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Toxicity of Organic Compounds to Marine Invertebrate Embryos and Larvae: A Comparison Between the Sea Urchin Embryogenesis Bioassay and Alternative Test Species

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Abstract. This study investigated the toxic effects of the insecticides lindane and chlorpyrifos, the herbicide diuron, the organometallic antifoulant tributyltin (TBT), and the surfactant sodium dodecyl sulfate (SDS) on the early life stages of *Paracentrotus lividus* (Echinodermata, Euechinoidea), *Ciona intestinalis* (Chordata, Ascidiacea), *Maja squinado* and *Palaemon serratus* (Arthropoda, Crustacea) in laboratory acute toxicity tests. The assays studied embryogenesis success from fertilized egg to normal larvae in *P. lividus* (48 h incubation at 20 °C) and *C. intestinalis* (24 h incubation at 20 °C), and larval mortality at 24 and 48 h in *M. squinado* and *P. serratus*. For *P. lividus*, the median effective concentrations (EC_{50}) reducing percentages of normal larvae by 50% were: 350 μg l^{-1} for chlorpyrifos, 5500 μg l^{-1} for diuron, 4277 μg l^{-1} for SDS, and 0.309 μg l^{-1} for TBT. For *C. intestinalis*, the EC_{50} values affecting embryogenesis success were 5666 μg l^{-1} for chlorpyrifos, 24,397 μg l^{-1} for diuron, 4412 μg l^{-1} for lindane, 5145 μg l^{-1} for SDS, and 7.1 μg l^{-1} for TBT. The median lethal concentrations (LC_{50}) for *M. squinado* larval survival were 0.84 μg l^{-1} (24 h) and 0.79 μg l^{-1} (48 h) for chlorpyrifos, 2.23 μg l^{-1} (24 h) and 2.18 μg l^{-1} (48 h) for lindane, and 687 μg l^{-1} (48 h) for SDS. For *P. serratus* the LC_{50} values obtained were 0.35 μg l^{-1} (24 h) and 0.22 μg l^{-1} (48 h) for chlorpyrifos, 3011 μg l^{-1} (24 h) and 3044 μg l^{-1} (48 h) for diuron, 5.20 μg l^{-1} (24 h) and 5.59 μg l^{-1} (48 h) for lindane, and 22.30 μg l^{-1} (24 h) and 17.52 μg l^{-1} (48 h) for TBT. Decapod larvae, as expected, were markedly more sensitive to the insecticides than sea urchins and ascidians, and SDS was the least toxic compound tested for these organisms. Lowest observed effect concentrations (LOEC) of TBT for sea urchin and ascidian embryos, chlorpyrifos and lindane for crustacean larvae, and SDS, were similar to those found in many coastal areas indicating that there would be a risk to invertebrate embryos and larvae from exposure in the field to these pollutants.

Keywords: ecotoxicology; embryos; larvae; marine invertebrates; organic compounds

Introduction

Over the last few decades, the effects of industrialization, intensive agriculture, and urban development have lead to the occurrence of serious pollution problems in the marine ecosystems

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(McIntyre, 1992; Goldberg, 1995; Luoma, 1996). For example, the anthropogenic input of synthetic organic compounds is a problem of major concern. Insecticides, herbicides, and other industrial compounds such as surfactants and organometals can be broadly detected in the water, the sediments, and the biota, although annually used reported volumes of these compounds vary. Tilman et al. (2001) calculated the global production of pesticides to be 3.7×10^6 tons, and the flux of some organochlorines to the ocean has been estimated in 4754 tons y^{-1} for lindane, 239 for polychlorinated biphenyls (PCB's), 165 for dichlorodiphenyltrichloroethane (DDT), and 77 for hexachlorobencene (HCB) (GESAMP, 1989). The consumption of organotin compounds was 30×10^3 tons y^{-1} in the middle 80s (WHO, 1990), whereas the use of surfactants in 1989 was estimated to be 15×10^6 tons (Berth and Jeschke, 1989; Lewis, 1990).

Four pesticides were tested in the present work: chlorpyrifos, diuron, lindane and tributyltin (TBT). These substances are introduced in the marine environment constituting a threat to non-target marine species. Chlorpyrifos, diuron and lindane are pesticides with a broad use in agriculture and domestic labours, and they are introduced in coastal areas by spray drift, surface runoff or accidental spills (His and Seaman, 1993; Key and Fulton, 1993; Foster et al., 1998; Breivik et al., 1999; Carabias Martínez et al., 2000). Both diuron, an urea derived herbicide, and lindane, an organochlorine insecticide, are persistent pesticides (half life up to 6 months), chlorpyrifos is an organophosphate less persistent (half life less than 2 weeks) (Kennish, 1997). Diuron herbicidal action is based on the inhibition of Photosystem-II (Liu, 2001). Lindane inhibits neurotransmitters function in vertebrates (Brooks, 2001), and chlorpyrifos causes the inhibition of acetylcholinesterase, thus damaging the transmission of the nervous impulse of the central nervous system (Timchalk, 2001). Therefore, insecticides are not expected to be highly toxic to sea urchins, as compared to crustaceans, with a more complex nervous system. The TBT is an organometallic compound used in antifouling paints to prevent the attachment of marine organisms to the hulls of ships and other immersed surfaces. TBT has several mechanisms of toxicity, inhibiting ATP-ase activity in mitochondria, and other ATP-ases such as the Na^+/K^+ ATP-ase and Ca^{2+} ATP-ase (Cima et al., 1996a, b, 1998; Hollingworth, 2001). We have also conducted toxicity tests with a surfactant, sodium dodecyl sulfate (SDS), which is used nowadays for a variety of purposes, and is commonly used as a reference toxicant in ecotoxicological studies (Cardwell et al., 1977; Lewis and Suprenant 1983; Whiting et al., 1996).

The importance of marine invertebrates in the functioning of marine ecosystems has lead to their use as test species in biological assays; however, few invertebrate species have been used in toxicity studies. Furthermore, little work has been done on the comparison of sensitivity of different species to pollutants, relying hazard assessment of pollutants on single-species tests that can not detect the full range of pollutants entering the marine environment (Fliedner and Klein, 1996).

The embryonic and larval stages of marine invertebrates are less tolerant to toxicants than adults (e.g. Wisely and Blick, 1967; Connor, 1972; Dinnel et al., 1989; Marin et al., 1991; Ringwood, 1991, 1992) and have been used for assessing the biological quality of marine water and sediments. Although the impact of organic pesticides and detergents has been well documented, data on the effects of these compounds on marine early life stages of development are scarce in contrast with freshwater toxicology based on the use of *Daphnia* (Hutchinson et al., 1998; Leung et al., 2001). This toxicological information is needed to assess the degree of environmental risk posed by those pollutants, and to implement seawater quality standards protective for these marine organisms. We have done toxicity tests of selected organic compounds with embryos and larvae of four marine invertebrates, an echinoid (*Paracentrotus lividus*), an ascidian (*Ciona intestinalis*), and two crustacean species (*Maja squinado* and *Palaemon serratus*). These species were chosen due to their abundance, ecological importance, and commercial relevance.

The aim of the study reported here was to determine the toxic effects of representative organic compounds in order to provide biological criteria for the implementation of water quality standards to protect these organisms, and compare the sensitivity of the broadly used sea urchins with alternative species of marine invertebrates.

Materials and methods

Biological material

Ciona intestinalis and *Paracentrotus lividus* were collected in pristine sites from local populations in Ría de Vigo (Galicia, NW Spain) and were transferred into aquaria until the experiments. Mature females of *Maja squinado* and *Palaemon serratus* were purchased at the local market in Ría de Arousa (Galicia, NW Spain), transported to the laboratory and maintained in aquaria. The methods for maintenance of adults in laboratory and larval rearing are described elsewhere (Mariño-Balsa et al., 2000).

Experimental solutions

Stock solutions were made up by dissolving analytical grade chlorpyrifos (Chem Service), diuron (Aldrich Steinhem), lindane (Merck Schucdart) and TBT (Aldrich Steinheim) in a non-toxic organic dissolvent, DMSO (dimetylsulfoxide) approximately 4 h before the beginning of the experiments. The toxicity of the organic solvent DMSO on the embryonic development of *Ciona intestinalis* and *Paracentrotus lividus*, and on larvae of *Maja squinado* was previously investigated. According to these experiments we chose the concentration of DMSO to use in the stock solution, taking into account the toxicity threshold of the test species. SDS stock solutions were made up from analytical grade SDS (Merck Darmstadt) in ultra pure (Milli-Q) water to produce a concentration 10-fold the highest used. Experimental concentrations were chosen on the basis of preliminary trials and on data from the literature (e.g. Lewis and Suprenant, 1983; Bryan and Gibbs, 1991; Hill and Nelson, 1992; His and Seaman, 1993; Key and Fulton, 1993; Mansueto et al., 1993b; Whiting et al., 1996; Foster et al., 1998; Hartgers et al., 1999). The experimental concentrations were obtained by diluting the stock solution in artificial seawater (ASW). The ASW was prepared as in Zaroogian et al. (1969) but salinity was adjusted to 34 ppt adding ultra pure (Milli-Q) water.

In each experiment with pesticides ASW and the highest DMSO concentration used to prepare the pesticides solutions were used as controls. For the ascidians the 10% dilution of the nominal concentrations caused by adding 2 ml of egg suspension in 20 ml of experimental solution was taken into account.

Incubations were made in 25 ml glass vials with airtight teflon caps to avoid losses of the organic compounds. Five replicates of each experimental concentration, five controls with ASW and five controls with DMSO solutions were tested. All glassware was acid-washed (HNO_3 10% vol.) and rinsed with acetone and ultra pure water before the experiments.

Physico-chemical conditions of the experiments were 33.51 $\pm$ 0.37 ppt salinity, 6.33 $\pm$ 0.87 mg l^{-1} O_2 and 7.93 $\pm$ 0.16 pH (mean $\pm$ std, $n = 35$).

Paracentrotus lividus *embryonic development and larval length*

To test the effects of the toxic compounds on the embryonic development and larval length of *P. lividus* 600 fertilized eggs were added to each vial containing the experimental solutions. The vials were incubated at 20 °C until larvae reached four-arms pluteus stage (approximately 48 h after fertilization) (Fernández and Beiras, 2001). Larvae were then preserved by adding a few drops of 40% buffered formalin and the percentage of fully developed 4-arm pluteus larvae ($n = 100$) and the mean larval length ($n = 25$) were recorded.

Ciona intestinalis *embryonic development*

Embryos (2 ml, ca.150 embryos ml^{-1}) at 2-cell stage obtained by *in vitro* fertilization following the methods of Bellas et al. 2001 were delivered into experimental vials containing 20 ml of the studied compounds. These vials were incubated in a culture chamber at 20 °C for 20 h. After incubation a few drops of formalin were added and the percentage of normal hatched larvae ($n = 100$) was recorded.

Maja squinado *and* Palaemon serratus *larval survival*

The toxicity tests were performed with the zoea I stage of the spider crab (*Maja squinado*) and zoea I of the common prawn (*Palaemon serratus*). Ten

Table 1. Chlorpyrifos, diuron, lindane, SDS, TBT, and DMSO NOEC's and LOEC's for the species studied

	Lindane	TBT	Chlorpyrifos	SDS	Diuron	DMSO (ml l^{-1})
P. lividus						
NOEC	–	0.1	50	–	–	8
LOEC	750	0.2	100	400	3200	10
C. intestinalis						
NOEC	1600	2	1600	3200	6400	6.4
LOEC	3200	4	3200	6400	12,800	12.8
M. squinado						
24 h						
NOEC	0.8	n.t.	0.5	1000	n.t.	10,000
LOEC	4.0		2.5	–		–
48 h						
NOEC	0.8		0.5	100		10,000
LOEC	4.0		2.5	1000		–
P. serratus						
24 h						
NOEC	0.1	12.5	0.5	n.t.	1000	n.t.
LOEC	0.5	62.5	1.0		10,000	
48 h						
NOEC	0.1	12.5	0.1		1000	
LOEC	0.5	62.5	0.5		10,000	

Concentrations are given in ml l^{-1} for DMSO and in μg l^{-1} otherwise. n.t. not tested.

active larvae were transferred by pipette into each vial. The toxicity tests were conducted at 18 °C and a daily cycle of 14 h light: 10 h dark. Number of dead larvae was recorded at 24 and 48 h.

Statistical analysis

We have established dose–response relationships for tested compounds to calculate the EC_{50} and LC_{50}, defined here as the toxicant concentrations causing 50% reduction in the embryogenesis success and the toxicant concentration reducing percentages of larval survival by 50%. EC_{50} and their 95% confidence intervals were calculated according to the probit method for *Ciona intestinalis* and *Paracentrotus lividus* larvae, and the Litchfield–Wilcoxon method was employed to calculate LC_{50} for *Maja squinado* and *Palaemon serratus* data. The Lowest Observed Effect Concentrations (LOEC) and No Observed Effect Concentrations (NOEC) were determined by ANOVA and Tukey's test. When data did not meet the assumptions of normality and homocedasticity non-parametric Kruskall–Wallis test and Games–Howell test were employed to compare individual treatments. Previously to performing EC_{50} calculations, data were normalized to the control mean percentage of larval abnormality (larval mortality for *M. squinado* and *P. serratus*) 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. For analysis, data were firstly arcsine-transformed to achieve normality (Hayes, 1991). Statistical tests were performed according to Newman (1995) and Sokal and Rohlf (1995).

Results

Carrier (DMSO) experiments

DMSO showed toxicity on the embryonic development of *Paracentrotus lividus* and *Ciona intestinalis* only at concentrations above 8 and 6.4 ml l^{-1}, respectively (Table 1, Figure 1a,b), and the LOEC obtained for these species were 10 and 12.8 ml l^{-1}. The DMSO concentration chosen to

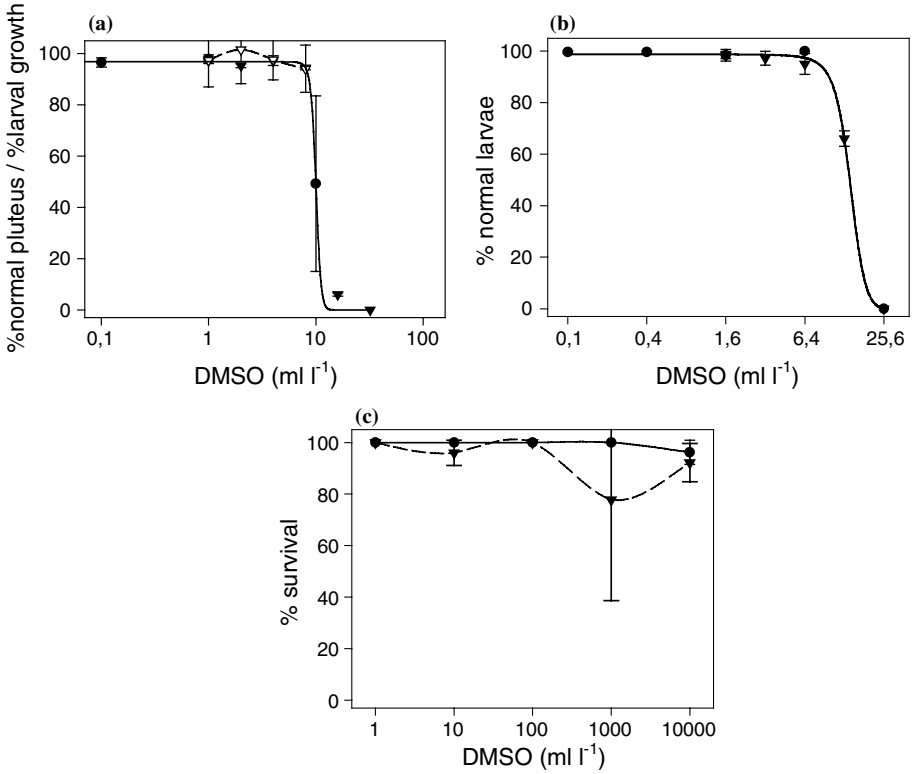

Figure 1. Effects of DMSO on the embryonic development (filled symbols) and larval growth (open symbols) of *Paracentrotus lividus* (a), on the embryonic development of *Ciona intestinalis* (b), and on the survival rate of *Maja squinado* larvae (c). Biological responses are expressed as mean percentage compared to the controls. (a,b) Different symbols indicate experiments carried out with different batches of embryos. (c) Circle: survival at 24 h; triangle: survival at 48 h. Error bars represent standard deviations.

make up the experimental solutions was 2 ml l^{-1}, and each pesticide experiment included a 2 ml l^{-1} DMSO-control. These controls did not show significant differences with ASW controls. Since DMSO did not affect mortality of *Maja squinado* larvae at concentrations below 1000 ml l^{-1} (Table 1, Figure 1c) we prepared the pesticide solutions using also 2 ml l^{-1} DMSO.

Paracentrotus lividus *embryonic development and larval length*

Lindane had no toxic effects on the *Paracentrotus lividus* embryonic development at concentrations up to 90 mg l^{-1}, whereas TBT, chlorpyrifos, SDS and diuron significantly inhibited *P. lividus* embryogenesis (Table 1 and Figure 2). Tributyltin showed the highest toxicity, inhibiting completely the embryonic development at 0.4 μg l^{-1}. The effects of chlorpyrifos were not significant at concentrations below 300 μg l^{-1} but increased at higher values reaching 100% abnormalities at 400 μg l^{-1}, and yielding an EC_{50} of 350 μg l^{-1} (Table 2). Comparison of EC_{50} in molar units showed that TBT was approximately 900 times more toxic than chlorpyrifos, 15,000 more than SDS and 25,000 more than diuron (Table 2 and Figure 3).

Although lindane showed no toxic effects on the embryonic development at the concentrations tested, a dose–response relationship was found for larval length. A concentration of 10 mg l^{-1} caused a larval length reduction of 50% and the LOEC inhibiting larval growth was 14 times lower (Table 1, Figure 2). TBT reduced larval length by 25% at 0.2 μg l^{-1} and by 40% at 0.3 μg l^{-1}. For chlorpyrifos, an inhibition of 25% and 45% was observed at 200 and 300 μg l^{-1}, while diuron had a significant effect on *P. lividus* larval length only at concentrations above 3200 μg l^{-1}, causing a 50% reduction at concentrations above 6000 μg l^{-1}.

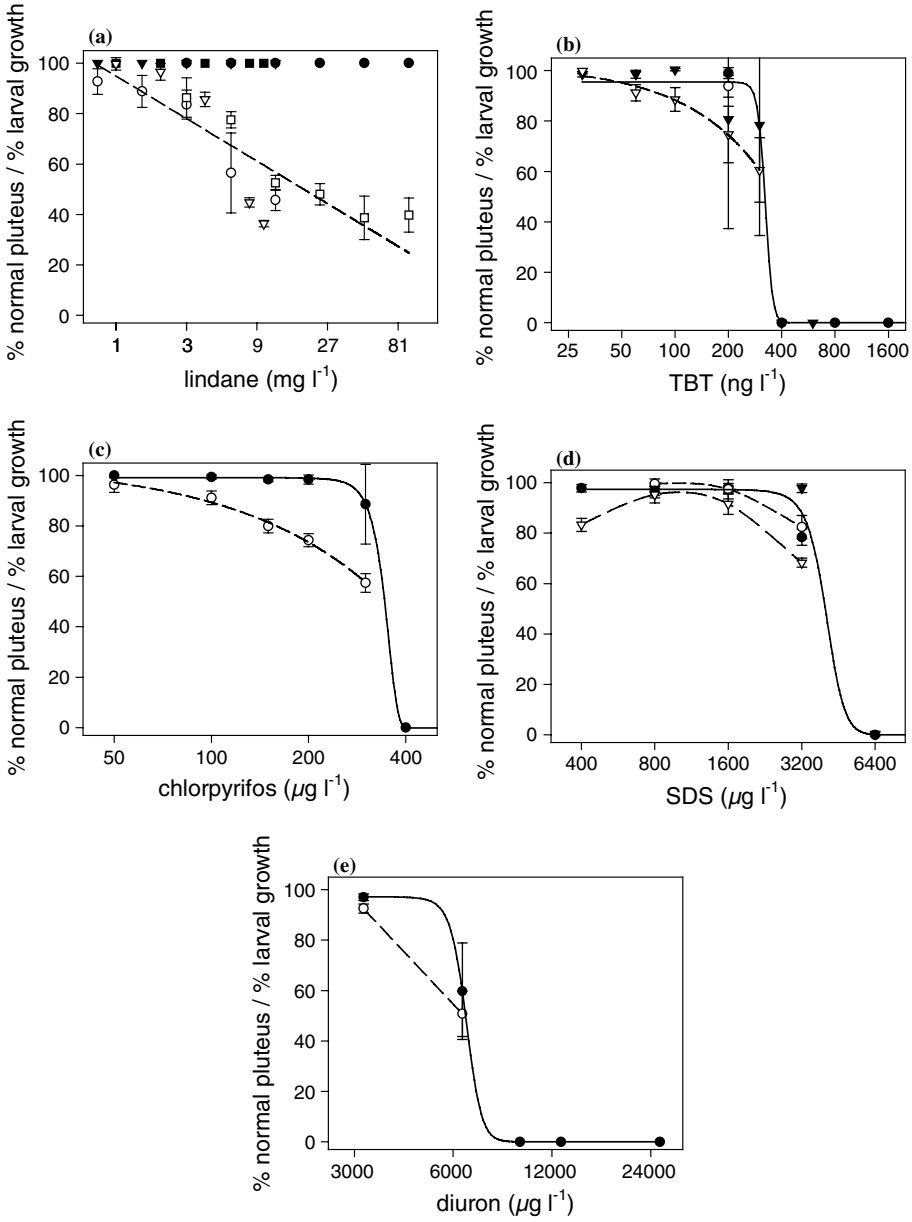

Figure 2. Percentage of normal 4-arm pluteus larvae (filled symbols) and larval growth (open symbols) obtained after 48 h exposure of *Paracentrotus lividus* fertilized eggs to different concentrations of lindane (a), TBT (b), chlorpyrifos (c), diuron (d), and SDS (e). Different symbols indicate experiments carried out with different batches of embryos. Error bars represent standard deviations.

Ciona intestinalis *embryonic development*

The organic compounds caused a significant decrease in the number of *Ciona intestinalis* hatched larvae within the range of concentrations tested (Table 1 and Figure 4). TBT was the most toxic substance for *C. intestinalis* embryos with a LOEC of 4 μg l^{-1} (Figure 4b), and impaired the embryonic development at concentrations above 16 μg l^{-1}. The EC_{50} values and their 95%

Table 2. Median effective concentrations (EC_{50}, μg l^{-1} and μM) and median lethal concentrations (LC_{50}, μg l^{-1} and μM) for the organic compounds tested

	Lindane	TBT	Chlorpyrifos	SDS	Diuron
P. lividus					
EC_{50} (μg l^{-1})	>91,000	0.309	300	4100	5600
	–	(0.2730–0.3627)	(350–400)	(3750–4580)	(5400–5700)
(μM)	>313.5	0.00096	0.83	14.2	23.9
	–	(0.000846–0.00112)	(0.969–1.108)	(12.97–15.84)	(23.11–24.39)
C. intestinalis					
EC_{50} (μg l^{-1})	4412	7.1	5666	5145	24,397
	(4082–4766)	(6.01–8.51)	(5087–6349)	(4939–5367)	(21,959–27,539)
(μM)	15.2	0.022	15.7	17.8	104
	(14.03–16.39)	(0.0185–0.0262)	(14.10–17.61)	(17.13–18.61)	(93.9–117.8)
M. squinado					
24 h					
EC_{50} (μg l^{-1})	2.23	n.t.	0.84	n.c.	n.t.
	(1.56–3.20)		(0.56–1.25)		
(μM)	0.0077		0.0023		
	(0.00537–0.01102)		(0.00155–0.00346)		
48 h					
EC_{50} (μg l^{-1})	2.18	n.t.	0.79	687	n.t.
	(1.53–3.12)		(0.52–1.19)	(566.6–834.2)	
(μM)	0.0075		0.0022	2.38	
	(0.00527–0.01074)		(0.00144–0.00329)	(1.960–2.886)	
P. serratus					
24 h					
EC_{50} (μg l^{-1})	5.20	22.30	0.35	n.t.	3011
	(4.94–5.48)	n.c.	(0.27–0.46)		(2805–3231)
(μM)	0.018	0.069	0.00097		12.8
	(0.0170–0.0189)	n.c.	(0.000748–0.001275)		(11.97–13.79)
48 h					
EC_{50} (μg l^{-1})	5.59	17.52	0.22	n.t.	3044
	(5.33–5.87)	n.c.	(0.16–0.29)		(2837–3265)
(μM)	0.019	0.054	0.00062		12.9
	(0.0184–0.0202)	n.c.	(0.000443–0.000804)		(12.11–13.93)

The 95% confidence intervals (95CI) are given in brackets. n.c. not calculated. n.t. not tested.

confidence intervals (95 CI) are shown in Table 2 after conversion into molar units for comparison. TBT was 700 times more toxic than lindane and chlorpyrifos, and 800 times more than SDS. Diuron was the least toxic of the compounds tested with an EC_{50} 4700 times lower than that of TBT (Figure 3).

Maja squinado *and* Palaemon serratus *larval survival*

Chlorpyrifos was the most toxic compound tested on *Maja squinado* larvae with a LC_{50} = 0.84 μg l^{-1} at 24 h and 0.79 at 48 h (Table 2 and Figure 5b). No significant effects were detected at 0.1 μg l^{-1}, but larval mortality strongly increased at higher concentrations being 100% at 2.5 μg l^{-1}. Lindane showed 3 times less toxicity in molar units, with a LC_{50} value of 2.23 μg l^{-1} at 24 h and 2.18 μg l^{-1} at 48 h, and a less severe dose–response relationship compared to chlorpyrifos. SDS was much less toxic to *M. squinado* larvae than pesticides, with a LC_{50} of 687 μg l^{-1} following a 48 h exposure (Figures 3 and 5c). The LC_{50} of chlorpyrifos for *Palaemon serratus* larvae was 0.35 μg l^{-1} after 24 h exposure and 0.22 μg l^{-1} at 48 h (Table 2), whereas lindane was 11 times less toxic with 24 and 48 h-LC_{50} values of 5.20 and 5.59 μg l^{-1}, respectively. TBT showed LC_{50} values of 22.30 μg l^{-1} (24 h) and 17.52 μg l^{-1} (48 h) and diuron was the less toxic

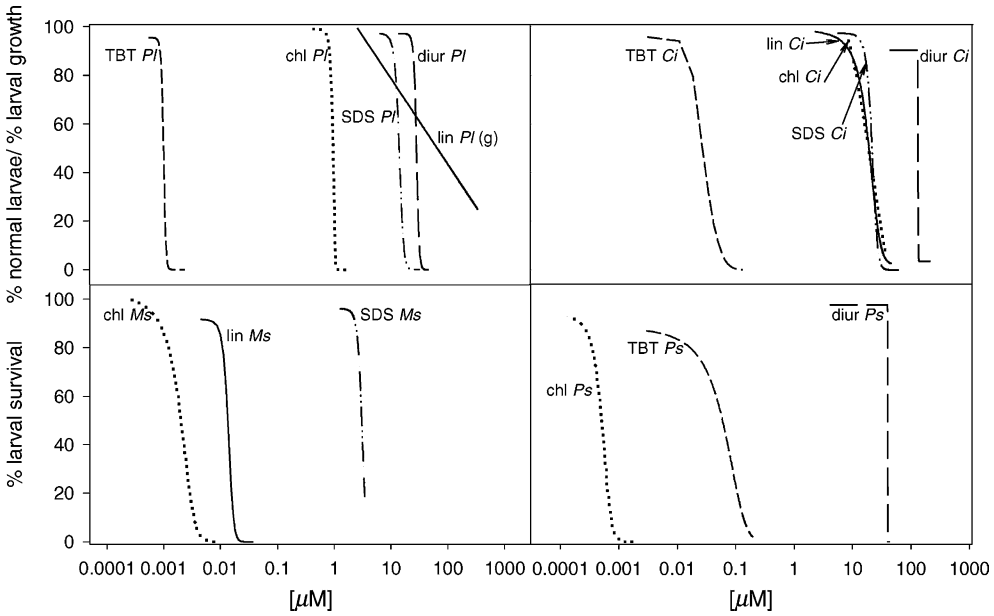

Figure 3. Dose–response curves of chlorpyrifos (chl), diuron (diur), lindane (lind), SDS, and TBT, for *Paracentrotus lividus* (*Pl*), *Ciona intestinalis* (*Ci*), *Maja squinado* (*Ms*), and *Palaemon serratus* (*Ps*).

compound tested for *P. serratus* larvae with LC_{50} values of 3011 μg l^{-1} (24 h) and 3044 μg l^{-1} (48 h) (Figures 3 and 6).

Discussion

Based on EC_{50} values from the present study, TBT was the most toxic compound to the early developmental stages of *Paracentrotus lividus* and *Ciona intestinalis*, followed in decreasing order of toxicity by chlorpyrifos, lindane, and SDS, while diuron was the least toxic. Despite the methodological variability among experiments, this order of toxicity is consistent with previous toxicity data reported for bivalve and sea urchin species (Kobayashi, 1995; His et al., 1999a, b). For larvae of sessile organisms, TBT has been usually found more toxic than insecticides, and these more toxic than surfactants. However, crustacean larvae yielded in this study different results, since the highest toxicity was found for chlorpyrifos, followed by lindane, TBT, SDS and diuron. Those data are in agreement with literature of crustacean ecotoxicology (Ramamoorthy and Baddaloo, 1995).

It has been well established that marine organisms are affected by low environmental concentrations of TBT. Shell malformations were found at sublethal concentrations (less than 10 ng l^{-1}) in the oyster *Crassostrea gigas* and the mussel *Mytilus edulis* (reviewed by Bryan and Gibbs, 1991). Copepods, echinoderms, polychaetes and tunicates are affected at TBT concentrations in the 10–100 ng l^{-1} range, whereas decapod crustaceans and fishes are relatively resistant to this biocide (Bryan and Gibbs, 1991). However, those data mainly correspond to adult stage animals and chronic exposure conditions. Reported effects of TBT in ascidian and sea urchin embryos and larvae include inhibition of the oxidative phosphorylation in the mitochondria and impairing of the intracellular Ca^{+2} homeostasis (Cima et al., 1996a, b, 1998; Girard et al., 1997, 2000; Mansueto et al., 2000). Furthermore, Lignot et al. (1998) reported effects of TBT on the osmoregulatory capacity of crustacean larvae.

Kobayashi (1995) reviewed the literature on sea urchin ecotoxicology and found EC_{50} values of TBT below 1.8 μg l^{-1} for the embryonic development. In our study, concentrations of TBT that inhibit *Paracentrotus lividus* embryonic

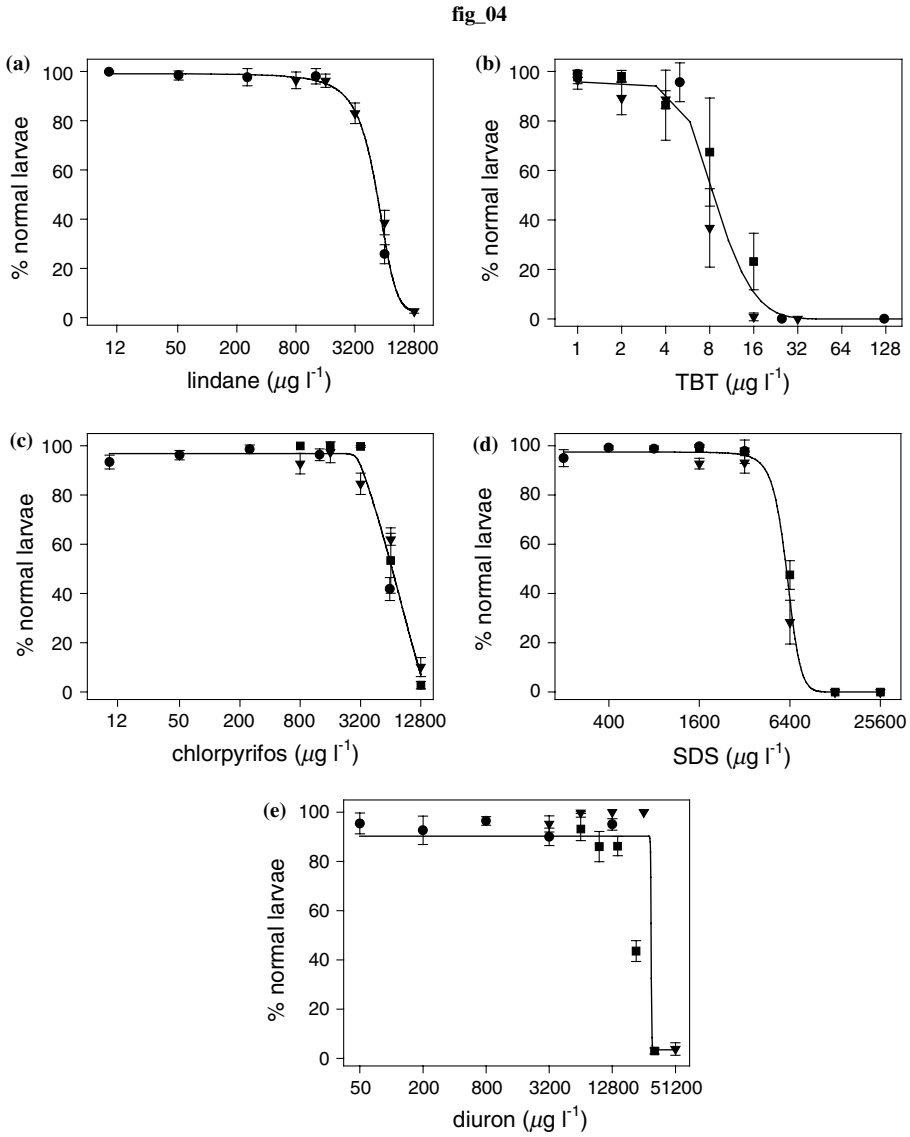

Figure 4. Percentage of *Ciona intestinalis* normal tadpole larvae obtained after 20 h exposure of 2-cell embryos to different concentrations of lindane (a), TBT (b), chlorpyrifos (c), diuron (d), and SDS (e). Different symbols indicate experiments carried out with different batches of embryos. Error bars represent standard deviations.

development are in agreement with those found in other studies with similar exposure times (Table 3). Moreover, Girard et al. (2000) obtained toxic effects on larval survival and growth of *P. lividus* larvae after exposure of eggs to 0.03–0.3 μg l^{-1} of TBT, suggesting that concentrations of TBT with no effect on the embryonic development could affect the sea urchin larval cycle and metamorphosis. Organoestanic compounds like TBT have been found to damage the embryonic and larval development of *Ciona intestinalis* in the 0.1–10 μM concentration range (Mansueto et al., 1993a, b; Maggio et al., 1994; Gianguzza et al., 1996; Pellerito et al., 1997, 1998; Mansueto et al., 2000). In contrast, our results showed toxic effects of TBT at concentrations of a nanomolar range (EC_{50} = 22 nM), although the incubation times in our toxicity tests are higher (20 h).

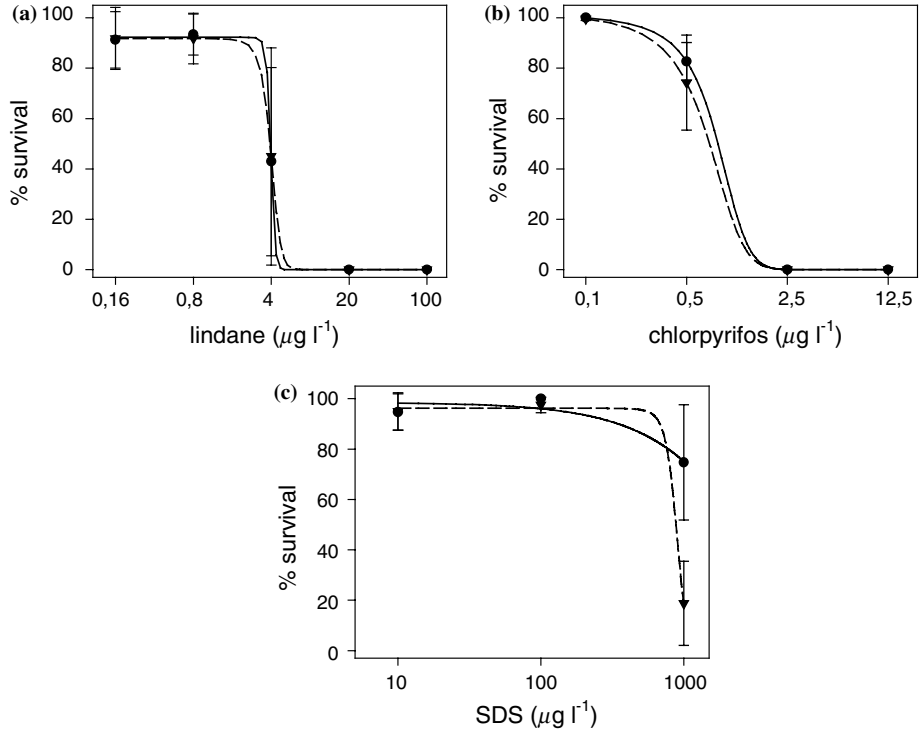

Figure 5. Survival rate (%) of newly released *Maja squinado* larvae after 24 h (circles) and 48 h (triangles) exposure to different concentrations of chlorpyrifos (a), lindane (b), and SDS (c). Error bars represent standard deviations.

Ramamoorthy and Baddaloo (1995) reported average LC_{50} of TBT of 8.2 μg l^{-1} for marine crustaceans. In the present study, we report a 48 h-LC_{50} of TBT for the zoea I stage of *Palaemon serratus* of 17.52 μg l^{-1}. Thus, in comparison with literature data for crustaceans (Table 3) *P. serratus* is among the most tolerant species to TBT.

The rise in the world production of organoestanic compounds for the last 30 years has given place to detecting concentrations of TBT of 2 μg l^{-1} in marinas and harbour areas of the United Kingdom, France, Italy, Portugal, Netherlands, United States, Canada, Australia, or Japan (Bryan and Gibbs, 1991; Waldock, 1994). Comparing these concentrations with those producing toxic effects on marine invertebrate early stages (< 0.2 μg l^{-1}, present work) we conclude that such concentrations are dangerous for early developmental stages of most marine invertebrates.

Some insecticides are selective, and act against a limited group of organisms because they affect some aspect of the specific metabolism of that group. Others are broad-spectrum, such as lindane and chlorpyrifos, affecting a broader range of organisms and they can easily pose a threat for non-target species. Both organochlorines and organophosphorous insecticides are neurotoxic agents. Crustaceans are within the most sensitive marine invertebrates to organochlorine and organophosphorous insecticides. Ramamoorthy and Baddaloo (1995) reviewed the sensitivity of crustacean marine species to different toxicants, and the average LC_{50} calculated from those data are 7.4 and 1.6 μg l^{-1} for lindane and chlorpyrifos, whereas the average LC_{50} for chlorpyrifos calculated with data from Roast et al. (1999) yielded 0.31 μg l^{-1}. We obtained high sensitivity of *Maja squinado* and *Palaemon serratus* larvae, with 24 h-LC_{50} of 2.23 and 5.20 μg l^{-1} for lindane, and 0.84 and 0.35 μg l^{-1} for chlorpyrifos. These data confirm the high toxicity of organochlorines and organophosphorous compounds on early developmental stages of crustaceans. In good agreement

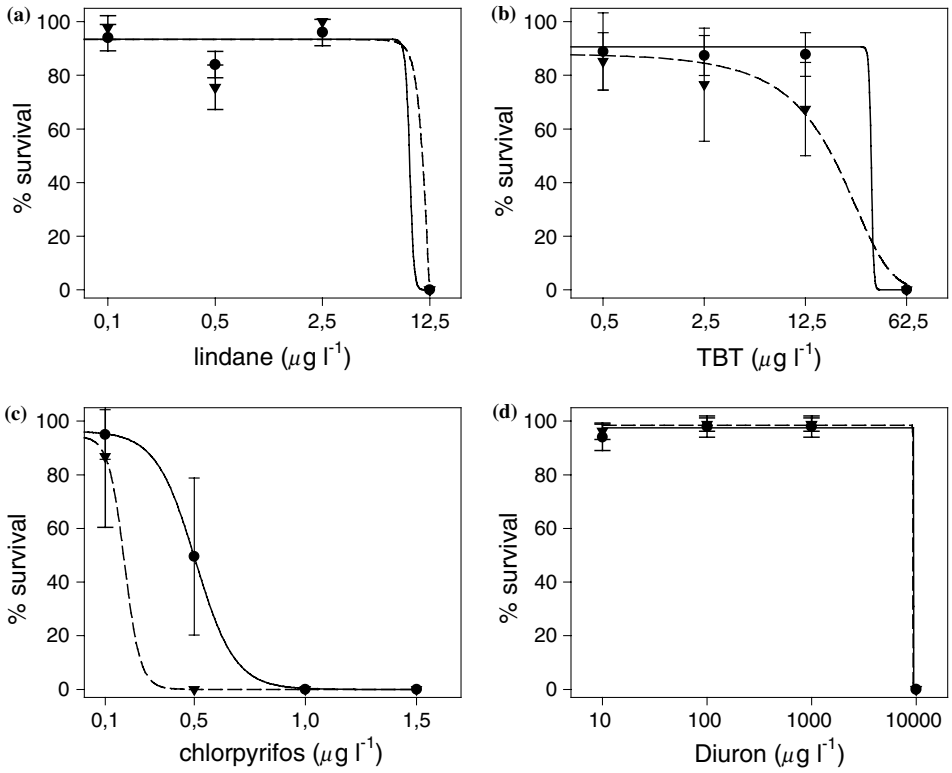

Figure 6. Survival rate (%) of newly released *Palaemon serratus* larvae after 24 h (circles) and 48 h (triangles) exposure to different concentrations of chlorpyrifos (a), lindane (b), and TBT (c). Error bars represent standard deviations.

with our results, previous studies with marine crustaceans showed a similar sensitivity to chlorpyrifos and lindane (Table 3). Therefore, *M. squinado* and *P. serratus* larvae appear to be good organisms for acute toxicity testing of organochlorine and organophosphorous pesticides since their LC_{50} are comparable with those of frequently used test species. On the other hand, EC_{50} values for lindane of 64–656 and 0.30 μg l^{-1} have been obtained for the freshwater cladocerans usually employed in ecotoxicological studies *Daphnia magna* and *Ceriodaphnia dubia* (Fliedner and Klein, 1996, Foster et al., 1998; Hartgers et al., 1999). Average LC_{50} obtained from data reviewed by Ramamoorthy and Baddaloo (1995) for freshwater crustaceans were 240 for lindane and 6.7 μg l^{-1} for chlorpyrifos, which suggests higher tolerance of freshwater than marine species to these toxicants. Some previous studies have shown higher sensitivity of marine than freshwater invertebrates to pesticides (Hutchinson et al., 1998; Leung et al., 2001), however, these comparisons were made on datasets of different taxonomic composition. Robinson (1999) also found higher sensitivity of the marine crustacean *Mysidopsis bahia* than *Daphnia* spp. to 8 pesticides.

As expected, the embryonic development of *Ciona intestinalis* and *Paracentrotus lividus* is not especially sensitive to organochlorine and organophosphorous insecticides as lindane or chlorpyrifos compared to crustaceans. To our knowledge, we report the first data on toxicity of chlorpyrifos and lindane on ascidian early stages. The EC_{50} of lindane for the embryogenesis of *C. intestinalis* was 4412 μg l^{-1}, whereas lindane had no toxic effects on the embryonic development of *P. lividus* at very high concentrations (90 mg l^{-1}), although 10 mg l^{-1} caused a 50% reduction in larval length. In contrast, chlorpyrifos was toxic to sea urchin embryos at lower values (EC_{50} = 300 μg l^{-1}) than to ascidian embryos (EC_{50} = 5666 μg l^{-1}). Lowest observed effect

Table 3. Toxicity of TBT, chlorpyrifos, lindane, diuron and SDS to marine invertebrates

Toxicants	Test species	Effect concentration ($\mu g\ l^{-1}$)		Endpoint	References
TBT	Echinoids				
	P. lividus (embryo)	16.1	2 h-LOEC	cleavage	Girard et al. (1997)
	P. lividus (embryo)	2	14 h-LOEC	DNA and echinochrome production rate	Ozretić et al. (1998)
	P. lividus (embryo)	0.5	24 h-LOEC	normal larva	Marin et al. (2000)
	P. lividus (embryo)	0.3	96 h-LOEC	survival	Girard et al. (2000)
	A. crassispina (embryo)	0.1	32 h-LOEC	normal larva	Kobayashi and Okamura (2002)
	Ascidians				
	C. intestinalis (embryo)	32	2 h-LOEC	cleavage	Gianguzza et al. (1998)
	S. plicata (embryo)	320	2 h-LOEC	cleavage	Cima et al. (1996a)
	Crustaceans				
	H. americanus (larva)	1	48 h-LOEC	survival	Laughlin and French (1980)
	G. oceanicus (larva)	0.3	96 h-LOEC	survival	Laughlin et al. (1984)
	R. harisii (larva)	13	96 h-LC_{50}	survival	Laughlin and French (1989)
	P. japonicus (larva)	1	24 h-LC_{50}	survival	Lignot et al. (1998)
	8 species of adult amphipods	1.2–23.1	48 h-LC_{50}	survival	Ohji et al. (2002)
Chlorpyrifos	Echinoids				
	S. droebachiensis (embryo)	5400	48 h-LOEC	normal larva	Buznikov et al. (2001)
	S. purpuratus (embryo)	5400	48 h-LOEC	normal larva	Buznikov et al. (2001)
	Crustaceans				
	P. pugio (adult)	0.37	96 h-LC_{50}	survival	Key and Fulton (1993)
	P. pugio (larva)	0.44	96 h-LC_{50}	survival	Key and Fulton (1993)
	P. pugio (larva)	0.37	96 h-LC_{50}	survival	Wilson (1997)
	P. pugio (embryo)	0.49	24 h-LC_{50}	cleavage	Lund et al. (2000)
	C. sapidus (embryo)	5.9	14 d-EC_{50}	hatching	Lee and Oshima (1998)
	N. integer (adult)	0.13	96 h-LC_{50}	survival	Roast et al. (1999)
	N. integer (juvenile)	0.19	96 h-LC_{50}	survival	Roast et al. (1999)
Lindane	Crustaceans				
	P. vannamei (larva)	0.19	7 d-LOEC	nuclei acid content	Galindo et al. (1996)
	G. pulex (juvenile)	6.1	14 d-LOEC	growth	Blockwell et al. (1996a)
	G. pulex (adult)	29.8	24 h-LOEC	changes in hepatopancreatic ceca	Blockwell et al. (1996b)
Diuron	Echinoids				
	A. crassispina (embryo)	1000	32 h-LOEC	normal larva	Kobayashi and Okamura (2002)
	Crustaceans				
	D. pulex (adult)	17900	96 h-EC_{50}	survival	Nebeker and Schuytema (1998)
	H. azteca (adult)	19400	96 h-EC_{50}	survival	Nebeker and Schuytema (1998)
SDS	Echinoids				
	P. lividus (embryo)	4157	24 h-EC_{50}	normal larva	Rolland et al. (1999)
	L. variegatus (embryo)	2700	24 h-EC_{50}	normal larva	Nipper et al. (1993)
	E. lucunter (embryo)	3000	36 h-EC_{50}	normal larva	Sampaio de Araújo et al. (1999)
	Crustaceans				
	C. sapidus (larva)	9800	48 h-LC_{50}	survival	Whitting et al. (1996)
	P. pugio (larva)	34000	48 h-LC_{50}	survival	Whitting et al. (1996)

concentrations of organochlorines and organophosphorous compounds reported on sea urchin embryos and larvae are within 1000 and 3000 $\mu g\ l^{-1}$ (Kobayashi, 1995), and recently, Buznikov et al. (2001) found toxic effects of chlorpyrifos on embryos of the sea urchins *Strogylocentrotus droebachiensis* and *S. purpuratus* at 15 μM (5400 $\mu g\ l^{-1}$) revealing lower levels of toxicity for this compound. In other studies EC_{50} values for bivalve embryos and larvae were above 5000 $\mu g\ l^{-1}$ for lindane, with exposure times of 48 h (His et al., 1999a). Following our own results

Table 4. Acute water quality criteria (μg l^{-1}) proposed for the species studied in the present work

	Lindane	TBT	Chlorpyrifos	SDS	Diuron
P. lividus	<750	0.003	3	41	56
C. intestinalis	40	0.07	57	51	244
M. squinado	0.02	n.d.	0.008	7	n.d.
P. serratus	0.05	0.2	0.002	n.d.	30

n.d. no data available.

and those from literature, reported concentrations of lindane and chlorpyrifos in estuarine and marine environments (Zhou et al., 1996, 2001; Lehotay et al., 1998) suggest a risk for crustacean early stages of development but not for sea urchins and ascidians.

Diuron was toxic for *Paracentrotus lividus* embryos above 6400 μg l^{-1} and for the embryonic development of *Ciona intestinalis* only at concentrations above 15000 μg l^{-1}. Literature data about diuron toxicity in aquatic invertebrates is scarce and present high variability. Kobayashi and Okamura (2002) found effects of diuron on embryos of the sea urchin *Anthocidaris crassispina* above 1000 μg l^{-1}. Long-term toxicity studies on freshwater organisms yielded EC_{50} values from 3300 to 27,100 μg l^{-1} (Nebeker and Schuytema, 1998). The average EC_{50} value of diuron for juvenile and adult stages of crustaceans was approximately 300 μg l^{-1} for marine species and 900 μg l^{-1} for freshwater species, with exposure times within 48 and 96 h (Ramamoorthy and Baddaloo, 1995) which are lower than the LC_{50} found in our experiments for *Palaemon serratus* larvae (3044 μg l^{-1}). Diuron belongs to the phenylureas, a group of photosynthetic inhibitor herbicides which biochemical mode of action is the inhibition of photosynthetic electron transport in chloroplasts (Moreland, 1980; Liu, 2001). Compared with insecticides, herbicides show a much lesser impact in estuarine and marine environments. In general, herbicides show low toxicity in marine animals because they usually inhibit metabolic activities specific of plants, although they have produced damages in the aquatic vegetation (Kennish, 1997). Maximum concentrations of 0.2–7 μg l^{-1} have been reported in marinas due to the use of diuron as biocide in antifouling paints (Ferrer and Barceló, 1999; Thomas et al., 2001), which indicates that diuron presents a low threat to marine invertebrates since reported concentrations in seawater are much lower than those causing toxicity.

Surfactants intercalate within cellular membranes solubilizing and substituting lipids and proteins, and producing impairment of the ion exchange and of the calcium metabolism of the cell (Hansen et al., 1997). The toxicity level of SDS for *Ciona intestinalis* and *Paracentrotus lividus* embryos (EC_{50} = 5145 and 4277 μg l^{-1}) is lower than that of *Maja squinado* larvae (48 h-LC_{50} = 687 μg l^{-1}). In a previous work with *P. lividus* Rolland et al. (1999) obtained a very similar EC_{50} value whilst other studies with sea urchins showed lower EC_{50} values for SDS (Nipper et al., 1993; Sampaio de Araújo et al., 1999). Similar toxicity was also reported in *P. lividus* embryos exposed to the surfactant linear alkylbenzene sulphonate (LAS) (Bressan et al., 1991). The lowest observed effect concentration of LAS on larvae of the ascidian *Molgula* was within 1000–3000 μg l^{-1}, with similar sensitivity than other marine invertebrates like echinoids, bivalves, annelids and sponges (reviewed by Lewis, 1991). It has also been found a significant inhibition of metamorphosis in larvae of the ascidian *Botryllus schlosseri* and *Botrylloides leachi* exposed to 1000 and 2000 μg l^{-1} of LAS, respectively (Marin et al., 1991). These authors concluded that *Botryllus* and *Botrylloides* larvae were slightly more resistant to this toxicant than *C. intestinalis* larvae, which LC_{50} was 1000 μg l^{-1}. The high sensitivity of *M. squinado* larvae to SDS observed in the present work is in contrast with previous data for crustaceans (Table 3), which indicated that the sensitivity of crustaceans to SDS is highly variable. The EC_{50} values of SDS for bivalve embryos and larvae are within the 840–6000 μg l^{-1} range (His

et al., 1999a) while fish embryos and larvae were more resistant to surfactants (reviewed by Lewis, 1991). Surfactant concentrations that have been used in the present work and previously in other works are similar to those found in rivers and coastal zones (Lewis, 1991). Therefore, in certain polluted zones surfactants can produce alterations of the population dynamics of marine invertebrate species indicating a risk for their preservation in coastal ecosystems.

Based on the results of the present work and using a protection factor of 100 (Länge et al., 1998) maximum permissible concentrations were calculated (Table 4). In comparison, current US-EPA water quality criteria (US-EPA, 2002) (0.16 $\mu g\ l^{-1}$ for lindane, 0.011 $\mu g\ l^{-1}$ for chlorpyrifos, and 0.37 $\mu g\ l^{-1}$ for TBT) are above the proposed levels and therefore would not provide protection for those organisms. No water quality criteria were found for SDS and diuron in the literature.

In summary, experimental data are presented here supporting high levels of toxicity of TBT on early life stages of marine invertebrates. We also report evidence of the high toxicity of lindane and chlorpyrifos on crustacean larvae, whilst ascidian and sea urchin early stages showed lower sensitivity to those compounds. The concentrations of SDS found to be toxic for marine invertebrates in the present work have been measured in polluted aquatic ecosystems, representing a threat to the environment. The lack of toxicity of diuron to the early developmental stages of *Ciona intestinalis*, *Paracentrotus lividus* and *Palaemon serratus*, and the low concentrations detected in seawater indicate that this compound should not endanger marine invertebrate populations. However, when assessing the environmental risk of diuron it must be taken into account that photosynthetic organisms are much more sensitive to this compound.

Finally, as Table 3 shows, there is a substantial amount of variation within the range of effective concentrations reported by different authors, which depends on the test species, life cycle stages, bioassay methodology and toxicity criteria. This makes urgently necessary the standardization of those methods. We also need to keep in mind that there is not single method or species suitable for measuring marine pollution, but a battery of bioassays could be adequate for the assessment of particular pollution events.

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