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Toxic Effects of Irgarol and Diuron on Sea Urchin *Paracentrotus lividus* Early Development, Fertilization, and Offspring Quality

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Abstract. Irgarol and Diuron are the most representative "organic booster biocides" that replaced organotin compounds in antifouling paints. It cannot be assumed beforehand that their use will have no environmental impact: more ecotoxicological data and a significant environmental monitoring are required. Spermio and embryotoxicities of the biocides Irgarol and Diuron were investigated on *Paracentrotus lividus*, the dominant echinoid species of the Mediterranean Sea. Spermiotoxicity was studied by assessing the effects of sperm exposure on fertilization rate as well as on the induction of transmissible damages to the offspring. Embryotoxicity was studied by assessing the developmental defects in the exposed larvae. The experimental results show a Diuron EC50 of 2.39 ($\pm$0.21) mg/L with a NOEL of 0.25 mg/L for embryos, and of 5.09 ($\pm$0.45) mg/L with a NOEL of 0.5 mg/L for sperms, respectively. Data obtained from the embryotoxicity test on Irgarol [EC50 0.99 ($\pm$0.69) mg/L] are of the same order of magnitude as the literature data about Japanese urchins. Spermiotoxicity tests show an Irgarol EC50 of 9.04 ($\pm$0.45) mg/L with a NOEL of 0.1 mg/L. These data show the different sensitivities of the two tests: embryos are more sensitive than sperms for both the tested chemicals and Diuron seems to be the less toxic one. Moreover, as a major output of the experimental work, tested herbicides exert transmissible damage to spermatozoa evidenced by larval malformations in the offspring, mainly P1 type (skeletal alterations). The comparison of the endpoints results offers an interesting indication of a probable different mode of action (Irgarol seems to interact with calcium homeostasis) of the two biocides.

TBT-based antifouling paints were largely used to inhibit the growth of fouling organisms, i.e., barnacles and tubeworms among others. Since the early 1980s, it was pointed out that TBT and its derivatives had deleterious effects on non-target organisms, some of which (*e.g.,* oysters) were economically important (Maguire 1987; Thompson *et al*. 1985). Furthermore, many authors found that even very low TBT concentrations (ng/L) caused sublethal effects (such as poor grow rates and low reproductive success) in a wide range of marine organisms (Alzieu *et al*. 1982; Andreae *et al*. 1983; Chiavarini *et al*. 1996; Hall and Bushong 1996; Laughlin *et al*. 1996; Marin *et al*. 2000; Waldock and Thain 1983). As an example, these compounds were held responsible for the near collapse of oyster farming in France and declines in the population of dogwhelks in British waters (Evans 1999). However, regulations, which were introduced by many countries in the last decade, restricting the use of these antifoulings, have greatly reduced the TBT contamination levels (Evans 1999). Furthermore, in 2001 the IMO (International Maritime Organization) decided that the use of organotin antifoulants would be totally banned by the year 2003 and the TBT coatings on ships should be prohibited from 2008 onwards (international convention on the control of harmful antifouling system on ships, adopted on 5/10/2001). Consequently, many organotin-free antifouling paints have been developed.

Tin-free antifouling paints, composed of seawater-soluble matrices containing tin-free biologically active ingredients (a copper compound and/or organic biocides), are now available. The copper component (typically cuprous oxide or even metallic copper) alone, in fact, is not effective against diatoms and algae. Therefore, secondary biocides must be incorporated into the paints (Brady 2000). These compounds are termed "organic booster biocides" and some of them originated for agricultural use (Voulvoulis *et al*. 1999). The mainly used organic boosters are, until now, Irgarol (2-methylthio-4-t-butylamino-6-cyclopropylamino-s-triazine) and Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) (Voulvoulis *et al.* 1999).

The introduction of these and other compounds in the antifouling products obviously generates the necessity to investigate any possible adverse effect on the marine ecosystem. It seems, in fact, that Irgarol does not undergo to rapid biodegradation and the persistence in water is probably due to the low affinity to partition onto sedimentary material and high resistance to degradation (Thomas *et al*. 2002).

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Irgarol residues have been found in European coastal and lake waters and in Japanese coastal waters suggesting the possibility that Irgarol may become a ubiquitous contaminant of the aquatic environment (Okamura *et al*. 2000), mainly in the areas of high yachting activity and low water exchange rates (Omae 2003; Thomas *et al*. 2002).

In 2001, Thomas *et al.* wrote ''a simple risk assessment indicates that at present Irgarol 1051 and Diuron represent a lower threat to the environment than TBT. However, it cannot be assumed that their use will have no environmental harm, and further data are required on the fate and effects of all antifouling paint booster biocides.''

Van Wezel and van Vlaardingen (2004) proposed environmental risk limits for Irgarol 1051 and some other TBT substitutes, derived from data on ecotoxicology and environmental chemistry of these substances. For Irgarol 1051, plants appear to be especially sensitive. The authors concluded that the plant species composition, and thereby the complete ecosystem functioning, cannot be considered as protected.

A comprehensive review of very recent Irgarol and Diuron toxicity data for aquatic organisms was proposed by Kostantinou and Albanis (2004); other studies about the toxic effects of Diuron have been recently published about corals (Jones *et al*. 2003), sea grass (Maccinnis-Ng *et al*. 2003), *V. fisheri* and *D. magna* (Hernando *et al*. 2003). Other data are reported in the U.S. EPA Pesticide Database.

Gaps in available data make difficult the evaluation of biocides impact on the aquatic environment. Therefore, further ecotoxicological studies especially about marine non-target organisms still seems to be necessary.

In 2002 Kobayashi and Okamura assessed, comparatively, the effects of various biocides on Japanese *H. pulcherrimus* and *A. crassispina* sea urchin eggs and embryos. In this study, the maximum concentration at which Irgarol showed no toxic effects (No Observed Effect Concentration: NOEC) on the early juvenile stages of sea urchin was 10 μg/L. The NOEC value (1 mg/L) was reported also for Diuron, in the same study.

In this report, we show our experimental data on the toxic effects of the two booster biocides Irgarol and Diuron on sea urchin *Paracentrotus lividus* early development, fertilization, and offspring quality. The sea urchins toxicity test has been utilized for several decades to evaluate a number of xenobiotics and their future in the marine ecosystem (Bay *et al*. 1993; His *et al*. 1999; Kobayashi and Okamura 2002; Manzo 2004; Marin *et al*. 2000; Pagano *et al*. 1996a, 1996b). *P. lividus* is one of the most commonly used organisms in biomonitoring studies, which require simple, rapid, and inexpensive but sensitive methods (Bougis *et al*. 1979; Chapman and Long 1983; Kobayashi 1991; Manzo and Torricelli 2000; Pagano *et al*. 1989). In particular *P. lividus* early life stages are very sensitive to many pollutants (His *et al*. 1999; Ringwood Huffman 1992).

Materials and Methods

Test Organisms

Adult *Paracentrotus lividus* (Lamark) were collected from the Tyrrhenian Sea (Bay of Naples) by the staff of the Zoological station. The sea urchins were then stabulated for 24 h in natural Filtered SeaWater (FSW) at 18 ± 1°C (Salinity 38%, pH 8 ± 0.2).

In fact, it was noted that the utilization of the animals immediately after the collection produces a decrease of normal plutei in the control, probably due to the stress induced by the collection activity itself. An abrut increase in temperature or salinity might not only induce spawning, but seriously harm the gametes (ASTM 2004).

Test Solutions

The herbicides Irgarol (2-(tert-butylamino)-4-(cyclopropylamino)-6-(methylthio)-s-triazine), and Diuron (3-(3,4-dichlorophenyl)-1,1dimethylurea) at the highest available purity (Residue Analysis-Pestanal®) were purchased by Sigma Aldrich, Milano, Italy, from a Fluka catalogue. The choice of pure chemicals was based on the fact that the technical products mentioned in the literature always have a quite high level of purity (97–99,8%). This substantially reduces the problem to the possible presence of highly toxic intermediates of synthesis or metabolites; on the other hand, pure chemicals guarantee a higher reproducibility and comparability of the results.

Since both chemicals have a relatively low water solubility (7 mg/L for Irgarol and 42 mg/L for Diuron), the stock solutions were prepared dissolving 10 mg of the herbicide in 1 mL of dimethyl sulfoxide (DMSO, 97% purchased from Merk, Dusseldorf, Germany).

Test solutions were then obtained diluting the stock solution in natural FSW as follows. For Irgarol the following concentrations were used for test solutions: 0.01, 0.1, 0.5, 1, 5, 7.5, and 10 mg/L for the embryotoxicity test; 0.01, 0.1, 0.5, 1, 5, and 10 mg/L for the spermiotoxicity test.

For Diuron the following concentrations were used for test solutions: 0.2, 0.25, 0.5, 1, 2, 2.5, 5, and 7.5 mg/L for the embryotoxicity test and 0.25, 0.5, 1, 2, 2.5, 5, and 7.5 mg/L for the spermiotoxicity test.

Controls

Seawater used for the test solutions was sampled in an uncontaminated area far from the coast and was already frequently used in the laboratory for ecotoxicological tests and optimisation of analytical methods. As a consequence, seawater samples from this area were analysed several times for trace elements and organic micropollutants using wide-spectrum-screening analytical methods. Test solutions were always controlled for Irgarol and Diuron concentrations before the test execution, checking those at the lowest and highest concentration. The analytical control was performed using solvent extraction and GC/MS determination for Irgarol, and solvent extraction and LC/MS for Diuron according to Lamoree *et al*. (2002). A sufficient amount of seawater, necessary for the experiments and negative (blank) controls, was then filtered (0.45 μm). Standard control was carried out with equivalent volumes of DMSO at a final concentration of 0.1% (that is, the higher one in the test solutions) and exhibited no observable effect on the studied organisms.

Toxicity Test

Gametes were harvested and embryos were reared as described by Pagano *et al*. (1986). Spawning was induced in sea urchin by injection of 1 mL of 0.5 M KCl through the perioral membrane. Eggs were collected by separately placing each spawning female in a different 250-mL beaker with FSW while ''dry'' sperm from each male was collected with an automatic pipette and stored in a sterile tube

placed on ice. For each experiment, six individual females were selected for their appropriate egg quality (no immature forms, no debris, no fertilised eggs) and amount. Males were selected for sperm motility (checked under the microscope) and amount. Then, the best three male and three females gametes were pooled and filtered through nylon cheesecloth (Ø = 200 μm for eggs and 50 μm for sperm). The eggs suspension (stock solution) was diluted in order to obtain the final concentration of 250–300 eggs/mL.

Embryotoxicity Test

In the embryotoxicity protocols, fertilization was carried out by adding 1 mL of pooled-sperm, diluted 1:1000 in FSW, to the egg suspension and by incubating if at 18 ± 1°C for 20 min. Fertilisation success in the stock solution was verified by the presence of the fertilization membrane in a random sample of 100 eggs. An excess of sperm was removed by decanting zygotes and resuspending them in FSW.

The experiments consisted of the exposition of a volume of the egg suspension corresponding to 250–300 fertilized eggs to the test solution (10 mL). Three replicates for each treatment were prepared. Each experiment was performed at least three times. The eggs were incubated at 18 ± 1°C, for 48–50 h. After this period, 100 μL of 40% buffered formalin was added in each vessel and developmental abnormalities were determined in each replicate by direct observation of 100 individuals, randomly chosen.

For each treatment schedule, 100 plutei were scored for the frequencies of: (1) normal (N) larvae, according to their symmetry, shape, and size; (2) retarded (R) larvae with shape and symmetry the same as normal, but with reduced size (<1/2 N); (3) malformed larvae (P1), affected in skeletal and/or gut differentiation and/or pigmentation; (4) pre-larval arrest (P2), embryos unable to go to larval differentiation, as abnormal blastula or gastrulae (Pagano *et al*. 1986, US EPA 1995).

Spermiotoxicity Test

Aliquots (10 μL) of concentrated sperm from the pool of three males were diluted in 10 ml of testing solutions. After the exposition of 30 min at room temperature, 50 μL of treated sperm suspension were added to 10 ml of FSW containing untreated eggs (20/30 eggs/mL) obtained from stock solution. Experimental wells, three replicates for each treatment, were incubated at 18 ± 1°C for 20 min. Each experiment was performed at least three times. The fertilization rate was determined on a random sample of 100 eggs. The zygote cultures were incubated at 18 ± 1°C, for 48–50 h. Then offspring quality, expressed as frequency of developmental defects, was assessed as described before.

Statistical Analysis

Differences in fertilization success (comparisons between the control group and each of the experimental groups) were tested for significance using the multiple comparison Dunnett's test.

Mean percentage abnormalities and 95% confidence limits were calculated for all the samples and compared to the results obtained for the controls. If abnormalities in the controls were 20% or more, the test was judged invalid and repeated.

The EC50 was calculated using the Linear Interpolation Method (Inhibition Concentration procedure or ICp) (Cesar *et al*. 2004, US EPA 1993). The bootstrap method is used to obtain the 95% confidence interval, because standard statistical methods for calculating confidence intervals are not applicable, Analysis of variance (ANOVA) was applied, using raw data, to test for significant differences in effects among treatments (significance level was always set at $\alpha = 0.05$); then NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect Concentration) were calculated with Dunnett's procedure.

Results

Diuron

Embriotoxicity. The effect mean percentage (Figure 1A) shows values with an increasing trend up to the maximum effect (SD = 0) at 7.5 mg/L. EC50 is 2.39 (± 0.21) mg/L and NOEL 0.25 mg/L. The developmental defects in treated *P. lividus* larvae (Figure IB) are mainly of the P1 type (larvae affected in skeletal or gut differentiation), with an increasing trend until 2.5 mg/L, at 5 mg/L, the effect becomes drastic, with the total arrest at prelarval stadium (P2) at 7.5 mg/L.

Spermiotoxicity. Significant effects on fertilization rate (FR) were observed for this herbicide (Figure 2A). FR shows a significant progressive decrease due to a reduction of the fertilization ability of exposed sperms. The EC50 is 5.09 (± 0.45) mg/L, and NOEL 0.5 mg/L. However, it can be noted that the spermiotoxic effect is not complete, thus permitting the assessment of embryo cultures. The offspring of treated sperm (Figure 2B) shows mainly malformations of P1 type and a dose-dependent increase in the effect percentage curve. So the treated sperm that retain fertilization ability produce offspring that develop abnormally. Diuron first produces toxic effects on the fertilization process and, then, offspring damage. However, at the last two concentrations, 7.5 and 10 mg/L, the data were biased by quite low FR (7% and 17%, respectively).

Irgarol

Embryotoxicity. The Irgarol toxic effect exerted on *P.lividus* embryos is reported in Figure 3A. The values quickly increase up to EC50 0.99 (± 0.69) mg/L, and seem to stabilize from 1 to 5 mg/L dose (laying around 60% toxic effects) and at higher concentrations a corresponding increase is observed, with the maximum at 10 mg/L. The induced developmental defects are reported in Figure 3B. The abnormal individuals are mainly P1 malformed larvae, showing a dose-dependent trend up to 7.5 mg/L (90%). At a higher tested concentration (10 mg/L), massive prelarval arrest (P2) effects are observed (80%).

Spermiotoxicity. The toxicity pattern of this herbicide on sperm fertilization ability is shown in Figure 4A. Significant ($\alpha = 0.05$) effects can be evidenced already at 0.01 mg/L concentration (NOEL < 0.01 mg/L), but then the effects remain under 25% up to 5 mg/L. The EC50 is 9.04 (± 0.45) mg/L.

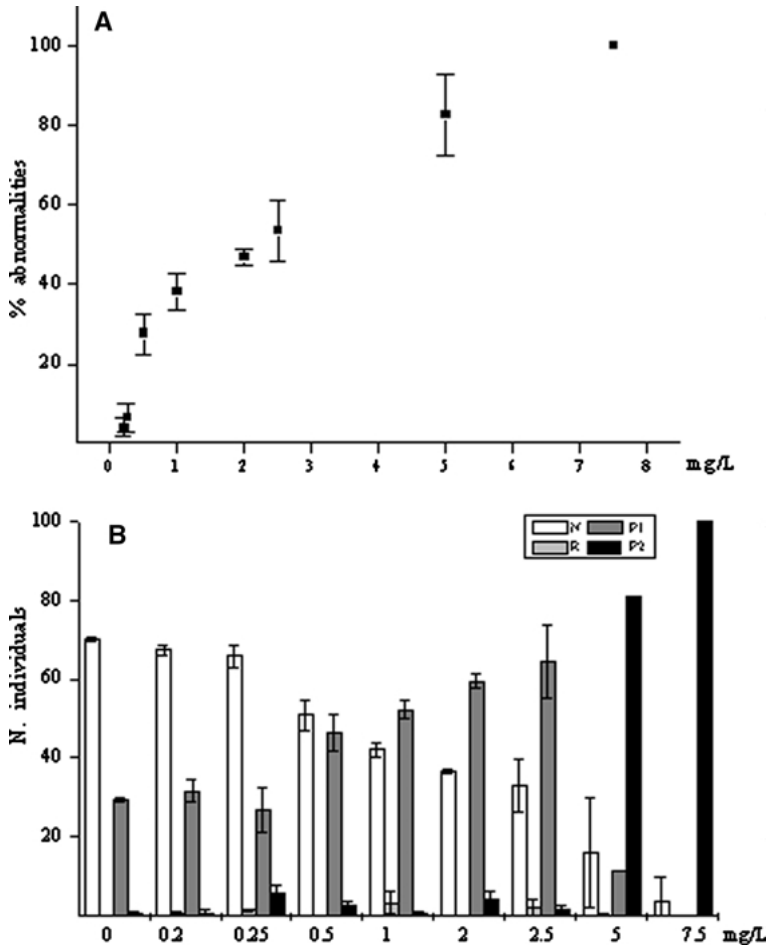

Fig. 1. Diuron embryotoxicity in *P. lividus*. A: % of malformed out of 100 individuals normalized with respect to control, as a function of tested concentrations (mean of at least three experiments, each in triplicate ± SE,). B: Number of individuals with different developmental anomalies obtained after 48-h exposure. N = normal plutei; R = retarded larvae; P1 = malformed larvae; P2 = blastulae or gastrulae (developmental arrest). See also Materials and Methods.

Irgarol-induced effects on offspring quality following sperm exposure are reported in Figure 4B. Malformed larvae (P1) are almost exclusively present. It is interesting to note the increment of P1 also at the lowest concentration even if the fertilization rate is not affected. Then, the rate between P1 malformed larvae and normal offspring is almost the same (and only the decrease of fertilization success can be seen). So, the maximum defect on offspring is already obtained at the lowest test concentration (0.01 mg/L). After that, the herbicide affects only the sperm fertilization ability.

Discussion

Sea urchin embryos seem to be very sensitive to Irgarol. The measured embryotoxicity on *P. lividus* (Table 1) is comparable with raw data reported by Kobayashi and Okamura (2002) for the Japanese urchin embryos (EC50 is not reported). Spermiotoxicity test shows an EC50 value in the same range as those reported for crustacean (Kostantinou and Albanis 2004) and a NOEL of 0.10 mg/L.

Being an herbicide, Irgarol is much more toxic to algae, its prevalent effect being the inhibition of photosynthesis, in particular acting on Photosystem-II (PSII) (Dahl and Blanck 1996). Toxicity effects are particularly evident for marine species (*Enteromorfa intestinalis* zoospores) for which growth inhibition is significant at concentrations as low as 100 ng/L (Scarlett *et al*. 1997). A moderate toxicity is reported for the marine bacterium *V. fischeri* EC50 = 50.8 ± 7.8 mg/L (Fernandez-Alba *et al*. 2002) and for marine crustacean *A. salina* (> 40 mg/L, Okamura *et al*. 2000).

To our knowledge, the mode of action of triazine upon aquatic invertebrates is not well known. In our spermiotoxicity and embriotoxicity tests, we observed a predominance of P1 malformed larva, mainly affected by skeletal alterations.

From these results, it seems possible to hypothesize that in *P. lividus*, this compound could interact with calcium homeostasis. At higher concentrations, it could modify cytoskeleton assembly during blastomere division, producing P2-type alterations, while, at lower concentrations, it could alter the deposition of the larval skeleton giving rise to malformed larvae (P1).

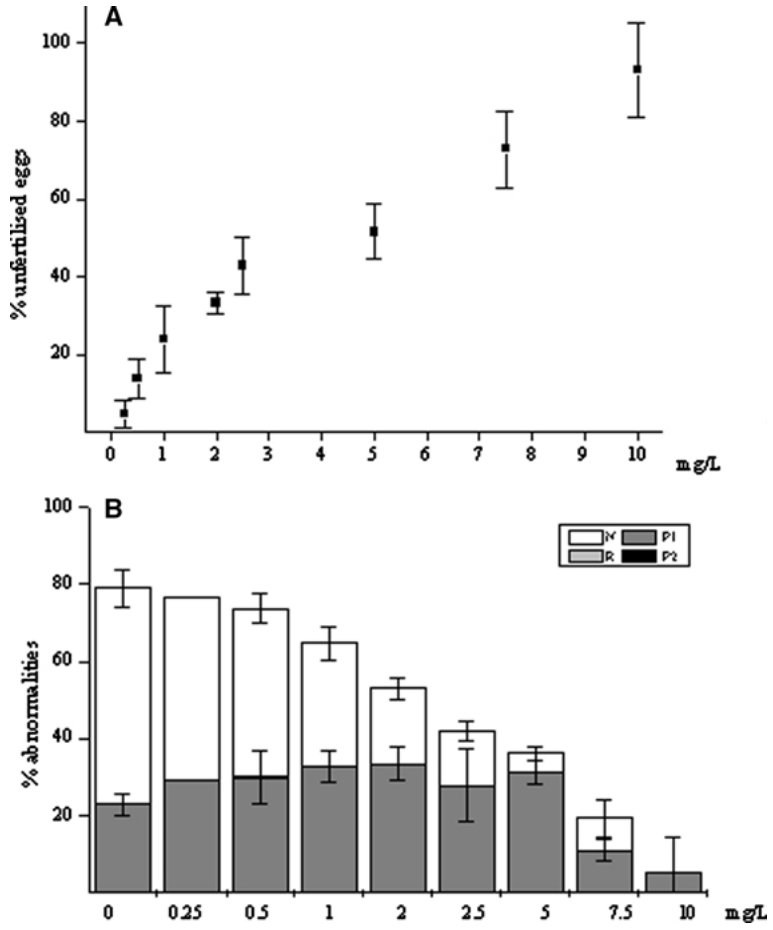

Fig. 2. Diuron spermiotoxicity in *P. lividus*. A: % of unfertilised eggs out of 100 eggs, as a function of tested concentrations (mean of at least three experiments, each in triplicate ± SE). B: Offspring quality: % of developmental anomalies in the fertilized eggs after 48-h exposure. N = normal plutei; R = retarded larvae; P1 = malformed larvae; P2 = blastulae or gastrulae (developmental arrest). See also Materials and Methods.

Exposed sperms show a dose-related decrease in fertilization ability (Figure 2A) but with less sensitivity than for embryos, probably because they are differentiated cells. On the contrary, the maximum defect in offspring is obtained at the lowest test concentration (0.01 mg/L). After that, the herbicide affects only the sperm fertilization ability; it actually exerts an acute spermiotoxicity.

Diuron, even if belonging to a different chemical class of pesticide (phenylureas) than Irgarol, is a photosynthesis inhibitor too, but the mode of action at the biochemical level, up to now, has not been precisely determined.

Diuron ecotoxicological data in the recent literature are quite scarce, particularly with reference to marine species. Hernando *et al*. (2003) reported toxicity effects on *D. magna* (EC50 8.6 mg/L ± 1.3) and *V. fisheri* (EC50 100 mg/L ± 7.8). The chronic effect upon algae (*S. capricornutum*, 72 h test) was reported by Fernandez Alba *et al*. (2002) (0.0045 mg/L ± 0.0079). Kobayashi and Okamura (2002) reported only the NOEL for the Japanese sea urchin (1 mg/L). *P. lividus* NOEL (0.5 mg/L, Table 1) is lower than those sea urchin species and LOEL and EC50 higher only than those reported in the literature for algae.

Diuron is less toxic (LOEL 0.5 mg/L) to *P. lividus* embryos than Irgarol but its toxic effects appear earlier. In fact, a 65% effect level can be observed at a concentration of 2. 5 mg/L (Figure 3A) and almost exclusively P2 damage can be seen at a concentration of 5 mg/L (this happens only for Irgarol concentrations >10 mg/L: 80% P2).

The spermiotoxicity test is less sensitive than the embryotoxicity test for Diuron (NOEL 0.5 mg/L). It is possible to note from the comparison of the two herbicide spermiotoxicity effect curves (Figures 2A, 4A) that Diuron is more toxic from a concentration of 1 mg/L onwards. The developmental defects on exposed sperms is mainly of the P1 type for both herbicides, but for Diuron we can read a dose-related trend of P1 malformations as well as for the inhibition of fertilization rate. So, this biocide exerts quickly, producing on exposed sperm populations toxic effects such as fertilization inhibition and permanent damage transmissible to offspring, that is to say acute and chronic effects.

Fortunately, the NOECs determined are much higher than the concentrations reported in different world seawaters up to now (for a review, see Kostantinou and Albanis 2004). It is important to consider that, from 2008 onwards, tributyltin-

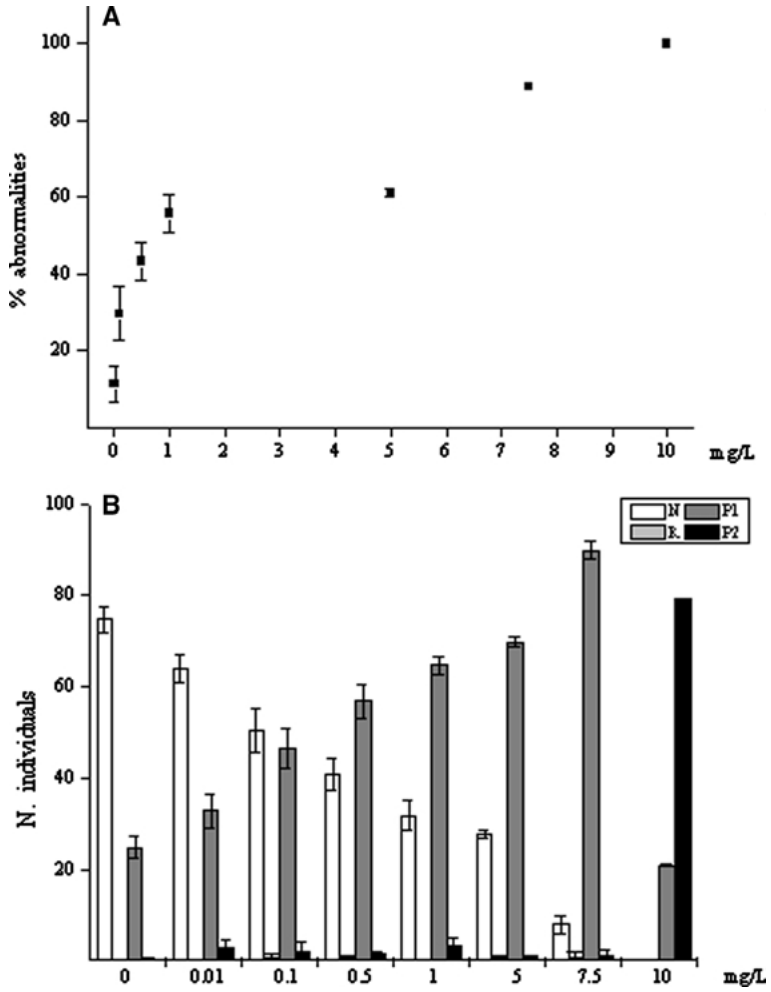

Fig. 3. Irgarol embryotoxicity in *P. lividus.* A: % of malformed out of 100 individuals normalized with respect to control, as a function of tested concentrations (mean of at least three experiments, each in triplicate ± SE). B: Number of individuals with different developmental anomalies, obtained after 48-h exposure. N = normal plutei; R = retarded larvae; P1 = malformed larvae; P2 = blastulae or gastrulae (developmental arrest). See also Materials and Methods

based paints will be totally banned and the replacing organotin-free biocide environmental levels can considerably increase. Moreover, these active compounds can accumulate in marine sediments especially if introduced as paint particles (Thomas *et al*. 2002).

As for antifouling, various chemicals are used. Mixture toxicity, in particular at low concentrations, certainly plays a role in the environmental situation.

Conclusions

The known sensitivity of sea urchins to numerous environmental pollutants environmental is noticeable also in the chemicals (Irgarol and Diuron) tested in this report.

Diuron is less toxic (LOEL 0.5 mg/L) to *P. lividus* embryos than Irgarol but its toxic effects appear earlier and produce prelarval arrest (P2).

Irgarol induces a predominance of larvae with skeletal alterations (P1). It seems possible to hypothesize that in *P. lividus*, this compound could interact with calcium homeostasis. Further experiments are in progress in our laboratory in order to assess the toxicity effects on *P. lividus* of Irgarol and Diuron mixtures at real contamination levels.

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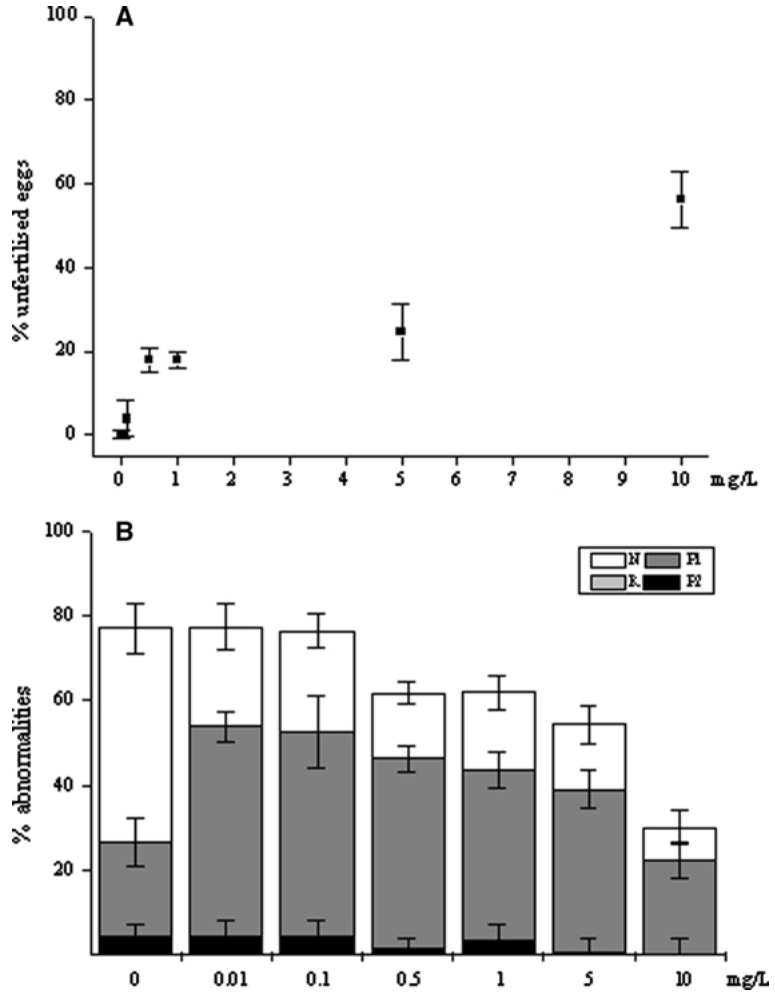

Fig. 4. Irgarol spermiotoxicity in *P. lividus.* A: % of unfertilised eggs out of 100 eggs, as a function of tested concentrations (mean of at least three experiments, each in triplicate ± SE). B: Offspring quality: % of developmental anomalies in the fertilized eggs after 48-h exposure. N = normal plutei; R = retarded larvae; P1 = malformed larvae; P2 = blastulae or gastrulae (developmental arrest). See also Materials and Methods.

Table 1. Herbicides toxic effects on *P. lividus*

		NOEL (mg/L)	LOEL (mg/L)	EC50 (mg/L)
Diuron	Embriotoxicity	0.25	0.5	2.39 ± 0.21
	Spermiotoxicity	0.5	1	5.09 ± 0.45
Irgarol	Embriotoxicity	< 0.01	0.01	0.99 ± 0.69
	Spermiotoxicity	0.10	0.5	9.04 ± 0.45

No Observed Effect Level (NOEL) and Lowest Observed Effect Level (LOEL) were determined with Dunnett's procedure. EC50 (50% Effective Concentration) ± SE (ICp approach: U.S. EPA, 1993).

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