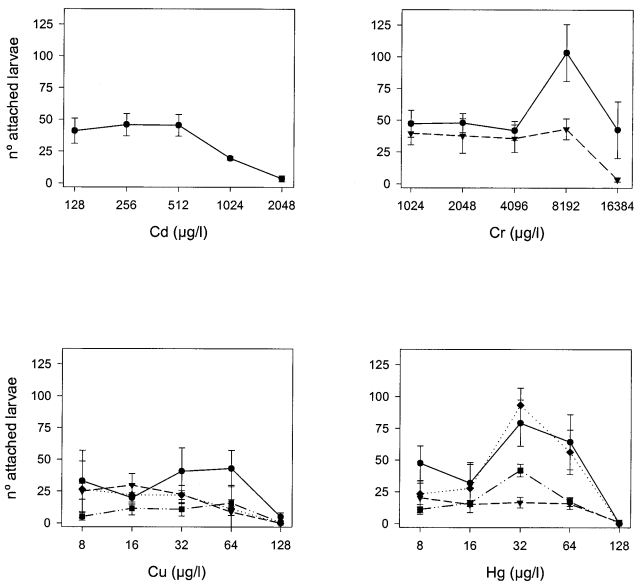

Fig. 2. Number of attached larvae of *Ciona intestinalis* after 70 h exposure of gametes throughout their development to the larval attachment to different concentrations of dissolved Cd, Cr, Cu, and Hg. Symbols indicate different experiments. Error bars represent the standard deviation for each treatment ($n = 5$).

a transmissible damage to the sperm of the bivalve *Mytilus galloprovincialis*, giving rise to malformations and embryonic and larval mortality without affecting the fertilization ability (Pagano *et al.* 1996). In contrast with those works, Warnau *et al.* (1996) did not find transmissible damage to gametes of the sea urchin *Paracentrotus lividus* exposed to Hg, Cu, Cd, and Ag.

Exposure of gametes of *Ciona intestinalis* to Cd, Cr, Cu, and Hg and the study of the effects of these metals on the fertilization and subsequent viability of embryos resulted in EC_{50} values similar to those obtained for the embryonic development of eggs fertilized in absence of metals and exposed at two-cell stage (793 μg/L [7.0 μM] for Cd, 10590 μg/L [204 μM] for Cr, 54.2 μg/L [0.853 μM] for Cu, and 50.2 μg/L [0.250 μM] for Hg), except for Cu, where higher toxicity was observed (Bellas 2002). Thus, exposure of gametes to Cd, Cr, and Hg before fertilization did not increase the sensitivity of the embryos to the trace metals tested. However, preexposure of gametes to Cu resulted in more pronounced toxic effects, which is in agreement with the decrease of the fertilization rate of *Ciona intestinalis* gametes exposed to Cu that was observed in our fertilization experiments (Bellas 2002).

Attachment and metamorphosis of ascidian and other marine invertebrate larvae, such as bivalves or bryozoans, has been already tested as a response to the effects of trace metal exposure (reviewed by Pawlik 1992). When we compare the effects of Cd, Cr, Cu, and Hg on the gametes, subsequent viability of embryos, and larval attachment, we observe that the EC_{50} values for Cd and Cr are lower or similar to those obtained when we exposed hatched larvae to the metals. However, Cu and Hg showed higher EC_{50} values when exposing gametes at different exposure periods. This effect may be due to the fact that abnormal larvae with functional adhesive papillae can attach and metamorphose. The number of attached larvae was significantly greater at 8192 μg/L for Cr and at 32 μg/L for Hg than in controls. Enhancement of a biological response at low concentrations of toxicants (hormesis) has been reported previously in the 1930s for the settlement of larvae of the bivalve *Ostrea virginica* in Cu-polluted water (Prytherch 1931), and the induction of early metamorphosis of ascidian *Polyandrocarpa tincta* and *P. gravei* larvae, at 64, 5600, and 2700 μg/L of Cu, Fe, and Al, respectively (Grave and Nicoll 1939). Later on, those positive effects have been observed in other marine invertebrates, for the attachment of bryozoan larvae exposed to Cu and Hg antifouling paints (Wisely 1963), and more recently on the metamorphosis of larvae of the oyster *Crassostrea gigas* exposed to 1 μg/L of Hg (Beiras and His 1994), the embryonic development of *Mytilus edulis* embryos exposed to 8 μg/L of Cu (Hoare *et al.* 1995), and for the embryos of *Paracentrotus lividus* exposed to 5 μg/L of Fe (Pagano *et al.* 1996). Thus, metal cations may be putative inductors triggering larval attachment. Alternatively, this effect might not be due to an induction of attachment or metamorphosis, but a sublethal toxic response of larvae to trace metals that consists of ceasing swimming, lying at the bottom, and subsequently causing the settlement (Dubilier 1988; Pawlik 1992).

It has also been suggested that adhesive papillae of ascidian larvae can have a chemoreception function (Cloney 1978; Torrence and Cloney 1988), thus *Ciona intestinalis* larvae could detect trace metals in the water and delay or inhibit the attachment. Although there is little information in the literature concerning the ability of larvae to detect trace metals in aqueous solution, Wisely and Blick (1967) proposed a repellent

effect of Hg and Cu on larvae of the bivalves *Crassostrea commercialis* and *Mytilus edulis* that caused the larvae to withdraw into their shells.

Kayser (1982) found that embryos and larvae of the ascidians *Ciona intestinalis* and *Ascidiella aspersa* developed and metamorphosed at concentrations of 5000 μg/L of Cd, but not at 10000 μg/L. This great difference with our experiments, ten times more resistant, can be due to differences in methodology, such as physicochemical parameters of incubation (temperature, salinity, pH), using natural filtered seawater that can give rise to decreasing metal concentration by complexation with organic constituents of marine water, or sorption of metals onto walls of glass vials.

Since all solutions were prepared from ASW, which is recommended for toxicity tests to avoid the presence of organic ligands that can reduce toxicity by complexation (Calabrese *et al.* 1973), complexation with organic matter can be considered negligible in our experiments. Although metal concentrations were not measured in the experimental solutions, in our previous work with trace metals we obtained actual concentrations in the experimental vessels that ranged 94–120% of the nominal concentrations (Bellas *et al.* 2001). Therefore, differences between nominal and actual concentrations would have been kept to a minimum.

Once the EC_{50} is expressed in molarity units, the four metals tested can be ranked from highest to lowest toxicity on early life stages of development of *Ciona intestinalis* in the order Hg > Cu ≫ Cd ≫ Cr, in agreement with previous data for early developmental stages of ascidians, bivalves, and sea urchins. It is remarkable that the classification of trace metals based on their toxicity on marine animals is the same as the classification based on the affinity of metals for sulfhydryl groups of metabolites and enzymes (Viarengo 1989; Warnau *et al.* 1996; Rainbow 1997), which could explain the high toxicity of Hg and Cu, since they are the trace metals with higher affinity for -SH groups (Viarengo 1989). This finding suggests that the induced toxic effects of these metals in *Ciona intestinalis* early stages are in part the result of a protein impairment. Our results are in agreement with data obtained before by many researchers for embryos and larvae of marine invertebrates commonly used in bioassays, as bivalves (*e.g.*, Calabrese *et al.* 1973; MacInnes and Calabrese 1978; Martin *et al.* 1981), and echinoids (*e.g.*, Waterman 1937; Kobayashi 1981; Dinnel *et al.* 1989; Warnau *et al.* 1996). Although toxicity tests done with embryos and larvae of crustacean have produced similar results, it has often been found that Cd is more toxic than Cu (Corner and Sparrow 1956; Ramachandran *et al.* 1997; Itow *et al.* 1998a, 1998b; Mariño-Balsa *et al.* 2000). Likewise, our data are in agreement with literature on fish ecotoxicology (reviewed by Von Westernhagen 1988).

In conclusion, our results suggest that environmentally realistic concentrations of trace metals, Cd, Cr, Cu, and Hg, showed toxicity on *Ciona intestinalis* early stages and could be a menace for populations in certain polluted coastal ecosystems. Based on these results and using a protection factor of 100 (Länge *et al.* 1998), maximum permissible concentrations for the protection of those organisms would be: 2 nM (0.5 μg/L) for Hg, 6 nM (0.4 μg/L) for Cu, 64 nM (7 μg/L) for Cd, and 2 μM (128 μg/L) for Cr.

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