ENVIRONMENTAL OCCURRENCE AND CONCERNS OF ANTIFOULING BIOCIDES

Undesirable effects of the antifouling biocides Irgarol and Diuron upon some non-target marine organisms

In this report, results obtained with organisms belonging to different phyla, at different levels of biological complexity and of the trophic chain, have been summarized. *Algae Dunaliella tertiolecta, Tetraselmis suecica, Isoscrysis galbana*, bacteria *Vibrio fischeri,* crustacean *Artemia salina*, echinoids *Paracentrotus lividus*, and fishes *Sparus auratus*, *Dicentrarchus labrax* were tested for their sensitivity to the antifouling biocides Irgarol and Diuron

DOI: 10.12910/EAI2014-48

Sonia Manzo

Introduction

The extensive use of antifouling (AF) biocides on boat shells and other submerged surfaces was often responsible for the contamination of water and sediments by many toxic substances, especially booster biocides such as Irgarol and Diuron [1], used in combination with other compounds such as copper salts [2].

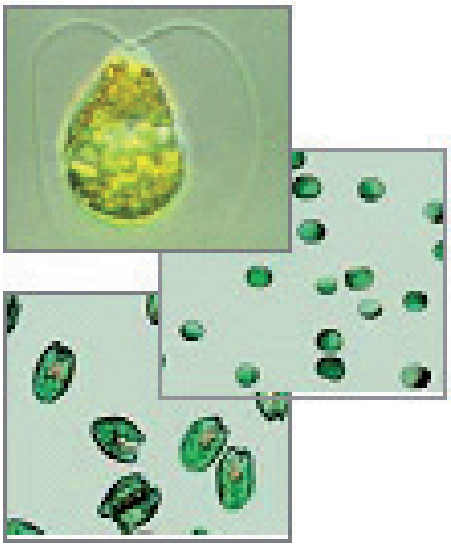
However, some of these chemicals have also been used as pesticides for agricultural use (e.g., Diuron), leading to possible confusion in identifying contamination sources [3]. There is evidence that these compounds were highly toxic for freshwater and marine autotrophs [4], influencing key species in both environments. The previous TBT experience (shell malformation in oyster, mortality of mussel larvae and imposex in gasteropods at ng/L concentration) generated the necessity to investigate any possible adverse effect on the marine ecosystem of these herbicides.

Ecotoxicological assessment of the adverse effects of these compounds in estuarine and coastal systems is, therefore, a matter of concern for many stakeholders involved in the conservation and exploitation of these areas (e.g., oyster or mussel farmers, fishermen).

Ecotoxicology is the science studying the contaminants effects on the biosphere constituents. Albeit a relatively new field, ecotoxicological research is rapidly developing due to concern induced by the industrial development. Ecotoxicology has therefore become an important part in environmental and ecological risk assessment and in the definition of environmental policies. As a matter of fact, unlike analytical chemistry approaches, ecotoxicological tests integrate all toxic signals, thus adding toxicity-based criteria to the currently adopted policies for a more comprehensive evaluation of the environmental hazard. Additionally, the laboratory results obtained with pure chemicals allow to evaluate the effects observed in the environmental samples and, then, to estimate the possible contribution of each biocide to the overall toxicity.

Sonia Manzo
ENEA, Technical Unit for Technologies Development - Portici Research Centre

The ecotoxicological approach is generally based on a battery of bioassays with organisms belonging to several species, since the use of a combination of assays and/or organisms increases the ecological reliability and easiness of interpretation of results, which in turn offers a powerful tool for assessing the potential bias of individual organisms and also the mode of action of contaminants.
This paper will explore the ecotoxicological effects of Irgarol and Diuron, registered for the marine environment, on non-target species at the ENEA's Portici Research Centre, during the last few years. Toxicity test with organisms (bacteria, microalgae, crustaceans, echinoids and fishes) belonging to different trophic levels were performed. The toxicity data was utilised to create concentration-response curves and to calculate EC50, LOEC and NOEC parameters. In addition, the toxicity results were fitted by interpolation models, in order to obtain continuous dose-response curves.



Algae

Algae test

Marine algae are highly diffused in coastal ecosystems [5], therefore they are particularly susceptible to contaminants associated with anthropogenic pollution.

The evaluation of the effects of AF biocides upon marine phytoplankton is a necessary step to predict their potential impact on coastal marine food webs and on the whole ecosystems they support.

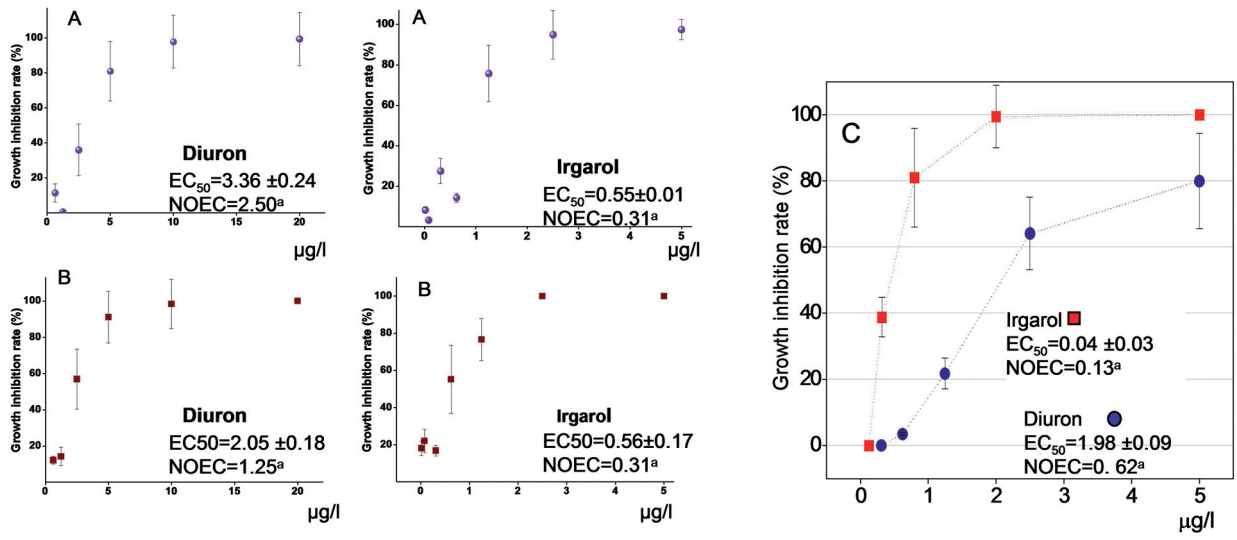


FIGURE 1 Dose-response curves (mean percentage of growth inhibition rate with respect to control) for Irgarol and Diuron with different marine algal species *I. galbana* (A), *T. suecica* (B), *D.tertiolecta* (C) after 96h incubation.a ($p<0.05$)

The chronic test was carried out according to UNI EN ISO 10253. Briefly, an algal suspension at concentration of $1x10^6$ was added to each replicate [3] to reach the final concentration of $1x10^4$. Artificial seawater [6] was used for sample dilution, and culture medium was utilized as negative control (6 replicates). The test flasks were placed in a thermostatic chamber at 20 °C with a light source in the 7000-8000 lux range for 72h. The cell density of each sample is measured after 72h by the Burker chamber. Growth inhibition percentage and EC50 were calculated for each sample with respect to the control.

Results

Being a herbicide, Irgarol is toxic to algae and its prevalent effect is the inhibition of photosynthesis, acting on Photosystem-II (PSII) in particular [7].
The tested microalgae showed a high sensitivity to Irgarol. However, the EC50 values, LOEC and NOEC resulted quite similar for the tested species.
Figure 1 showed the dose response curves of these phytoplanctonic species. It is possible to note effects starting from the lowest tested concentrations. The curves had an increasing trend, and for *D. tertiolecta* (C) and *I. galbana* (A) an 80% toxic effect at concentration of about 1 g/L Irgarol was observable.
Among the tested species, *D. tertiolecta* was the most sensitive one to both biocides.

Artemia Salina test

Crustaceans are frequently used as bioindicators and biomonitors in various aquatic systems. The brine shrimp *Artemia salina* is a zooplankton organism found in hypersaline habitats such as inland salt lakes, coastal salt pans and man-managed saltworks worldwide. Their life cycle begins by hatching dormant cysts where these cysts are inactive but, once in salt water, they become rehydrated and resume their development. Brine shrimp larvae are commonly used for toxicity assays.
A. salina cysts were hatched by using the procedure described in APAT-IRSA, 2003 [8]. The encysted organisms were first hydrated in a volume of artificial seawater [6] for 1h at 25 °C at 3000-4000 lux. Then they were incubated for 24h in the dark at the same temperature. Acute toxicity test (48h) was conducted according to APAT-IRSA. Ten nauplii were transferred in beaker with 40 ml of sample. Each sample was tested in triplicate. The negative control consisted of 6 artificial seawater replicates. The treatments were

Artemia Salina

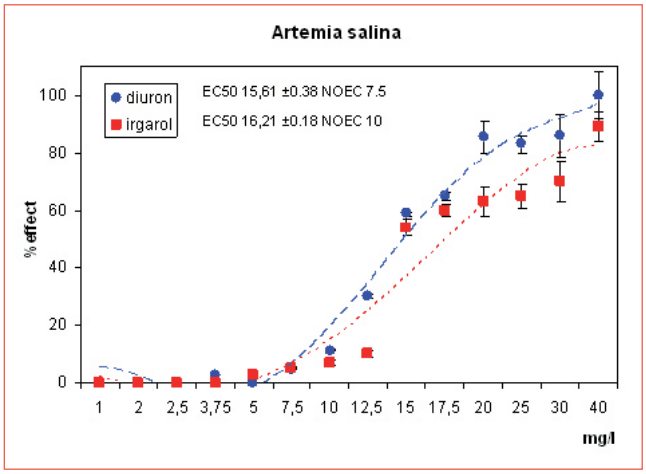


FIGURE 2 Dose response curves (mean percentage of effect (mortality) with respect to control) obtained for *A. Salina* exposed to Irgarol and Diuron solutions for 48 h

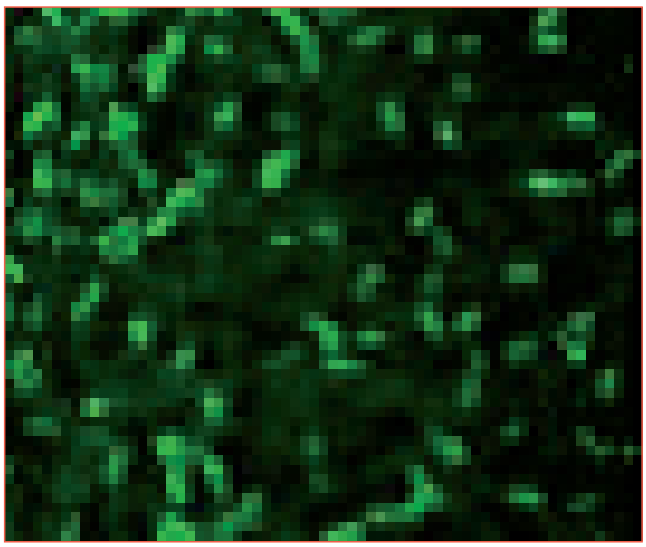
incubated at 25 °C, with a light regime of 14:10h light: dark. No food was provided during the course of the exposure. Every 24h the number of the live individuals was recorded. The effect percentage for each sample was calculated with respect to the control.

Results

In Figure 2, the dose response curves obtained for *A. salina* exposed to Irgarol and Diuron solutions for 48h are reported. As can be observed, this organism showed scarce sensitivity to these biocides also at the lowest concentrations, in fact the NOEC values are around 10 mg /L.

Vibrio Fischeri test

Microtox® is a standardised toxicity test system which is rapid, sensitive, reproducible, ecologically relevant and cost effective. It is recognised and used throughout the world as a standard test for aquatic toxicity testing. The Procedure employs the bioluminescent marine bacterium *(Vibrio fischeri)* as test organism. The bacteria are exposed to a range of concentrations of the material being tested. The reduction in intensity of light emitted from the bacteria is measured along with standard solutions and control samples. The change in light output and concentration of the toxicant produces a dose/response relationship. The results are normalised and the EC50 (concentration producing a 50% reduction in light) is calculated.



Vibrio Fischeri

V. fischeri bacteria were exposed to serial dilutions (1: 2) and to a negative control Microtox diluent (NaCl 2%). The luminescence decrease was evaluated after 5, 15 and 30 minutes of exposure. The luminescence was measured using a Microbics Model 500 Toxicity Analyzer and following the manufacturer's instructions (Microbics Corporation). The results were expressed as luminescence inhibition percentage with respect to the control.

Results

In Figure 3 are reported the dose response curves

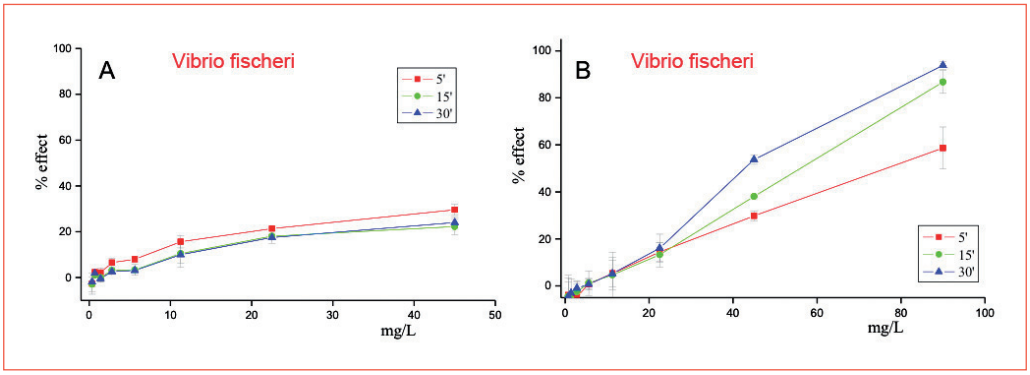


FIGURE 3 Dose response curves (mean percentage of effect (bioluminescence inhibition) with respect to control for *V. fischeri* at three different exposure times to A) Irgarol, B) Diuron

for the bacteria at three different exposure times. For Irgarol the trends did not show significant differences and NOEC and LOEC values were comparable (Figure 3 A). This biocide resulted slightly toxic for *V. fischeri* with the highest toxic effect of 25% with respect to the control. Actually Diuron exerted a lower toxicity with an EC50 of 73 mg /L, and NOEC and LOEC values over 10 mg/L (11.25 mg/L and 22.50 mg/L, respectively) (Figure 3B). In this case, starting from concentration of 30 mg/L a dependence with the exposure times was also observable.

Paracentrotus Lividus test

Sea urchin embryos and gametes are often utilized to assess the toxicity of chemicals in the marine ecosystem due to their sensitivity and availability. In addition, spermiotoxicity and embryotoxicity tests offer the possibility of comparing the effects of the same substance upon two different biological systems. Fertilization was carried out by adding pooled-sperm to the egg suspension and incubating it at 18 °C for 20 minutes. A volume of the egg suspension corresponding to 250–300 fertilized eggs was treated with 10 mL of test solution. Three replicates for each treatment were prepared. The eggs were then incubated at 18 °C, for 48–50 h. After this time, 100 µL of 40% buffered formalin was added in each vessel and developmental

Paracentrotus lividus

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: normal larvae, according to their symmetry, shape, and size, malformed larvae affected in skeletal and/or gut differentiation and/or pigmentation, embryos unable to go to larval differentiation, such as abnormal blastula or gastrulae.

Spermiotoxicity test was performed, according to

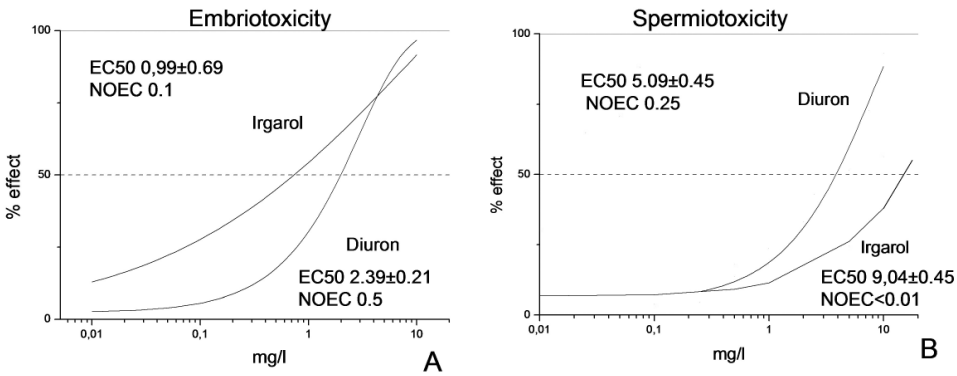


FIGURE 4 Dose response curves obtained for *P. lividus* embryos (A) and sperms (B) treated with Irgarol and Diuron

Manzo et al. 2006 [9].
Sperm was collected "dry" from each male and stored on ice. 10 µL of concentrated sperm was diluted in 10 mL of sample. The solution were incubated for 30 min at room temperature, then 50 µL of treated sperm was added to 10 mL of FSW containing untreated eggs. Experimental wells were incubated at 18 °C for 20 min. Three replicates were carried out for each sample. The fertilization rate was determined on a sample of 100 eggs. The effect percentage for each sample was calculated with respect to the control.

Results

Embriotoxicity

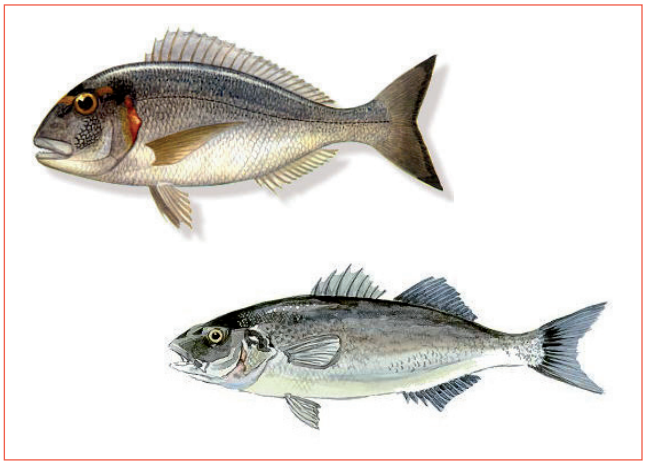
The embriotoxic effects of Irgarol and Diuron are reported in Figure 4.A.
The Irgarol toxicity values quickly increase up to EC50 0.99 (± 0.69) mg/L, and seem to stabilize from 1 to 5 mg/L dose (laying around 60% toxic effects) and at higher concentrations a corresponding increase is observed, with the maximum at 10 mg/L.
Diuron toxicity 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.

Spermiotoxicity

Spermiotoxicity values are reported in Figure 4B.
The toxicity pattern of Irgarol on sperm fertilization ability 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. Significant effects on the fertilization rate (FR) were observed for Diuron. FR shows a significant progressive decrease due to a reduction in the fertilization ability of exposed sperms. The Diuron EC50 is 5.09 (±0.45) mg/L, and NOEL 0.5 mg/L.

Fish test *(Sparus aurata, Dicentrarchus labrax)*

The purpose of the toxicity test with fish species is to help assess possible risk to similar species in natural environments, as an aid in determination of possible water quality criteria for regulatory purposes, and for use in correlation with acute testing of other species for comparative purposes.



Sparus aurata, Dicentrarchus labrax

Fish early life stage toxicity test [10]

Larvae were exposed to increasing concentrations of Irgarol and Diuron in static conditions and did not feed during the test. Filtered seawater was used to dilute the solutions and as control. In addition, a DMSO control has been also used. The experiments were conducted in triplicate using a testing volume of 200 ml and 20 individuals in each replicate. The experiment exposure was 24 and 48 h at T 18 °C with regular photoperiod.

Fish Acute Toxicity test [11]

Juveniles of *Sparus aurata* were exposed to increasing concentrations of Irgarol and Diuron. Filtered seawater was used to dilute the solutions and as control. In addition, a DMSO control has been also used. The experiments were conducted in triplicate using a testing volume of 8 L and 5 individuals for each replicate and exposure condition for 96 h at T 18 °C with regular photoperiod and continuous aeration.

Results

Fish larvae were also very sensitive to Irgarol (*D. labrax,* NOEC= 2.5 mg/L) with respect to Diuron (S. aurata larvae NOEC < 0.01mg/L), while the juveniles *(S. aurata)* showed a high resistance (NOEC= 2 mg/L): therefore, during a brief exposure (48h), larvae

		Irgarol			Diuron		
Test organism		**EC50**	**LOEC**	**NOEC**	**EC50**	**LOEC**	**NOEC**
Vibrio fischeri	*5 min*	>45	2.81	1.41	73.12	22.50	11.25
Dunaliella tertiolecta		[a]0.40±0.03	[a]0.32	[a]0.13	[a]1.98±0.09	[a] 1.25	[a] 0.62
Tetraselmis suecica		[a]0.56±0.17	[a]0.40	[a]0.31	[a] 2.05±0.18	[a] 2.50	[a] 1.25
Isochrysis galbana		[a]0.45±0.02	[a]0.31	[a]0.08	[a]3.36±0.24	[a] 5.00	[a] 2.50
Artemia salina		>15.00	15.00	10.00	15.61±0.38	10.00	7.50
Paracentrotus lividus	*spermiotox*	0.99±0.69	0.01	<0.01	2.39±0.21	0.5	0.25
	embriotox	9.04±0.45	0.50	0.10	5.09±0.45	1	0.5
Sparus auratus (juveniles)		-	-	[b]2	>1	-	[a] < 10
Dicentrarchus labrax (larvae)		[a]134.59±23.96	[a]12.50	[a]2.50	-	-	-

a: µg/L; b: max concentration tested

TABLE 1 Toxic effects of Irgarol and Diuron for different marine organisms

resulted to be more sensitive compared to juveniles, probably due to an easier absorption. However, some observations about the insurgence of sublethal effects were evidenced also in the juveniles, such as scarce reactivity, and altered orientation.

Conclusions

The sensitivity range of the different organisms to Irgarol and Diuron was quite similar. The two biocides showed a high toxicity for algae species, whereas they resulted moderately toxic toward the other species. The highest sensitivity of algae is linked to the mechanism of action of these compounds; both compounds are photosynthesis inhibitors acting upon transport electron chain in the photosystem II.

The NOECs were between 0.08-0.31 mg/L and 0.62- 2.5 mg/L for Irgarol and Diuron, respectively. Among the tested species, *T. suecica* was the less sensitive to Irgarol while I. galbana to Diuron. Similarly, looking at the EC50s, Irgarol always resulted the most toxic compound.

Sea urchin embryos seem to be very sensitive to Irgarol. The spermiotoxicity test shows an EC50 value in the same range as those reported for crustaceans [12] and a NOEC of 0.10 mg/L. 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 malformed larva, mainly affected by skeletal alterations.

Exposed sperms show a dose-related decrease in fertilization ability 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). The herbicide affected only the sperm fertilization ability, producing an acute spermiotoxicity. Although belonging to a different chemical class of pesticide (phenylureas) than Irgarol, Diuron is a photosynthesis

inhibitor, too, but the mode of action at the biochemical level has not been precisely determined so far. Diuron ecotoxicological data in recent literature are quite scarce, particularly with reference to marine species. Hernando et al. [13] reported toxicity effects on *D. magna* (EC50 8.6 mg/L ± 1.3) and *V. fisheri* (EC50 100 mg/L ± 7.8).

Although the sensitivity of tested organisms indicated that concentrations necessary to cause severe toxicity are higher than the reported environmental levels reviewed by Konstantinou and Albanis 2004 [12], this may not indicate any absence of risks, since interactions and synergic effects with other contaminants can take place [14].

It is also important to consider that, from 2008 onwards, tributyltin-based paints have been totally banned and the environmental levels of the replacing organotin-free biocide can considerably increase. Moreover, these active compounds can accumulate in marine sediments, especially if introduced as paint particles [15]. Compared with leached biocides, those bound to particles are considerably more persistent and, therefore, likely to pose a longer term threat to marine organisms.

references

1. L.W. Hall, P. Gardinali (2004), "Ecological risk assessment for Irgarol 1051 and its major metabolite in United States surface waters", *Human and Ecological Risk Assessment,* 10, 525-542.
2. I. Omae (2003), "Organotin antifouling paints and their alternatives", *Applied Organometallic Chemistry,* 17, 81-105.
3. G. Di Landa, L.Parrella, S. Avagliano, G. Ansanelli, E. Maiello, C. Cremisini (2009), "Assessment of the potential ecological risks posed by antifouling booster biocides to the marine ecosystem of the Gulf of Napoli (Italy)", *Water Air and Soil Pollution,* 200, 305-321.
4. H. Okamura, T. Nishida Y. Ono, W.J. Shim (2003), "Phytotoxic effects of antifouling compounds on nontarget plant species", *Bulletin of Environment Contamination and Toxicology,* 71, 881-886.
5. M.J. Behrenfeld, RT, O'Malley , D.A., Siegel C.R,.McClain, J.L. Sarmiento, G.C. Feldman, et al. (2006), "Climate-driven trends in contemporary ocean productivity", *Nature*, 444,752–5.
6. ASTM Standard guide for acute toxicity test with the rotifer Brachionus. *Annual Book of ASTM Standards Philadelphia* (1998),1440-1491.
7. B. Dahl, H. Blanck (1996), "Toxic effects of the antifouling agent Irgarol 1051 on periphyton communities in coastal water microcosms", *Marine Pollution Bullettin,* 32, 342–350.
8. APAT-IRSA (2003). APAT-IRSA – METODI ECOTOSSICOLOGICI. 8060. Metodo di valutazione della tossicità acuta con Artemia sp.
9. S. Manzo, S. Buono, C. Cremisini (2006), "Toxic effects of Irgarol and Diuron on sea urchin Paracentrotus lividus early development,fertilization, and offspring quality". *Archives of Environmental Contamination and Toxicology,* 51, 61-68.
10. USEPA, (1996), "Fish early stage toxicity test Ecological Effects Test guidelines", *OPPTS* 850.1400.
11. USEPA, (1996), "Fish acute toxicity test, freshwater and marine. Ecological Effects Test guidelines". OPPTS 850.1075.
12. I.K. Kostantinou, T.A. Albanis (2004), "Worldwide occurrence and effects of antifouling paint booster biocides in the aquatic environment: a review", *Environment International,* 30, 253–248
13. M.D. Hernando, M. Ejerhoon, A.R. Fernandez-Alba, Y. Chisti (2003), "Combined toxicity effects of MTBE and pesticides measured with Vibrio fisheri and Daphnia magna bioassays", *Water Research,* 37, 4091–4095.
14. S. Manzo, S. Buono and C. Cremisini (2008), "Predictability of Copper, Irgarol and Diuron combined effects on sea urchin Paracentrotus lividus", *Archives of Environmental Contamination and Toxicology,* 54, 57-68.
15. K.V. Thomas, M. McHugh,, M. Waldock (2002), "Antifouling paint booster biocides in UK coastal waters: inputs, occurrence and environmental fate", *Science of Total Environment,* 293, 117–127.