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Effects of Organotin Alternative Antifoulants on Oyster Embryo

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Abstract In September 2008, organotin (Ot) compounds were prohibited from being used worldwide. From 1997 onward in Japan, the production of paints containing TBT (tributylin) compounds was prohibited, and thus alternatives to Ot antifoulants have been used since then. It has been said that the decomposition characteristics of these materials are better than those of Ot compounds. The toxicity of alternative Ot antifoulants (e.g., diuron, irgarol 1051®, and Sea-Nine 211®) and Ot compounds (TBT and TPT (triphenyltin)), using oysters that inhabit a large area of Hiroshima Bay, were evaluated. The results showed that the toxicity of diuron and irgarol 1051 is very low, and the toxicity of Sea-Nine 211 is almost the same as that of TPT. Sea-Nine 211's effect was stronger on oysters than other shellfish, causing concern about the extent of Sea-Nine 211's impact on oyster development.

Organotin (Ot) compounds, used for many years as antifouling biocides on ships, marine structures, and fishing nets, have become a problem because of their toxicity and accumulation characteristics. Many advanced countries limited the use of Ot compounds in the latter half of the 1980s, and in Japan, administrative regulation was strengthened in 1990. Based on activities related to the Chemical Substances Control Law, Ot compounds were designated as Class II Specified Chemical Substances. After 1997, the production of paints containing TBT compounds was prohibited.

After international restrictions on the use of Ot-based antifoulants were imposed, paint manufacturers developed many alternative products. In Japan, research was performed by the Japan Shipbuilding Research Association for 3 years (beginning in 1991) to find alternative Ot compounds, and >20 chemical substances were proposed as alternative compounds. It was thought that these compounds could be safely used on marine vessels.

Up until recently, the antifoulant materials had not been disclosed, but the Japan Paint manufacturers Association has now disclosed registered data on their homepage. According to this information, the most frequently used compounds are TPBP (triphenylborane-pyridine), diuron (3-(3,4-dichlorophenyl)-1,1-dimethyl urea), Sea-Nine 211® (4,5-dichloro-2-*n*-octyl-3(2H) isothiazolone), chlorothalonil (2,4,5,6-tetrachloro isophthalo nitrile), ZnPT (zinc-2-pyridinethiol-1-oxide), CuPT (copper, *bis*(1,hydroxyl-2(1H)-pyridine thionatoO,S), and irgarol 1051® (2-methylthio-4-*tert*-butylamino-6-cyclopropylamino-*s*-triazine).

The toxicity of TPBP, diuron, Sea-Nine 211, chlorothalonil, ZnPT, CuPT, and irgarol 1051 used as alternative Ot antifoulants has been studied previously in microalgae (Okamura et al. 2000a), crustaceans (Ernst et al. 1991; Okamura et al. 2000a), shellfish (Manzo et al. 2006), fish (Okamura et al. 2002), and seaweed (Okamura et al. 2000b). Studies of oysters, like those of mussels (Beiras and Bellas 2008; Nadella et al. 2009), have yielded much toxicity-related data. However, most of these data are focused on the effects of heavy metals (Calabrese et al. 1973; Coglianese 1982; Glickstein 1978; His et al. 1996; Okazaki 1976) and other chemical substances (Cruz et al. 2007; His et al. 1999); only a few studies have looked at alternative Ot antifoulants.

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Oysters are native to Hiroshima Bay. Recently, the growth and embryo implantation rates of these oysters have decreased. It was decided to use these oysters in the experiment because of their high sensitivity to chemical compounds (Stebbing et al. 1980; Wikfors et al. 1994; Fichet et al. 1998) as well as the fact that oysters exist worldwide.

Woelke (1972) introduced the method that first employed oyster embryos, and His et al. proposed a revision of the standard American Society for Testing and Materials (ATSM) method (1989). This method enabled testing almost all year round by inducing spawning through thermal stimulation. However, there are many unfavorable factors in this test if one has limited time and facilities. Because there was limited time for the present research, we used oysters that were already spawning. This meant simulating conditions similar to those in an oyster nursery facility.

The rate of fertilization, development after fertilization, rate of deformity in D-shaped embryos, and number of underdeveloped embryos were measured. Then the effects of alternative Ot antifoulants on the oysters' embryology were evaluated.

Materials and Methods

Reagents and Materials

The alternative Ot antifoulants irgarol 1051®, diuron, and Sea-Nine 211® were used for the toxicity tests, and TBT and TPT were used to compare these alternative Ot antifoulants with Ot compounds. Irgarol 1051 was obtained from Chiba Specialty Chemicals K. K. (Japan). Diuron, TBT, and TPT were purchased from Tokyo Kasei Industries. Sea-Nine 211 (95%) came from Rhom and Hass (Philadelphia, PA). Dilute stock solutions (1000 mg/l) were made by dissolving the standard materials in dimethyl sulfoxide (DMSO), and then we made standard solutions (1.0–1000 μg/l) by diluting the dilute stock solutions with artificial seawater. The DMSO (for biochemistry) and 10% formalin solution (for tissue fixation) used were purchased from Wako Pure Chemical Industries (Japan). The artificial seawater was made by dissolving Daigo's artificial seawater SP, which was purchased from Nihon Seiyaku Kogyo (Japan). An alkaline formalin solution was made by further diluting the dilute solutions with the artificial seawater. The oysters used in the experiments were gathered from the breakwater in Itsukaichi Nishi Ward in Hiroshima City, Japan. Kenji Torigoe, who is affiliated with the Department of Education at Hiroshima University, identified the oysters used in the experiments. All the oysters used were *Crassostrea gigas* species.

Equipment

An Olympus CK40 biologic microscope with a magnification of 100× was used to photograph the oyster eggs, and Motic Images Plus 2.2S image editing software package was used to count the number of oyster eggs.

Oyster Toxicity Tests

After some initial trial-and-error tests, it was decided to use the procedure performed at the Fisheries Experimental Station in Hiroshima Prefecture, which simulates the conditions in a nursery. The artificial seawater was bubbled for 1–2 h to oxygenate the solution with dissolved oxygen. The oyster's shell was cut with a scalpel, and the sex organs were removed. Then a slight incision was made in the sex organs and a sample collected; the sample was observed under the microscope; and the sex of the oyster was determined. The scalpel used in the experiments was washed under running water each time a sample was removed. The male oysters were placed in a laboratory dish and kept in a refrigerator. This cold-storage preservation stage was a new stage introduced in the experiments. Because of this, it was possible to use these oysters in the experiments for >1 day. As in many cases, it was important to use samples freely.

In the case of female oysters, beakers filled with artificial seawater were covered with a fine net; the sex organs were placed on top of the beaker; and then the sex organs were cut. The eggs were collected in the beakers and then washed in artificial seawater. The eggs were washed with artificial seawater several times to separate the mature eggs from the immature eggs. Only the mature eggs that settled at the bottom of the beaker were used, unlike the reports by His et al. (1997, 1999), who used the ASTM process to select the eggs (ASTM 1989). A volume of 1 ml standard solution was placed into a 24-hole microplate. Three wells with the same concentration were prepared. Approximately 200 mature eggs were added to each well along with a volume of 25 μl artificial seawater. The sperm, which was preserved in a refrigerator, was diluted with artificial seawater 1000 times; a volume of 25 μl artificial seawater was added to each well; and then the well was used to fertilize the samples. This marked the beginning of the toxicity tests. A constant temperature tank, maintained at 25°C, was used for the microplates during cultivation. Each well was observed under the microscope at 2 and again at 24 h. The development stages of 200 oyster eggs or embryos (normal and abnormal) were identified, and at the same time the oyster eggs or embryos were photographed. After the experiments ended, the 10% lethal concentration (LC_{10}) and 50% lethal concentration (LC_{50}) values were calculated using the Ecotox-Statics software program developed by the Japanese Society of Environmental Toxicology.

Results and Discussion

As shown in Figs. 1 and 2, different morphologic patterns were observed in oyster embryos reared in the alternative Ot antifoulants (irgarol 1051, diuron, and Sea-Nine 211) or in the Ot compounds (TBT and TPT) at concentrations ranging from 1 to 1000 μg/l. Using these images, the toxicity of antifouling biocides was evaluated by examining cell division at 2 h after fertilization and checking embryology (i.e., for D-shaped embryos) at 24 h after fertilization.

Acute Effects

Figure 3 shows survival and deformity rates after a development period of 24 h. In this report, the survival rate of the eggs and the occurrence of deformity in D-shaped embryos was investigated. In the report by His et al. (1997), four types of deformity were shown: convex hinge, indented shell margin, incomplete shell, and protruding mantle. In our experiments, only protruding-mantle deformities were observed.

In the case of irgarol 1051 and diuron, the oyster eggs had developed favorably after of 2 and 24 h, and no evidence of any influence on the development of the oyster eggs was found, even at the maximum concentration studied (1000 μg/l).

In the case of Sea-Nine211, all of the oyster eggs in the 100 μg/l treatment died after 2 h. This happened before any cell division could take place (Fig. 1a). Approximately 10% of the oyster eggs in the 10 μg/l treatment died. This also happened before any cell division had occurred. In the eggs that survived, there were no signs of deformity or delayed development (Fig. 1b). Approximately 5% of the oyster eggs in the 1 μg/l treatment died before any cell division took place. The surviving eggs showed no signs of deformity or delayed development (Fig. 1c).

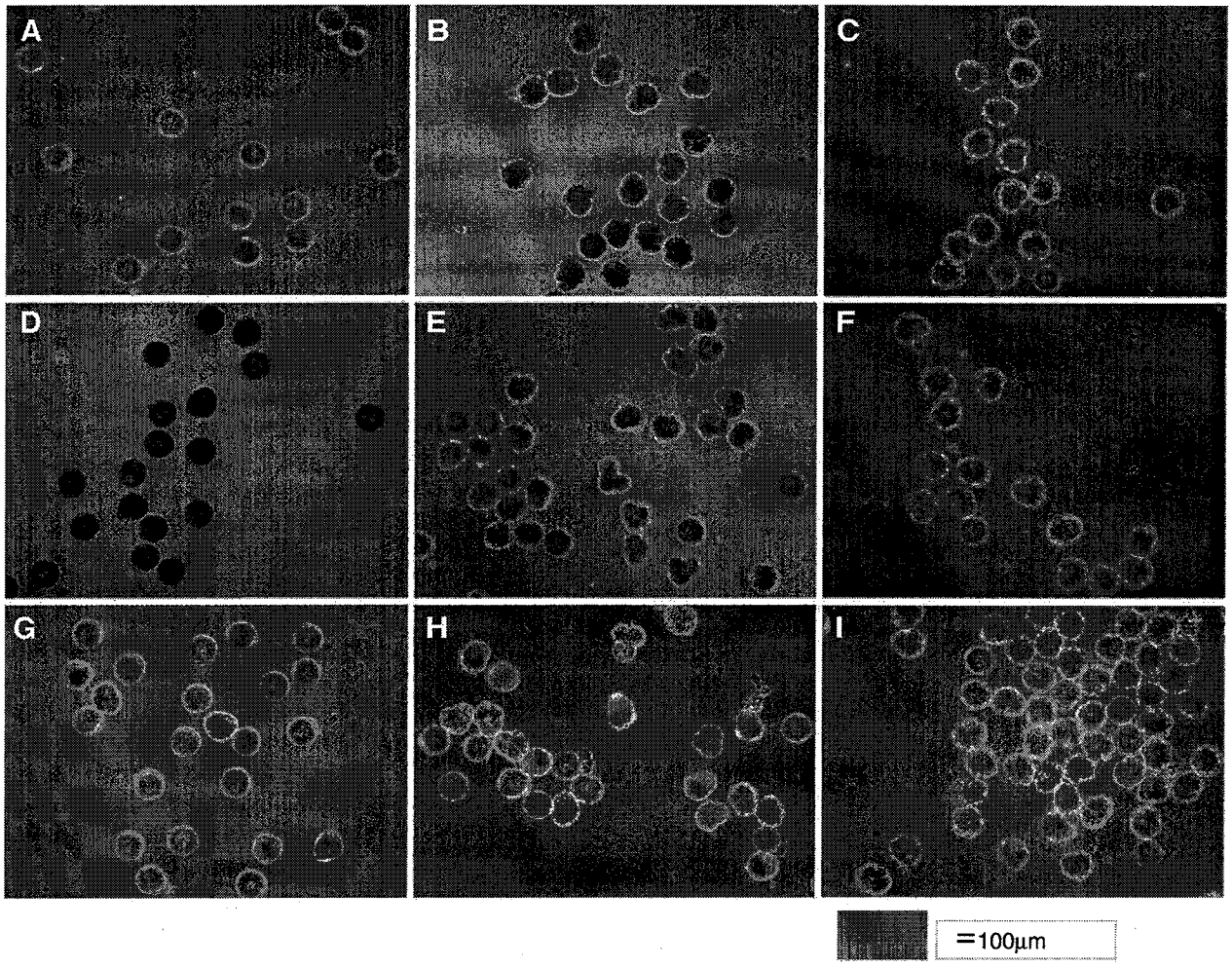

Fig. 1 Fertilized eggs after a period of 2 h. **a** Sea-Nine 211, 100 ppb, **b** Sea-Nine 211, 10 ppb, **c** Sea-Nine 211, 1 ppb, **d** TBT, 100 ppb, **e** TBT, 10 ppb, **f** TBT, 1 ppb, **g** TPT, 100 ppb, **h** TPT, 10 ppb, **i** TPT, 1 ppb

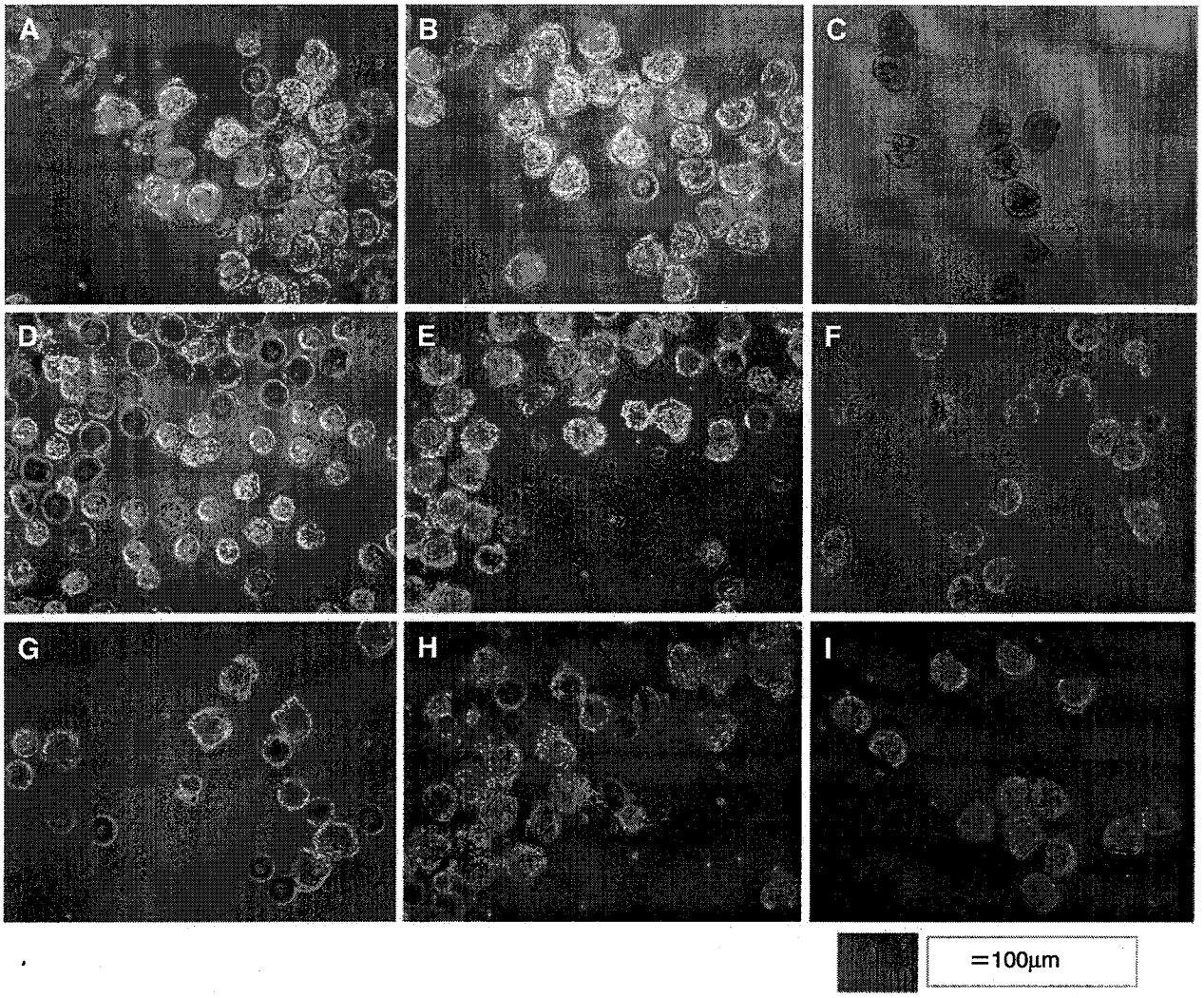

Fig. 2 Fertilized eggs after a period of 24 h. **a** Sea-Nine 211, 10 ppb, **b** Sea-Nine 211, 1 ppb, **c** Sea-Nine 211, 0.1 ppb, **d** TBT, 10 ppb, **e** TBT, 1 ppb, **f** TBT, 0.1 ppb, **g** TPT, 10 ppb, **h** TPT, 1 ppb, **i** TPT, 0.1 ppb

Approximately 30% of the oyster eggs in the 10 μg/l treatment died after 24 h. This happened before any cell division took place. All of the surviving oyster eggs, which became D-shaped embryos, developed protruding-mantle deformity (Fig. 2a). More than 90% of the oyster eggs in the 1 μg/l treatment survived. The surviving oyster eggs became D-shaped embryos. All of these embryos developed protruding-mantle deformity (Fig. 2b). Almost all of the oyster eggs in the 0.1 μg/l treatment survived. All surviving eggs became D-shaped embryos. Most D-shaped embryos were normal, but slightly more than 10% developed protruding-mantle deformity (Fig. 2c).

In the TBT samples, all of the oyster eggs in the 100 μg/l treatment died after 2 h before any cell division could take place (Fig. 1d). Slightly more than 30% of the oyster eggs in the 10 μg/l treatment died before any cell division took place. Approximately 20% of the eggs that survived showed signs of deformity or delayed development (Fig. 1e). Approximately 5% of the oyster eggs in the 1 μg/l treatment died. No cell division had taken place. There were no signs of deformity or delayed development in the surviving eggs (Fig. 1f).

All of the oyster eggs in the 10 μg/l treatment died after 24 h. Some eggs showed no signs of cell division, but in most cases cell division had occurred before death (Fig. 2d). Slightly more than 20% of the oyster eggs in the 1 μg/l treatment died. Every oyster egg that survived became a D-shaped embryo. All D-shaped embryos developed protruding-mantle deformity (Fig. 2e). After cell division had occurred, approximately 10% of the eggs

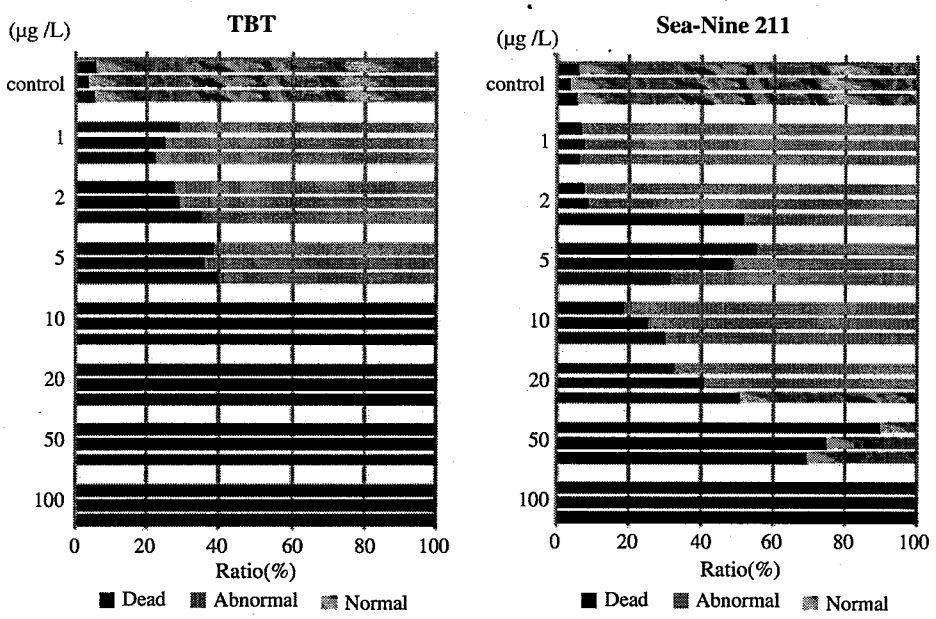

Fig. 3 Survival and deformity rates after a period of 24 h

in the 0.1 μg/l treatment died. Slightly more than 10% of the D-shaped embryos developed protruding-mantle deformity (Fig. 2f).

In the TPT samples, all of the oyster eggs in the 100 μg/l treatment died after 2 h before any cell division could take place (Fig. 1g). Slightly more than 40% of the oyster eggs in the 10 μg/l treatment died before any cell division took place. Slightly more than 30% of the eggs that survived showed signs of deformity or delayed development (Fig. 1h). Approximately 10% of the oyster eggs in the 1 μg/l treatment died. A small number of the eggs that survived showed signs of deformity or delayed development (Fig. 1i).

Approximately 50% of the oyster eggs in the 10 μg/l treatment died after 24 h. In some cases cell division had occurred before death, but most eggs showed no signs of cell division. All surviving oyster eggs, which became D-shaped embryos, developed-protruding mantle deformity (Fig. 2g). Approximately 10% of the oyster eggs in the 1 μg/l treatment died. The surviving eggs became D-shaped embryos. All D-shaped embryos developed protruding-mantle deformity (Fig. 2h). Almost all of the eggs in the 0.1 μg/l treatment survived. Every surviving oyster egg became a D-shaped embryo. Approximately 15% of the D-shaped embryos developed protruding-mantle deformity (Fig. 2i).

LC_{10} and LC_{50} Values

The LC_{10} and LC_{50} values of each compound were calculated from the survival rate of the fertilized oyster eggs after exposure times of 2 and 24 h using the Ecotox-Statics software package. The results are listed in Table 1. It can be seen that the toxicity of irgarol 1051 and diuron was not influential on fertilized oyster eggs at high concentrations (1000 μg/l). In the case of the other compounds, at 24 h the LC_{10} values of Sea-Nine 211, TBT, and TPT were 0.90, 0.36, and 0.52 μg/l, respectively. At 24 h, the LC_{50} values of these compounds were 17, 3.9, and 3.7 μg/l, respectively.

These results were compared with the previously reported analytic 24-h LC_{50} data on the Ot compound TBTO from various marine organisms. The values for *Acartia* sp. (Hall and Pinkney 1985), *Balanus amphitrite* (Hall and Pinkney 1985), *Eurytemora* sp. (Hall and Pinkney 1985), and *C. gigas* (Osada et al. 1989) were 1.0, 4.0, 0.7, and 7.0 μg/l, respectively. The toxicity of TBTO on *Acartia* sp. and *Eurytemora* sp. was a little higher than the

Table 1 Lethal effects of antifouling compounds to oyster embryos

Compound	2 h		24 h	
	LC_{10} (CI)	LC_{50} (CI)	LC_{10} (CI)	LC_{50} (CI)
TBT	2.6 (2.5–2.7)	16 (14–18)	0.36 (0.31–0.39)	3.9 (3.3–4.5)
TPT	2.4 (2.3–2.5)	14 (12–15)	0.52 (0.48–0.54)	3.7 (3.2–4.2)
Sea-Nine 211	7.4 (6.8–7.7)	28 (26–31)	0.90 (0.89–0.91)	17 (14–21)
Diuron	>1000	>1000	>1000	>1000
Irgarol 1051	>1000	>1000	>1000	>1000

values obtained in out experiments. TBTO's toxicity on *Balanus amphitrite* and *C. gigas* was at almost the same level as the values in our experiments. In the case of TPTCl (TPT-chloride), the 24-h LC_{10} value for *C. gigas* was 0.11 μg/l (Sugiyama et al. 1991), which was higher than in our tests.

The alternative antifoulants' 24-h LC_{10} (EC_{10}) analytic data were also compared with that from other marine organisms previously reported. The toxicity of Sea-Nine 211 and irgarol 1051 toward *Mytilus edulis*, *Paracentrotus lividus*, and *Ciona intestinalis* were 7.1 and 800, 5.9 and 2900, and 5.8 and 930 μg/l, respectively (Bellas 2006). Those results showed the same tendency as the results of our experiments, i.e., that Sea-Nine 211's toxicity was high and that of irgarol 1051 was low. In other experiments, the no observed–effect concentration (NOEC) of Sea-Nine 211 toward *Paracentrotus lividus* was found to be 6.5 μg/l (Bellas 2008). The NOEC of Sea-Nine 211 and irgarol 1051 toward the green alga *Scenedesmus vacuolatus* (Arrhenius et al. 2006) and fucoid alga *Fucus serratus* (Braithwaite and Fletcher 2005) was 27 and 0.51 and 8.0 and 8.0 μg/l, respectively. These results showed a different tendency from our results in that the toxicity of Sea-Nine 211 was high, whereas that of Irgarol 1051 was low.

Environmental Risk

In the evaluation of the environmental impact of these chemicals, a risk factor using the residual environmental concentration divided by its NOEC value was employed. When this value exceeded a risk factor of 1.0, it was decided the chemical had an influence on the environment (Hampel et al. 2007). In the case of Sea-Nine 211, toxicity toward the oyster eggs was the highest of the three chemicals studied. The residual environmental concentrations in Barcelona, Spain, and Osaka, Japan are 3.5 μg/l (Martinez et al. 2000) and 0.02 μg/l (Harino et al. 2005), respectively, and their risk factors divided by their NOEC values, 0.90 μg/l (in this case, the LC_{10} value was used), are 3.9 and 0.022, respectively. In the case of Barcelona, the risk factor was much higher than a value of 1.0, thus indicating that Sea-Nine 211 will influence the marine environment.

Conclusion

In our experiments, the ASTM methods were not used because of time and facility restrictions, but a simplified method, which simulated conditions similar to those in an oyster nursery, was employed. By adding a process that preserved the male sex organs after extraction and washing the eggs, the insemination rate was maintained at near 100%. Moreover, it was possible to scale down the experimental facilities by changing the volume of the examination solution to 1 ml. The temperature of the samples was controlled by using a constant temperature tank.

The toxicities of alternative Ot antifoulants (diuron, irgarol 1051, and Sea-Nine 211) were evaluated using the oysters' embryology. The results showed that the toxicity of diuron and irgarol 1051 was very low, whereas the toxicity of Sea-Nine 211 was high. Sea-Nine 211's effect was stronger on oysters than other shellfish, causing concern about the extent of Sea-Nine 211's impact on oyster development.

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