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TOXICITY EVALUATION OF SINGLE AND MIXED ANTIFOULING BIOCIDES USING THE *STRONGYLOCENTROTUS INTERMEDIUS* SEA URCHIN EMBRYO TEST

HENG WANG,†‡ YAN LI,§ HONGHUI HUANG,‡ XUE XU,† and YONGHUA WANG*†
†College of Life Sciences, Northwest A and F University, Yangling, Shaanxi, China
‡Key Laboratory of Fishery Ecology and Environment, South China Sea Fisheries Research Institute, Guangzhou, China
§School of Chemical Engineering, Dalian University of Technology, Dalian, Liaoning, China

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Abstract—The present study evaluated the single and mixed toxicities of commonly used antifouling biocides (copper pyrithione, Sea nine 211, dichlofluanid, tolylfluanid, and Irgarol 1051) on the early embryogenesis of sea urchin *Strongylocentrotus intermedius.* Their toxicities were quantified in terms of the median effective concentration (EC50) reducing the embryogenesis success by 50%. For individual biocides to the embryos, the toxicity was in order of copper pyrithione > Sea nine 211 > tolylfluanid > dichlofluanid > Irgarol 1051. The toxicities of mixture (binary, ternary, quaternary, and quinary) of compounds, evaluated by toxic unit, additivity index, and mixture toxicity index, showed that the copper pyrithione–Sea nine 211 combination was the most toxic with the EC50 value of 7.87 nM in all mixtures. Synergistic enhancements of toxicity were observed for all mixtures except the combination of tolylfluanid–Sea nine 211, revealing antagonistic effect. Both the concentration addition and independent action concepts failed to accurately predict the mixture toxicities of the antifouling combinations; thus, a new log K_{OW}-based model was developed to predict the combined toxicities of these antifouling chemicals, which were capable of predicting the mixture toxicities of antifouling biocides ($R^2 = 0.33$). Environ. Toxicol. Chem. 2011;30:692–703. © 2011 SETAC

Keywords—Antifoulants Embryo Mixture toxicity Log K_{OW} Sea urchin

INTRODUCTION

The widespread use of antifouling paints, such as tributyltin, to prevent the attachment of fouling organisms on submerged structures has caused sublethal effects such as poor growth rates and low reproductive success in a wide range of marine organisms [1]. Therefore, some new environment-friendly organic booster biocides have been developed as tributyltin-free coating alternatives, such as Irgarol 1051, copper pyrithione, dichlofluanid, tolylfluanid, and Sea nine 211 [2].

The *s*-triazine herbicide Irgarol 1051 was the first booster biocide gaining prominence as an environmental contaminant [3]. The presence of Irgarol has been found at concentrations up to 1,700 ng/L in the surface waters of marinas on the Côte D'Azur, France, by Readman et al. [4]. Among the substitutes for organotin compounds, metal pyrithiones, such as copper pyrithione (CuPT; bis-[hydroxy-2(*H*)-pyridine thionate-*O*, *S*]-copper, introduced in 1996), are marketed as environmentally neutral, nonpersistent antifouling biocides because they can undergo photolysis and rapidly degrade into less toxic compounds [5]. In early studies, concentrations of marina water samples for CuPT were found to be below the limit of detection (<20 ng/L; [6]), and few studies reported the effects of CuPT on marine organisms [7].

Sea nine 211 is another highly effective biocide against a wide range of fouling organisms. Significant concentrations (49–3,700 ng/L = 0.2–13 nM) of Sea nine 211 have been found in Spanish, Danish, and Greek marinas because of its use as a biocide in antifouling applications [2]. Dichlofluanid and tolylfluanid, fungicides primarily used as crop preservatives, are currently applied to ship hulls to prevent the growth of bacteria and invertebrates [8]. Similarly, because of their common use, the occurrence of dichlofluanid was monitored in seawater in the Mediterranean Coast (4–600 ng/L), and relatively high concentrations were found in marine sediment (7.2–688.2 ng/g). Only limited tolylfluanid toxicity data are available using sea urchin or *Daphnia magna* [9].

In response to the input of antifouling agents into aquatic ecosystems, the biocide contamination of water presents a serious environmental problem. Therefore, evaluation of the risk associated with the occurrence of those biocides in the marine environment and their monitoring in the coastal waters, as well as their toxicity to aquatic organisms, are urgently demanded.

Most of the research concerned the single toxicity of antifoulants in early studies; however, most of these booster biocides are used in combinations with paints. As shown by these studies, the aquatic organisms are typically not exposed to single substances but rather simultaneously to multiple mixtures of chemicals (e.g., Sea nine 211 < 1–3,000 ng/L approximately, and dichlofluanid < 4–600 ng/L in seawater of Catalonia). Additionally, a synergistic toxic response has been seen for a clear majority of the cases, suggesting that a consideration of individual component toxicity alone was not sufficient for determining the environmental impacts of toxicants. Therefore, the assessment of the mixture toxicity of antifoulants to aquatic organisms is also urgently needed.

The toxicity studies of antifouling biocides by embryo–larval bioassays have been ongoing for some decades [9]. The embryo–larval bioassays, in particular those with bivalves and sea urchins, were sensitive, simple, and reliable tools for assessing and monitoring marine pollution. Therefore, the bioassays with early developmental stages of the intermediate (short-spined) sea urchin *Strongylocentrotus intermedius*

* To whom correspondence may be addressed (yh_wang@nwsuaf.edu.cn).
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(Echinodermata, Echinoidea; A. Agassiz, 1863) were conducted in the present work. *Strongylocentrotus intermedius* is an economically important sea urchin inhabiting the northwest Pacific region of Asia and reported in a previous study [10]. The species is usually found from the littoral and upper sublittoral zone to a depth of 25 m, playing key ecological roles in the general functioning of ecosystems by removing algal communities or by preventing their establishment, leading to dramatic changes in the structure of benthic assemblages.

In an early study, Placket and Hewlett [11] identified three types of actions in analyzing the joint toxicity of binary mixture; to further quantitatively assess the degree of mixture effect, three toxicity indexes indicators have been proposed in the toxicity studies to quantify and analyze the mixture effects: toxic unit (TU), additivity index (AI), and mixture toxicity index (MTI) [12].

Because the occurrence of antifouling agents in marine environment varies both qualitatively and quantitatively, the experimental testing of every mixture is simply impossible. Consequently, several models have been developed for the prediction of combination effects on the basis of the concentration–response relationships of individual mixture components. The general idea is that the mixture toxicity of substances with a common target site and a similar mechanism of action can be described by the concept of concentration addition (CA) [13], and substances with dissimilar mechanisms of action and different target sites by the concept of independent action (IA) [14]. Thus, both concepts are suitable for a predictive assessment of mixture toxicity [15].

However, in some research, the two models (CA and IA) cannot denote the toxicity of compounds incorporating an organic phase and are only partially suitable for describing the mixture toxicity [16]. A good indicator of toxicity, the hydrophobicity of a compound, which is expressed as its log K_{OW} (octanol/water partition coefficients) value, was selected by us to build a log K_{OW}-based model for prediction of the mixture toxicity of antifoulants.

Thus, in the present study, we set out to experimentally test the individual and mixture toxicity of five selected dissimilarly acting antifouling agents to the embryonic development of a new species of sea urchin that was not reported in previous studies, assess the degree of the mixture effect with TU, AI, and MTI and compare the observed mixture (binary, ternary, quaternary, and quinary) toxicity with the predicted mixture toxicity according to IA and CA model, and further develop a new log K_{OW}-based model to evaluate the relationship between log K_{OW}s and the pEC50 ($-$log EC50) values of the biocides.

MATERIALS AND METHODS

Biological material

Experiments were carried out from June 16 to July 6, 2009, with mature *S. intermedius* (diameter $= 6.2 \pm 0.5$ cm, $\sim$two years old). Before the experiment, the sea urchins were fed with natural algae and acclimated for one week in flow-through filtered natural seawater (FSW) (0.45 μm filter) system at $15 \pm 1^{\circ}$C in the pond with 70 L.

For each experiment, the females and males were selected with high egg quality (no immature forms, no dilapidation, and no fertilization) and good sperm motility, respectively. Gametes were harvested and embryos were reared according to the standard protocol as described by Pagano et al. [17]. Spawning was induced in sea urchin by injection of 1 ml 0.5 M KCl (3.73 g KCl in 100 ml distilled water) through the perioral membrane. Gametes were filtered through nylon cheesecloth (D = 200 μm for eggs and 50 μm for sperm). Eggs were transferred into a measuring cylinder of FSW, and were filtered and washed three times with FSW. Then eggs were transferred into a measuring cylinder of FSW and stored at 20°C until use. The egg suspension (stock solution) was diluted to obtain a final concentration of 100 to 120 eggs/ml. The sperm solution was stored at 4°C until use.

Test chemicals

The selected five antifouling biocides were analytical-grade copper pyrithione (CuPT; bis-[hydroxy-2(H)-pyridine thionate-O,S]-copper), Irgarol 1051 (2-methylthio-4-tertiary-butylamino-6-cyclopropylamino-*s*-triazine), dichlofluanid (1,1-dichloro-*N*-[(dimethylamino)sulfonyl]-1-fluoro-*N*-phenylmethanesulfenamide), tolylfluanid (1,1-dichloro-*N*-[(dimethylamino)sulfonyl]-1-fluoro-*N*-(4-methylphenyl) methanesulfenamide), and Sea nine 211 (4,5-dichloro-2-*n*-octyl-4-isothiazolin- 3-one) obtained from Riedel-de Haën, Sigma-Aldrich. The properties including the structures of these compounds are shown in Table 1.

The stock solutions of the five antifouling biocides were freshly prepared in FSW approximately 1 h before the beginning of the experiments. The solutions contained one organic nontoxic dissolvent 1‰ dimethylsulfoxide for CuPT and Sea nine 211, and the other organic nontoxic dissolvent 1‰ acetone for dichlofluanid, tolylfluanid, and Irgarol 1051. The range of experimental concentrations was chosen on the basis of literature toxicity data and the solubility of the compound [9].

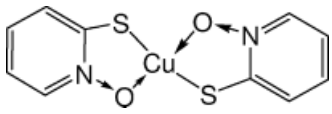
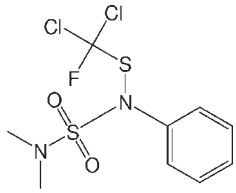
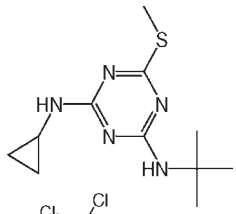
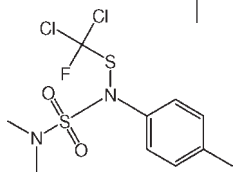
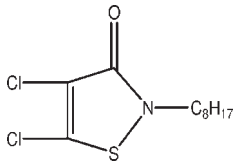
All glassware was acid-washed (HNO_3 10% volume) and rinsed with acetone and distilled water before the experiments. Three replicates of each experimental concentration, three controls with FSW, three dimethylsulfoxide, and three acetone controls were tested. Physicochemical conditions of the experiments were 32.90 ± 0.23 ppt salinity, 6.55 ± 0.35 mg/L dissolved O_2, and 8.2 ± 0.05 pH (mean + standard deviation, $n = 15$) during the entire test.

Single toxicity experiments

The effects of five antifouling biocides on the embryonic development of *S. intermedius* were evaluated by exposing eggs and sperm to a range of concentrations of these compounds as follows: the solutions of these compounds were diluted to get the final 10 concentrations for CuPT the experimental concentrations (from 0.05 to 911.25 nM), for Irgarol 1051 (from 25 to 492,075 nM), for dichlofluanid (from 50–25600 nM), for tolylfluanid (from 5 to 91,125 nM) and for Sea nine 211 (from 6 to 3,200 nM). The actual concentrations in solutions were not checked because they were almost the same as the calculated concentrations; the reasons might be that the numbers of experimental embryos were very low in the test solution, and the exposure time was relatively short (50 h); thus, the adsorption and metabolism of the organic compounds during the exposures could be ignored. In addition, the measurement errors were huge for those extremely low concentrations, which would cause great errors if we applied the measured concentrations.

The vials were incubated at 20°C. After exposure of the gametes to the experimental concentrations, the following crosses were made: untreated sperm × untreated eggs (control), treated sperm × untreated eggs, untreated sperm × treated eggs, and treated sperm × treated eggs. The treated sperm × untreated eggs was that after sperm was exposed to the solutions for 1 h at room temperature, 50 μl treated sperm suspension was added to 10 ml FSW containing untreated eggs (50–60 eggs/ml) obtained

Table 1. Five antifouling biocides studied and their characteristics

Antifouling biocides	CAS No.	CAS Name	Structural formula	Log K_{OW}	Molecular weight
Copper pyrithione[a]	14915–37–8	bis-(hydroxy-2(*H*)-pyridine thionate-*O*,*S*)-copper		0.97	315.86
Dichlofluanid[b]	1085–98–9	1,1-dichloro-*N*-[(dimethylamino)sulfonyl]-1-fluoro-*N*-phenylmethanesulfenamide		3.70	333.23
Irgarol 1051[c]	28159–98–0	2-(tert-Butylamino)-4-(cyclopropylamino)-6-(methylthio)-*s*-triazine		3.95	253.36
Tolylfluanid[b]	731–27–1	1,1-dichloro-*N*-[(dimethylamino)sulfonyl]-1-fluoro-*N*-(4-methylphenyl) methanesulfenamide		4.10	347.26
Sea nine 211[d]	64359–81–5	4,5-Dichloro-2-*n*-octyl-4-isothiazolin -3-one		4.90	282.23

[a] Arch Chemicals.
[b] Chemservice.
[c] Ciba Specialty Chemicals.
[d] Rohm and Haas.

from stock solution. The untreated sperm × treated eggs was obtained by a modified procedure [18]: 50 μl untreated sperm suspension were added to 10 ml FSW containing untreated eggs (50–60 eggs/ml) that had been exposed for 4 h to the solutions at 20°C before fertilization. The treated sperm × treated eggs was made as follows: After exposing sperm for 1 h and eggs for 4 h to the solutions at 20°C, 50 μl of the treated sperm suspension was added to glass vials containing 10 ml of FSW, and 250 to 300 treated eggs were carefully stirred to allow fertilization. The untreated sperm × untreated eggs followed the same exposure conditions. After successive divisions and developmental changes, the ratios of normal four-arm pluteus larvae were checked at 50 h after fertilization. One hundred to 120 embryos were fixed with 5% formaldehyde at the time of these observations.

Mixture toxicity experiments

To detect the interaction in binary mixtures, two antifouling biocides were combined in predetermined proportions to give the same total effect (50% mortality). The individual compounds were mixed at the ratio of their median effective concentrations (EC50s). The binary mixtures consisted of 1:1 of the EC50 for one chemical, with the other chemical constituting the remaining percentage by its EC50 times the fraction amount, to determine the combination ratio. For the ternary, quaternary, and quinary mixtures, the proportions tested were 1:1:1 (33% of the EC50 of each chemical), 1:1:1:1 (25% of the EC50 of each chemical), and 1:1:1:1:1 (20% of the EC50 of each chemical). The combined effects among the five antifouling biocides were estimated in the present study, and 26 mixtures were prepared: CuPT-Irgarol 1051, CuPT-tolylfluanid, CuPT-dichlofluanid, CuPT-Sea nine 211, Irgarol 1051-Sea nine 211, Irgarol 1051-tolylfluanid, Irgarol 1051-dichlofluanid, tolylfluanid-dichlofluanid, tolylfluanid-Sea nine 211, dichlofluanid-Sea nine 211, CuPT-Irgarol 1051-Sea nine 211, CuPT-Irgarol 1051-tolylfluanid, CuPT-Irgarol 1051-dichlofluanid, CuPT-tolylfluanid-Sea nine 211, CuPT-dichlofluanid-Sea nine 211, CuPT-tolylfluanid-dichlofluanid, Irgarol 1051-tolylfluanid-Sea nine 211, Irgarol 1051-tolylfluanid-dichlofluanid, Irgarol 1051-dichlofluanid-Sea nine 211, tolylfluanid-dichlofluanid-Sea nine 211, CuPT-Irgarol 1051-tolylfluanid-Sea-nine 211, CuPT-Irgarol 1051-tolylfluanid-dichlofluanid, CuPT-Irgarol 1051-dichlofluanid-Sea nine 211, CuPT-tolylfluanid-dichlofluanid-Sea nine 211, Irgarol 1051-tolylfluanid-dichlofluanid-Sea nine 211, and CuPT-Irgarol 1051-tolylfluanid--dichlofluanid-Sea-nine 211. While keeping all the above mixtures' ratios constant, the total concentrations of each mixture were varied so that complete concentration–response relationships could be obtained experimentally and the EC50s could be determined. In the mixture

toxicity experiments, the exposed embryos of sea urchin were used to evaluate the mixture toxicities. For every treated concentration, the tests were conducted in triplicate.

Statistical analyses

Control embryogenesis success was always above 95% normal larvae. All experiments were replicated three times ($n = 3$), and data were expressed as mean ± SD. The EC50 (median effective concentration), defined here as the toxicant concentrations causing 50% reduction in the embryogenesis success, and their 95% confidence intervals were calculated according to the Bliss probit analysis, using response and toxicant concentration data for all solutions [19].

To further quantitatively assess the degree of mixture effect, three toxicity index indicators have been proposed in the toxicity studies to quantify and analyze the mixture effects toxic unit (TU), additivity index (AI), and mixture toxicity index (MTI).Mathematically, TU can be formulated as [20]

$$\mathrm{TU} = \sum_{i=1}^{n} \frac{c_i}{\mathrm{EC}_{x_i}} \tag{1}$$

where n is the number of mixture components, $\mathrm{EC}x_i$ is the concentration of the ith mixture component that provokes x% effect when applied singly, and c_i is the concentration of the respective component in the mixture. Each fraction of $c_i/\mathrm{EC}x_i$ represents the concentration of a mixture component scaled for its relative toxicity and is generally termed the TU of that component. Simple addition is characterized by $\mathrm{TU} = 1$, $\mathrm{TU} > 1$ represents antagonism, and $\mathrm{TU} < 1$ indicates synergism.

The AI was extracted as described by Marking [21], with its equation as follows:

$$\mathrm{AI} = \begin{Bmatrix} 1/M - 1 & \text{if} M \le 1 \\ 1 - M & \text{if} M > 1 \end{Bmatrix} \tag{2}$$

where M is the sum of the concentrations that was expressed as equal fractions of the EC50 of each component ($M = \mathrm{sum}\mathrm{TU}_i$). Simple addition is characterized by $\mathrm{AI} = 0$, $\mathrm{AI} < 0$ represents antagonism, and $\mathrm{AI} > 0$ indicates synergism.

The MTI is determined using methodology originally described by Könemann [22], according to the equation:

$$\mathrm{MTI} = 1\text{-}(\log M/\log M_0) \tag{3}$$

where M is the sum of the concentrations that was expressed as equal fractions of the EC50 of each component ($M = \mathrm{sum}\mathrm{TU}_i$). M_0 is M divided by the largest fraction in the mixture ($M_0 = M/\max(\mathrm{TU}_i)$). For determination of the interaction from the numerical value determined, Table 2 was employed as a measure of the mixture toxicity scale [23].

Three models were used for predicting the mixture effects: the concentration addition (CA), independent action (IA), and log K_{OW}–based models.

Concentration addition assumed that all components in a mixture were similarly acting substances sharing an identical mechanism of action in the exposed organism. This model can be formulated as

$$\mathrm{EC}_{x\mathrm{mix}} = \left(\sum_{i=1}^{n} \frac{p_i}{\mathrm{EC}_{xi}} \right)^{-1} \tag{4}$$

where $\mathrm{EC}x_i$ was the concentration of the ith mixture component that induced a x% effect when applied singly. $\mathrm{EC}x\mathrm{mix}$ was the total concentration of the mixture provoking an x% effect, and p_i denoted the fraction of component i in the mixture. According to CA, each individual compound in the mixture contributes equally to the total toxicity, and any mixture component can be replaced by an equi-effective concentration of another component without affecting the overall effect.

Another model was IA, which assumes that the mixture components present a dissimilar mechanism of action, and which was based on the effects of the mixture components. Mixture toxicity according to IA was based on the effects of the components and can be calculated by

$$\mathrm{E}(C_{\mathrm{mix}}) = \mathrm{E}(C_1 + \cdots + C_n) = 1 - \prod_{i=1}^{n} [1 - \mathrm{E}(C_i)] \tag{5}$$

where $\mathrm{E}(C_{\mathrm{mix}})$ denoted the effect (scaled from 0 to 1) of a component mixture, and $\mathrm{E}(C_i)$ denoted the effect of the ith mixture component when applied singly at the concentration C_i. Accordingly, if the components of the mixture were present at which they did not produce an effect when applied singly, that is, $(\mathrm{E}(C_i) = 0)$, the mixture toxicity was expected to be zero.

The last model was the regression analysis of $\mathrm{pEC50}_{\mathrm{mix},i}$ versus $1\log K\mathrm{ow}_i$ for all the chemicals under study, resulting in the following QSAR equation:

$$\mathrm{pEC50}_{\mathrm{mix},i} = -\sum_{i=1}^{n} a_i \log K_{\mathrm{OW}_i} \tag{6}$$

where $\mathrm{EC50}_{\mathrm{mix},i}$ is the concentration of the mixture component that induce a 50% effect, $\log K_{\mathrm{OW}_i}$ was the logarithm of octanol/water partition coefficients of the ith ($I = 1,2,3,4$) mixture component (in descending order of log K_{OW} values), and a_i is the control modulus. The variable $\log K_{\mathrm{OW}_i}$ s were used to establish the linear regression models for describing the mixture effect toxicity ($\log K_{\mathrm{OW}_i}$ of each chemical in Table 1).

Table 2. Mixture toxicity scale for MTI (mixture toxicity index)

MTI	Classification for toxicity of mixtures
$\mathrm{MTI} < 0$	Antagonism
$\mathrm{MTI} = 0$	No addition (independent action)
$0 < \mathrm{MTI} < 1$	Partial addition
$\mathrm{MTI} = 1$	Concentration addition (sample similar action)
$\mathrm{MTI} > 1$	Synergism (potentiation of the toxic action(s) of one or more of the compounds of the mixture)

RESULTS

Single biocide toxicity

Significant single toxicity effects of the five antifouling biocides on four-arm pluteus were observed after 50 h fertilization. The concentration–response curves and the EC50 values for the different toxicants tested in each bioassay are shown in Fig. 1. It presents the effect of the same compound after concentrations were log transformed for complete visualization of all curves.

The toxicities (EC50 values) of the five compounds tested on the embryos of *S. intermedius* are reported in Table 3.

Among the five organic chemicals, CuPT was found to be the most highly toxic compound; it could affect the embryonic development of *S. intermedius* even at very low concentrations (0.15–33.75 nM) (Fig. 1C and Table 3). For the treated sperm × untreated eggs test, the calculated EC50 for four-arm pluteus (50 h) was 209.26 nM (66.10 μg/L) (Table 3), and its toxicity is sixfold lower than that of the exposed embryos test. For the untreated sperm × treated eggs test, it was 167.69 nM

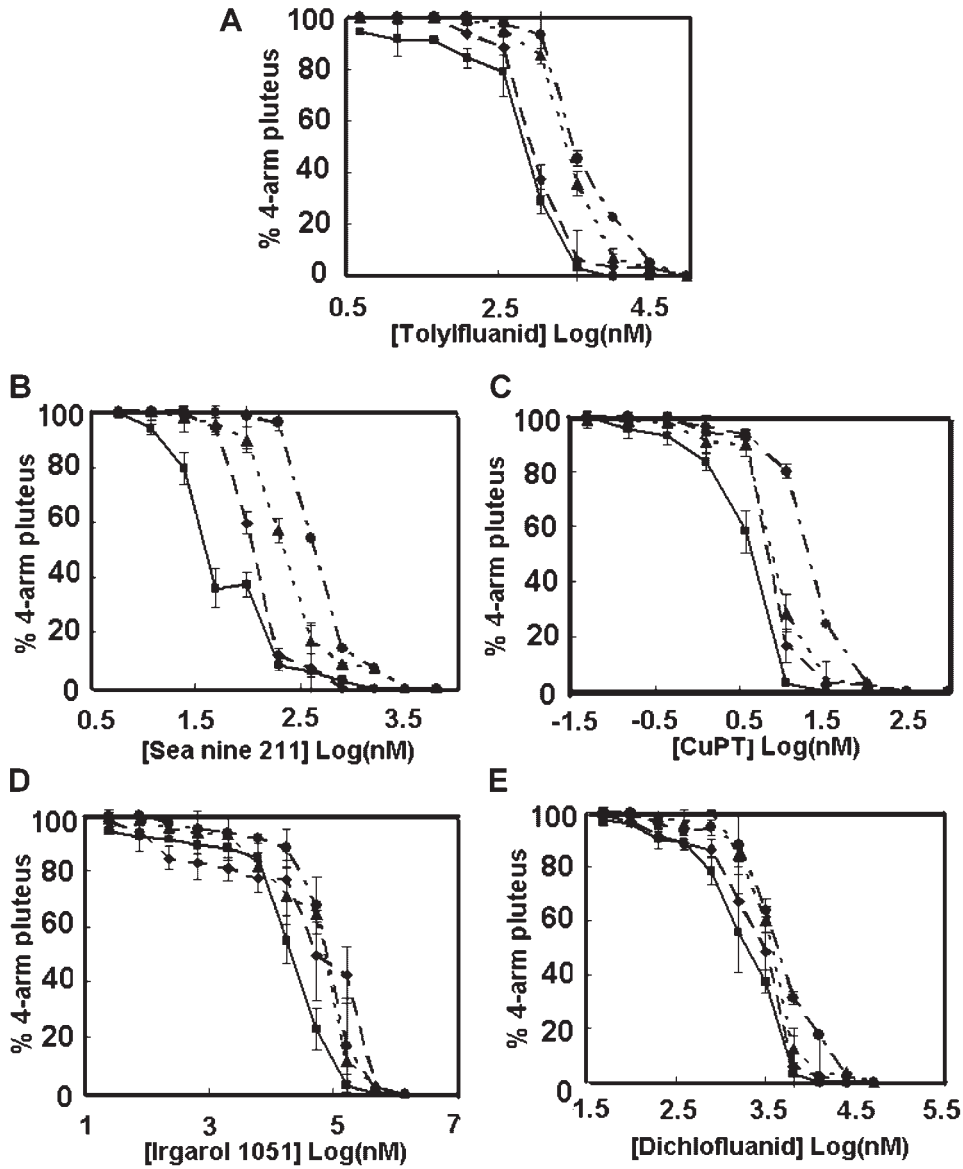


Fig. 1. Percentage of four-arm pluteus in *Strongylocentrotus intermedius* after 1 h exposure of sperm, 4 h exposure of eggs, 1 h exposure of sperm and 4 h exposure of eggs, and exposed embryos to Log_{10}-transformed values of different concentrations (nM) of five antifouling biocides. Circles represent treated sperm × untreated eggs test, triangles are untreated sperm × treated eggs test, squares are exposed embryos (untreated sperm × untreated eggs) test, and diamonds are treated sperm × treated eggs test. Error bars represent the standard deviations.

(52.97 μg/L) (Table 3), which was fivefold lower. When treated eggs were fertilized with treated sperm, the percentage of four-arm pluteus decreased significantly, with an obtained EC50 value of 74.03 nM (35.46 μg/L), which is twofold higher than that of the exposed embryo test. Consequently, we concluded that the embryos of *S. intermedius* were more sensitive to CuPT than other tests, and the sperm was the least sensitive to CuPT.

For Sea nine 211, the calculated EC50 for four-arm pluteus was 50.95 nM (14.38 μg/L) for the exposed embryos test, 112.28 nM (31.69 μg/L) for treated eggs and sperm test, 203.8 nM (57.52 μg/L) for treated eggs test, and 402.46 nM (113.59 μg/L) for treated sperm test, respectively. The sensitive order to Sea nine 211 was the same as CuPT. The tolylfluanid yielded EC50 was 641.45 nM (222.75 μg/L) for the exposed embryos test, 890.17 nM (309.12 μg/L) for treated eggs and sperm test, 2,531.57 nM (879.11 μg/L) for treated eggs test, and 1,337.36 nM (464.41 μg/L) for treated sperm test, respectively. Therefore, the sperm of sea urchin exhibited larger sensitivity to tolylfluanid than eggs in the present study.

The EC50 of dichlofluanid was 1,647.97 nM (549.15 μg/L) for the exposed embryos test, 2,263.18 nM (754.16 μg/L) for treated eggs and sperm test, 3,337.04 nM (1,112 μg/L) for treated eggs test, and 4,418.25 nM (1472.3 μg/L) for treated sperm test, respectively. The embryonic development showed similar sensitivity to dichlofluanid and tolylfluanid as well. The lowest toxic compound was Irgarol 1051, with an EC50 value of 19,089.45 nM (5.89 mg/L) for the exposed embryos, 31,959.6 nM (8.10 mg/L) for treated eggs and sperm, 41,428.63 nM (1.05 mg/L) for treated eggs, and 4,418.25 nM (17.27 mg/L) for treated sperm, respectively. The sensitive order of the four crosses tests was the same as that for CuPT and Sea nine 211.

Therefore, the toxicity EC50s of the tested chemicals toward the exposed embryos of *S. intermedius* was as follows (in descending order of toxicities): copper pyrithione > Sea nine 211 > tolylfluanid > dichlofluanid > Irgarol 1051. This order was the same regardless of exposure to affected sperm, eggs, or both.

Table 3. EC50 (median effective concentration) (nM) of single biocide for the biological responses tested on the embryos of *Strongylocentrotus intermedius*[a]

Antifouling compounds	Test	EC50(nM)
Copper pyrithione	Treated sperm (1 h)	209.26(142.19−277.32)
	Treated eggs (4 h)	167.69 (NC)
	Treated eggs and sperm	74.03(55.58−94.83)
	Exposed embryos (50 h)	32.93(21.34−44.29)
Sea nine 211	Treated sperm (1 h)	402.46(360.42−444.3)
	Treated eggs (4 h)	203.8(178.41−227.98)
	Treated eggs and sperm	112.28(99.13−125.44)
	Exposed embryos (50 h)	50.95(38.39−65.51)
Tolylfluanid	Treated sperm (1 h)	1337.36(935.74−1834.34)
	Treated eggs (4 h)	2531.57(2115.82−2962.61)
	Treated eggs and sperm	890.17(631.88−1183.44)
	Exposed embryos (50 h)	641.45(156.1−1030.32)
Dichlofluanid	Treated sperm (1 h)	4418.25(3461.45−5426.49)
	Treated eggs (4 h)	3337.04(2945.49−3701.95)
	Treated eggs and sperm	2263.18(1443.8−3023.8)
	Exposed embryos (50 h)	1647.97(1077.37−2237.36)
Irgarol 1051	Treated sperm (1 h)	68149.35(47258.75−90608.85)
	Treated eggs (4 h)	41428.63(20787.84−68599.87)
	Treated eggs and sperm	31959.6(12034.05−102162.77)
	Exposed embryos (50 h)	19089.45(8980.38−30786.3)

[a] The 95% confidence intervals (95% CI) are given in parentheses. NC = not calculated.

Mixed biocide toxicities

Toxicities of the five antifoulants to the embryonic development of sea urchin were presented in various combinations in Figure 2.

Binary mixtures toxicity

Ten combinations of binary mixtures of antifouling biocides were found in the present study, such as CuPT-Sea nine 211, Irgarol 1051-tolylfluanid, CuPT-dichlofluanid, and so forth. According to the three toxicity indicators (TU, AI, and MTI) for various antifouling chemical compositions of the binary mixtures, all of the mixtures exhibited synergistic interaction, except for the tolylfluanid-Sea nine 211 mixture, which was the only combination showing antagonistic effect in the present study (Fig. 3).

Figure 2 shows the concentration–response curves for the binary antifoulants, tested individually and in combination. For the CuPT trial (Fig. 2B), the abnormal embryos began to appear at 2.57 nM (811.76 ng/L) in the binary mixture that was higher than CuPT in the single solution with a factor of 17. Consequently, a binary mixture of CuPT with other biocides was less toxic than CuPT singly in the solutions, and other biocides may reduce the toxicity of CuPT. For Irgarol 1051 (Fig. 2A), a comparison of the EC50 values of Irgarol 1051 and its mixtures with other biocides indicated an enhancement of toxicity in all of the cases in which the antifoulants were combined. For Sea nine 211, tolylfluanid, and dichlofluanid (Figs. 2C, D, and E), all of their mixtures showed larger toxicity than their single state. In the whole test for all binary mixtures, the CuPT–Sea nine 211 combination was the most toxic, with an EC50 value of 7.87 nM (Fig. 4).

Ternary mixtures toxicity

The 10 combinations of ternary mixtures, such as the CuPT-Irgarol 1051-Sea nine 211, tolylfluanid-dichlofluanid-Sea nine 211, and Irgarol 1051-tolylfluanid-dichlofluanid, are shown in Figures 2 and 3. For ternary mixtures, test additivity occurred at one-third TU concentrations for each of the biocides. All of the combinations of ternary mixtures of antifouling biocides revealed synergistic effects according to the toxicity indices (Fig. 2). The EC50 values of CuPT varied from 0.36 to 4.41 nM in the ternary mixtures, lower than in the CuPT only test, from 1.42 to 6.74 nM for Sea nine 211, 17.91 to 220.44 nM for dichlofluanid, 6.89 to 102.69 nM for tolylfluanid, and from 207.5 to 3,052.32 nM for Irgarol 1051. The most toxic ternary mixture was the CuPT-tolylfluanid-dichlofluanid, with an $EC5_{mix}$ value of 56.27 nM.

Quaternary and quinary mixture toxicities

Information on the quaternary and quinary toxicity of the five biocides can be found in Figures 2 and 3. The mixtures were tested by the ratios of the individual EC50 values.

Among the six combinations, which all indicated synergistic combined effect (Fig. 3), the CuPT-tolylfluanid-dichlofluanid-Sea nine 211 combination showed the highest toxicity in the quaternary and quinary mixtures, reducing the larval settlement by 50% at the mixture concentration of 181.11 nM. The results also showed that toxic effects of the combinations with five or four antifoulants were not significantly different, because the two response curves were almost overlapped (Fig. 2).

Model adequacy prediction

A comparison of the predicted versus observed mixture toxicities according to the CA, IA, and log K_{OW}-based models of the mixture components with four-arm pluteus of *S. intermedius* is depicted in Figure 4. The CA and IA models underestimated the toxicity of the mixtures using Equations 4 and 5. Regression analysis of the experimental and predicted $EC50_{mix}$s for all of the combinations under study resulted in the following equations. Equations 7 through 9 used the IA, CA, and log K_{OW} -based models, respectively.

$$\begin{gathered}EC50_{mixIApre.}=4.66\times EC50_{mix\,exp.}+7812.8\\(R^2=0.29, F=8.69, SEE=967.48, p<0.01)\end{gathered}\quad(7)$$

$$\begin{gathered}EC50_{mixCApre.}=-0.0023\times EC50_{mix\,exp.}+9.54\\(R^2=0.11, F=2.55, SEE=1086.28, p<0.1)\end{gathered}\quad(8)$$

$$\begin{gathered}EC50_{mixlogP\text{-}basedpre.}=0.25\times EC50_{mix\,exp.}+343.12\\(R^2=0.33, F=10.34, SEE=941.66, p<0.01)\end{gathered}\quad(9)$$

The IA model ($R^2 = 0.29$) was better than the CA model ($R^2 = 0.11$), as revealed by the correlation coefficients between

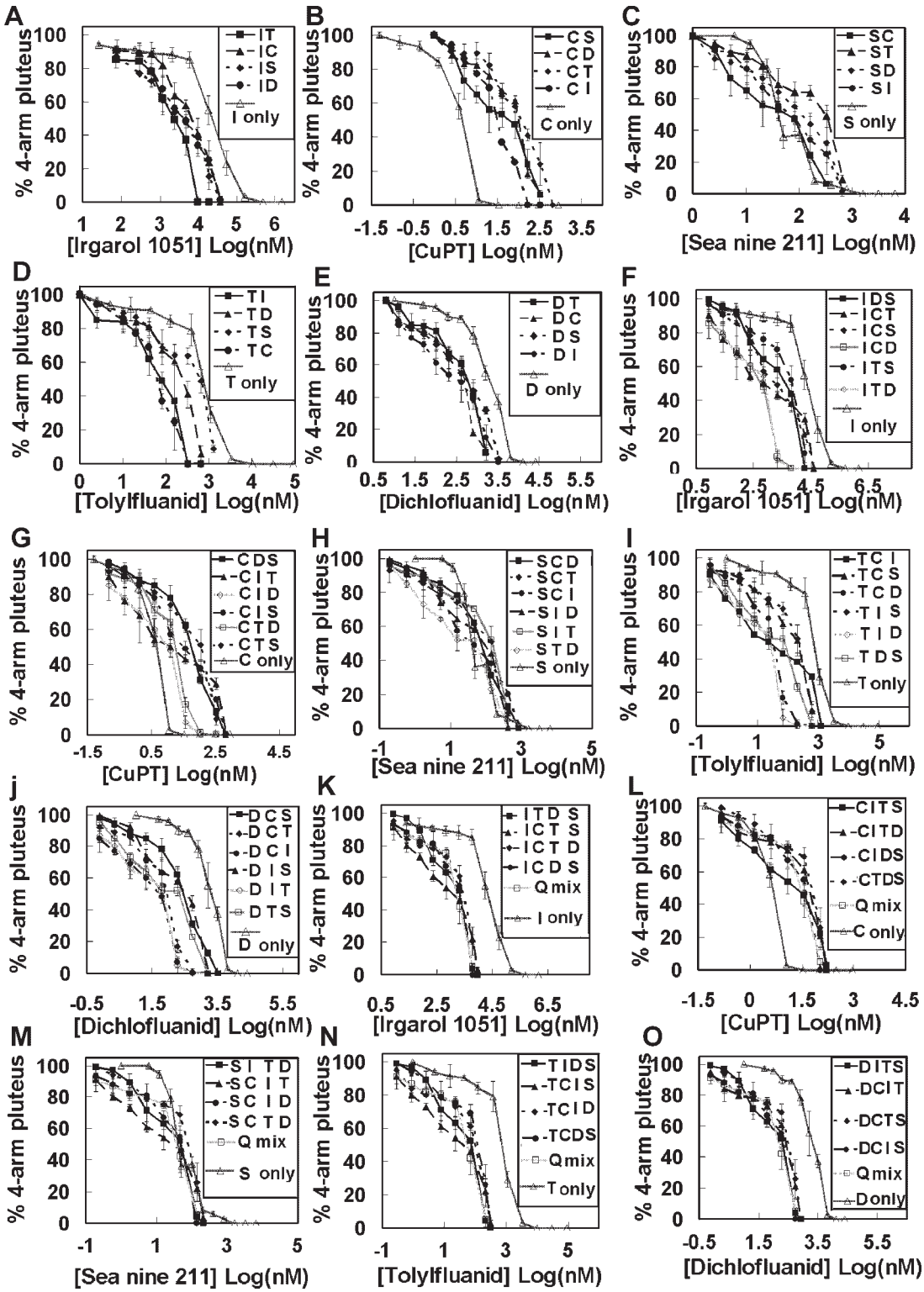


Fig. 2. Percentage of successful four-arm pluteus of *Strongylocentrotus intermedius* after 50 h exposure to Log_{10}-transformed values of different concentrations (nM) of five antifouling biocides in the mixture. Error bars represent the standard deviations. (**A–E**) were binary mixtures, (**F–J**) were ternary mixtures, and (**K–O**) were quaternary and quinary mixtures. A.D., C = CuPT; I = Irgarol 1051; D = dichlofluanid; T = tolylfluanid; S = Sea nine 211; Q mix = quinary mixtures.

the predicted ($EC50_{mixIApre}$) and the experimental toxicity ($EC50_{mixIApre}$). The IA model underestimated the mixture toxicities of these chemicals, and both CA and IA failed to accurately predict the toxicity of the mixtures (Fig. 4). As compared with the CA and IA models, the predicted joint toxicities of compounds using the log K_{OW}-based model were much more parallel with the experimental toxicities ($R^2 = 0.33$) (Fig. 5).

Thus, assessing the relationship between log K_{OW}s and EC50s of the five antifouling biocides to measure the toxic

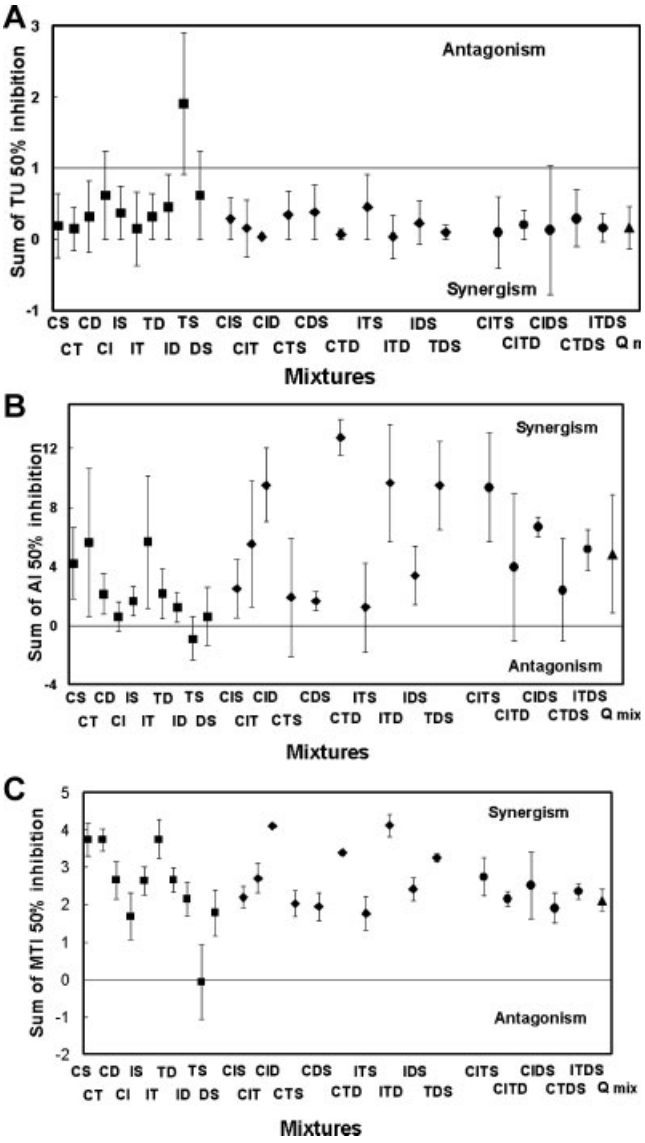


Fig. 3. Toxic unit (TU), additivity index (AI), and mixture toxicity index (MTI) values for various antifouling chemical compositions of the binary, ternary, quaternary, and quinary mixtures' toxicity on the embryos of *Strongylocentrotus intermedius*, with 95% confidence intervals of the fit. Squares indicate the binary mixtures; diamonds, ternary mixtures; circles, quaternary mixtures; and triangles, quinary mixtures. (**A**) TU sum values: TU > 1 represents antagonism, and TU < 1 indicates synergism; (**B**) AI sum values: AI < 0 represents antagonism, and AI > 0 indicates synergism; (**C**) MTI sum values: MTI < 0 represents antagonism, and MTI > 1 indicates synergism. A.D., C = CuPT; I = Irgarol 1051; D = dichlofluanid; T = tolylfluanid; S = Sea nine 211; Q mix = quinary mixtures.

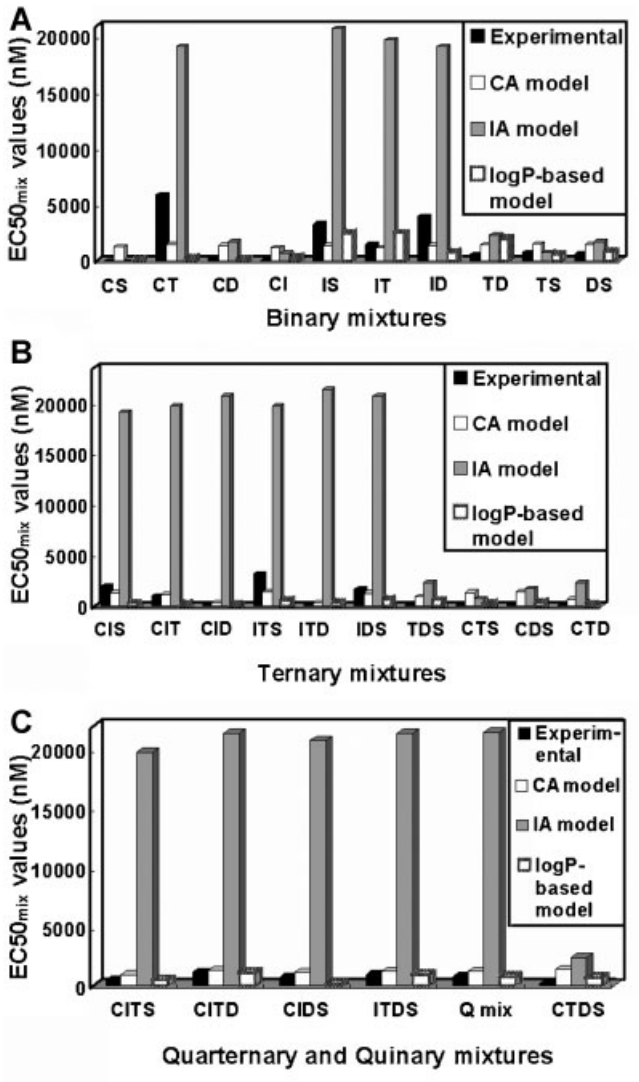


Fig. 4. The relationship between experiment and predicted $EC50_{mix}$ values by concentration addition (CA), independent action (IA), and logP-based models. A.D., C = CuPT; I = Irgarol 1051; D = dichlofluanid; T = tolylfluanid; S = Sea nine 211; Q mix = quinary mixtures. EC50 = median effective concentration.

effects is valuable. The log K_{OW}s of each biocide are shown in Table 1. The log K_{OW}-based models for the single, binary, ternary, and quaternary mixtures of the chemicals are shown in Table 4. In Table 4, a better relationship between EC50 and log K_{OW} (except for CuPT) ($R = 0.70$) was obtained than with other binary and ternary mixtures.

DISCUSSION

Since the international ban on the use of tributyltin in antifouling paints, a variety of other biocides, such as the copper pyrithione, Sea nine 211, tolylfluanid, dichlofluanid, and Irgarol 1051, have been introduced into the market as interesting replacements for traditional tributyltin-based antifouling paints. Therefore, assessment of the risk associated with the use of alternative antifouling compounds is urgently needed because of the increase of their use in the environment; however, limited research has been conducted to assess the toxicology of these compounds, with especially few studies investigating the toxicity of these antifoulants to marine invertebrates, as well as several models that failed in predicting the mixtures toxicity of the chemicals [24]. Therefore, in the present work, in view of some data on the mixture toxicity of antifouling compounds on the embryos of marine invertebrates, we investigated the effects of those selected biocides (copper pyrithione, Sea nine 211, tolylfluanid, dichlofluanid, and Irgarol 1051) on gametes, embryos, and larvae of sea urchin *Strongylocentrotus intermedius*, which are several orders of magnitude more sensitive to pollutants than the adults. We investigated not only the toxicity of the single compound, but also the binary, ternary, quaternary, and quinary mixtures toxicity of these biocides in terms of TU, AI, and MTI to quantify and analyze the mixture effects. For prediction of the

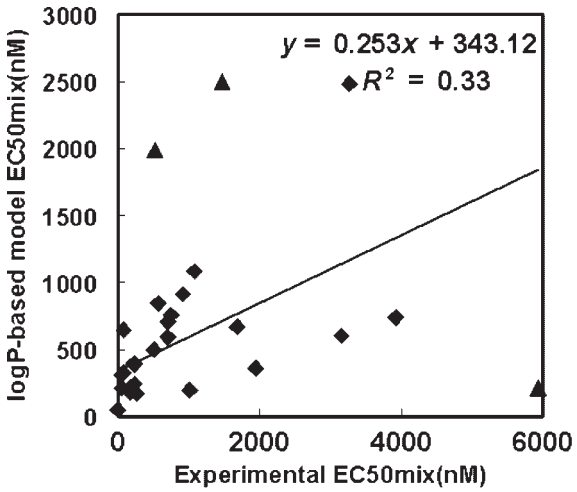


Fig. 5. Regression analysis of the experimental and predicted $EC5_{mix}$ values by the logP-based models. The triangles represent the off-line combinations, which are CuPT-Sea nine 211, CuPT-dichlofluanid, and Irgarol 1051-dichlofluanid combinations. EC50 = median effective concentration.

mixture toxicity of the chemicals, both the CA and IA were applied for the present investigation. To the best of our knowledge, this is the first report of the quinary mixtures toxicity of antifouling biocides to the embryos of the sea urchin *S. intermedius*, a new species that has never been used in previous studies, as well as building log K_{OW}-based models that incorporated an organic phase during the early stages of development of *S. intermedius*.

Single biocides toxicities

Table 5 lists the embryo toxicity EC50 values of the five antifouling biocides (CuPT, Sea nine 211, tolylfluanid, dichlofluanid, and Irgarol 1051), both that described in literature and that experimentally determined in the present study.

The EC50 value of 32.93 nM in the present study was approximately 30 times higher than those reported by Okamura et al. [25]. Nevertheless, based on the EC50 values obtained from the present work (Table 3), copper pyrithione was the most toxic compound to the embryos of the sea urchin *S. intermedius*, which may be because of the mechanism of the chemical. The mode of action for CuPT includes the disruption of cell membranes, disruption of pH-gradients, and complex binding with metals and proteins. These mechanisms lead to a disruption of adenosine triphosphate synthesis and transport through membranes, as well as a starvation of metals and other cations in the cell. Copper pyrithione likely so inhibits the transport through membrane electrical depolarization this depolarization results from inhibition of the primary electrogenic H1-adenosine triphosphatase at an intramembrane or internal site [26].

By a comparison of the toxicities of other four compounds, it was found that their toxicities were all settled in the range of previous studies on other sea urchins (including the *Paracentrotus lividus*, *Glyptocidaris crenularis, S. intermedius*, *Anthocidaris crassispina,* and *Lytechinus variegate*).

Sea nine 211 affected the 50% survival of *P. lividus* at 43 to 69 nM [9], and that of *G. crenularis* at 26 nM in our previous work (Xue Xu et al., Northwest A&F University, Yangling, Shaanxi, China, [27]). Isothiazolones use a two-step mechanism involving a rapid inhibition of growth and metabolism, followed by irreversible cell damage resulting in a loss of viability [28]. The biocide can quickly penetrate cell membranes, as indicated by its high hydrophobicity (log $K_{OW} = 4.9$), and it inhibits specific enzymes in the cell by reacting with intracellular thiols.

As to tolylfluanid and dichlofluanid, their EC50s for *P. lividus* were reported as 1,165 and 1,881 nM, respectively [9], and for *G. crenularis* as 280 and 530 nM by Xu, respectively. The toxicities of these two compounds were similar, and they were less than that of Sea nine 211. These toxicities to sea urchin were likely attributable to their actions on the biosynthesis enzyme dihydropteroate synthase, where sulfonamides act as analogs of one of the substrates, *para*-aminobenzoic acid [29].

For Irgarol 1051, the least toxic compound among the five selected antifouling agents, the EC50 for *P. lividus* ranged from 3,907 to 15,871 nM [9], and for *G. crenularis* at 16,500 nM in our previous work. However, levels of Irgarol 1051 in seawater vary from nondetectable to low parts per billion. Specifically, concentrations up to 1.7, 4.2, and 1.8 μg/L have been detected worldwide [30], which values were significantly less than the EC50 of Irgarol 1051 measured with the sea urchin. Thus, Irgarol 1051 was less toxic to the embryos and larvae of the sea urchin and the most frequently detected antifouling worldwide [3].

In the present study, we observed that the preexposure of *S. intermedius* gametes for 1 h (sperm) and 4 h (eggs) to five antifouling biocides also resulted in a significant decrease of the normal larva rate (Fig. 1), giving rise to embryonic and larval malformations and decreasing larval growth. Previously, the sea urchin (*P. lividus*) eggs suffered from being preexposed to heavy metals [31] and to the booster biocide zinc pyrithione [32]. The preexposure of sperm and eggs to Sea nine 211 also caused transmissible damage to the embryos. All of these reports are in good agreement with our experimental results, which demonstrate that the four-arm pluteus (50 h) of *S. intermedius* was more sensitive than gametes to the five antifouling agents in the test.

Mixed biocides toxicities

Various antifouling chemicals have been used, with main admittances in identical areas (e.g., shipping lanes, harbors); their coexistence and mixture toxicity will certainly play a role in the field situation. In the report by Riley and Zachara [33], various binary and ternary contaminant mixtures were measured in the ground at 91 waste sites at 18 U.S. Department of Energy facilities. No previous reports have been found on the assessment of the quinary mixture of the five antifoulants we selected, although several authors have investigated the binary, ternary, and quaternary mixtures toxicity of these compounds with other antifouling substances. Their studies reported syner-

Table 4. The regression analysis of $pEC_{50mix,i}$ versus $1\log Kow_i$ for all the chemicals following the equation: $pEC_{50mix,i} = -\sum_{i=1}^{n} ai \log Kow_i$ [a]

n	a_1	a_2	a_3	a_4	c	*R*	SEE	*P*
1	1.42	—	—	—	−8.93	0.70	0.40	< 0.1
2	0.36	−0.66	—	—	—	0.63	0.79	< 0.1
3	−0.15	−0.05	−0.19	—	—	0.31	0.74	< 0.1
4	0.49	−0.78	−1.08	−0.16	—	1.00	—	—

[a] *n* is the number of biocides (i.e., single agent $n = 1$, binary mixture $n = 2$, ternary mixture $n = 3$, and quaternary mixture $n = 4$), $\log K_{OWi}$ denoted the octanol/water partition coefficient of *i*th mixture component (in descending order of $\log K_{OW}$: $\log K_{OW1} > \log K_{OW2} > \log K_{OW3} > \log K_{OW4}$). (-) Denotes no data in the blanks; c = constant terms, *R* = relative coefficient, *p* = probability, and SEE = standard error of estimate.

Table 5. The EC50 (median effective concentration) values of the five compounds (copper pyrithione, Sea nine 211, tolylfluanid, dichlofluanid and Irgarol 1051) for the embryo toxicity from literature and this work[a]

Antifouling compounds	Species	Test duration and endpoint	Embryo toxicity EC50 (nM)	Reference
Copper pyrithione[b]	*A. crassispina*	27 h	0.007	[25]
	L. variegatus	3.5 h	1.67	[25]
	S. intermedius	50 h	32.93	Our current study
Sea nine211[c]	*P.lividus*	48 h	43	[9]
	P.lividus	48 h	69	[18]
	G. crenularis	53 h	26	Our previous study
	S. intermedius	50 h	50.95	Our current study
Tolylfluanid[d]	*P. lividus*	48 h	1,165	[9]
	G. crenularis	53 h	280	Our previous study
	S. intermedius	50 h	641.45	Our current study
Dichlofluanid[d]	*P.lividus*	48 h	1,881	[9]
	G. crenularis	53 h	530	Our previous study
	S. intermedius	50 h	1,647.97	Our current study
Irgarol 1051[e]	*P. lividus*	48 h	15,871	[9]
	G. crenularis	53 h	16,500	Our previous study
	S. intermedius	50 h	19,089.45	Our current study

[a] For each experiment, data were transformed to nM, respectively. Literature data regard the following species only: sea urchins *Paracentrotus lividus*, *Glyptocidaris crenularis*, *Strongylocentrotus intermedius*, *Anthocidaris crassispina* and *Lytechinus variegatus*. For each experiment, all data were transformed to nM.
[b] Copper pyrithione is supplied by Arch Chemicals (USA).
[c] Sea nine 211 is supplied by Rohm and Haas (USA).
[d] Dichlofluanid and Tolylfluanid are supplied by Chemservice (USA).
[e] Irgarol 1051 is supplied by Ciba Specialty Chemicals (USA).

gistic effects for the mixtures of copper-zinc pyrithione [34] and Irgarol 1051–2-(Thiocyanomethylthio) benzothiazole-dichlofluanid-Sea nine 211 [35]. Therefore, combined toxicity investigation is essential to the realistic assessment of their environmental impact, and just for this purpose we observed and evaluated the binary, ternary, quaternary, and quinary mixture toxicities of selected antifouling biocides that are used extensively in the world.

At equal molar concentrations of toxicants, the biocide mixtures were used to obtain an equal theoretical probability of competition of the various biocides for the binding sites of target molecules. Based on experimental EC50 values of constituent components, the expected toxicities of the mixtures can be calculated by summing the concentrations. The comparison of the EC50 values of single biocides and their mixtures indicates an enhancement of the toxicity in all of the cases when the antifoulants were combined (Fig. 2); that is, a ternary mixture of Irgarol 1051-tolylfluanid-dichlofluanid was more toxic than Irgarol 1051 by a factor of only 92, but a binary mixture of Irgarol 1051-CuPT showed three times more toxicity than Irgarol 1051 single, and the quinary mixture toxicity of the compounds was 24 times higher than a corresponding single compound in the experiments. In another words, the toxicities of individual components appear to be additive if the substances jointly act on common active sites of metabolic enzymes and if a constituent chemical acts as a diluent for the other components [36].

To quantify and analyze the mixture effects, three toxicity indexes indicators (TU, AI, and MTI) were used in our toxicity studies to see whether the toxic response was additive, synergistic, or antagonistic, which was formulated in Equations 1 through 3. In the present work, a synergistic toxicity for equitoxic mixtures was observed for the binary, ternary, quaternary, and quinary mixtures at EC50 combinations (Fig. 3), and most of the mixtures showed synergistic toxicity. However, the binary mixture of tolylfluanid-Sea nine 211 was the only exception that exhibited an antagonistic effect ($TU = 1.9$, $AI = -0.9$, and $MIT = -0.06$).

In binary mixture experiments, the combination of Sea nine 211 and Irgarol 1051 yielded different results when tested with the green algae *Scenedesmus vacuolatus* (antagonistic) [37] or with *Daphnia magna* (additive) [1], whereas in the present study the effect to the embryos of sea urchin *S. intermedius* was synergistic. An Irgarol 1051-dichlofluanid combination had been reported to be additive for *Vibrio fischeri*, but synergistic for *D. magna* [1], which was expected to have the same mode of action for *S. intermedius* in the present work.

For the ternary, quaternary, and quinary mixtures, synergistic effects were also observed. Because no previous data were available concerning the joint actions of the ternary, quaternary, and quinary mixture compounds belonging to the same chemical group, our work experimentally investigated and provided new data for the mixture toxicity of antifouling biocides coexisting in the seawater. Moreover, from 2008 onward, tributyltin-based paints will be totally banned and the replacing organotin-free biocide environmental levels will increase considerably.

Model adequacy prediction

From the environmental standpoint, toxicity researchers must predict the toxicity of pollutant mixtures, rather than to classify the combinations of toxicants by their types of interactions. The deviation from predicted combined effects may depend on the composition of a mixture. Variations in the type of combined effects for the different proportions of a mixture have been reported in the literature [38].

Because several studies have reported that the mixture toxicity of substances with a common target site and a similar mechanism of action was precisely described by the concept of concentration addition (e.g., Hermens et al. [39]), and that the joint toxicity of strictly dissimilarly acting compounds was also accurately predicted by the independent action (e.g., Backhaus et al. [40]), in our study, two models based on the CA and IA concepts were also built up using the experimental data obtained from our investigation, with an attempt see whether they were still applicable on this dataset to predict the combined

effects based on concentration–response curves of the individual components in mixtures.

However, unfortunately, both CA and IA failed to accurately predict the mixture toxicity of the five antifoulants over the analyzed effect range in the present study. The difference between the IA-predicted and CA-predicted EC50 values is statistically significant in all experiments (Fig. 4). The CA approach in the present study underestimated all of the mixtures of antifoulants exposed to the embryos of sea urchin *S. intermedius*. The obtained CA model is $EC_{50mixCApre.} = -0.0023 \times EC_{50mix\,exp.} + 9.54$, exhibiting relatively poor correlation between the predicted and experimental data ($R^2 = 0.11$, $p < 0.1$). Although CA has been proposed as the generally applicable concept for mixtures of similarly acting substances, CA can give a reasonable estimate of the mixture toxicity of substances with dissimilar mechanisms of action [41]. Thus, some previous studies concluded that CA offers a reasonable worst-case approach, only to be used in the predictive hazard assessment of mixtures [15], which also may be employed in the particular case of antifouling substances.

In contrast, the predictive values of IA were clearly much higher for our five-component antifouling mixture than their corresponding experimental values. The IA model is $EC_{50\,mix\,IApre.} = 4.66 \times EC_{50mix\,exp.} + 7812.8$ ($R^2 = 0.29$, $p < 0.01$) in the present study; as a consequence this implies that the application of IA is not entirely correct for the tested antifoulant mixtures, because the concept assumes a noninteractive type of joint action [42]. Interactions whereby one chemical affects the toxicity of others are possible because of not only the toxicant/target interactions but also the influences on the adsorption, distribution and excretion, biotransformation, and bioavailability [43]. Thus, the use of IA to estimate the toxicity of unknown acting antifouling substances may be incompatible with the precautionary principle.

Recently, several studies have discovered the impact of hydrophobicity on drug absorption, bioavailability, hydrophobic drug-receptor interactions, metabolism of molecules, as well as on toxicity. Thus the hydrophobicity descriptor log K_{OW} (K_{OW} = octanol/water partition coefficient) also has become a key parameter in studies of the environmental fate of chemicals to explain the variation of acute toxicity of chemicals [44]. However, until now the prediction reports of the joint toxicities of antifouling biocides with log K_{OW} descriptor have been unavailable; thus, in the present study a novel log K_{OW}-based model was established to give an organic-phase display of the types of interactions existing among the mixtures.

Comparing the different models applied in the present study for determining mixture toxicity, one can conclude that the predicated EC50 values by log K_{OW}-based model were in good agreement with the experimental results and were better than the CA and IA models in Figure 4, the regression line ($R^2 = 0.33$, $p < 0.01$) that removed the data of the combinations of CuPT-Sea nine 211, CuPT-dichlofluanid, and Irgarol 1051-dichlofluanid. In Table 4, a good correlation ($pEC_{50} = 1.42 \times \log_{Kow1} - 8.93$) was found between the chemical toxicity (of pEC50) of a single compound and its log K_{OW} factor, with $R = 0.70$, in which the toxicity increases when log K_{OW} value increases; that is, Sea nine 211 with log $K_{OW} = 4.9$ was more toxic than dichlofluanid with 3.7. Chemicals with larger log K_{OW} values could more easily cross the cell membrane and inhibit enzymes to disrupt the metabolic pathways. However, one exception exists for this equation: that CuPT was the most toxic compound but had the lowest log K_{OW} value of 0.9. This may be because of its mechanism, which was inhibition of the primary electrogenic H1-adenosine triphosphatase at an intramembrane or internal site, which was different from that of the other four antifoulants. For binary mixtures, the toxicity increases with the ascending of log K_{OW1} values, with the decrease of log K_{OW2} values, according to the weight before the term log K_{OW} in the equation. Therefore, log K_{OW2} (lower values) was a more important factor contributing to the toxicity pEC50 of binary mixtures than log K_{OW1} (higher values). However, in the ternary mixtures, a good correlation ($R = 0.31$) did not exist as in other mixtures. The most important parameter affecting the toxicity was log K_{OW3}, whose value was the lowest in the ternary mixtures, and all the depicted signs before the term log K_{OW} were minus. The toxicity increases with the decrease of all the log K_{OW}s. In addition, an interesting phenomenon was also found: a relative correlation $R = 1.00$ in quaternary mixtures, and the greatest contribution to the toxicity was log K_{OW3} with weight 1.08. The decreasing order was as follows: log K_{OW3} > log K_{OW2} > log K_{OW1} > log K_{OW4}.

CONCLUSIONS

The embryo–larval bioassay was used to assess the individual compound's deleterious effects on gametes, embryos, and larvae of the sea urchin *S. intermedius*, and the binary, ternary, quaternary, and quinary mixture toxicity of five commonly used antifouling agents (copper pyrithione, Sea nine 211, tolylfluanid, dichlofluanid, and Irgarol 1051). Our results show that when the biocide was individually used, the embryos and larvae exhibited larger sensitivities to toxicants than the gametes of *S. intermedius*, in which the copper pyrithione (EC50 = 32.93 nM) was the most toxic compound to the four-arm pluteus after fertilization and 50 h among the five antifouling agents. Irgarol 1051 (EC50 = 19,089.45 nM) was the least toxic at 580 times less toxic. For mixture toxicities of compounds, the tolylfluanid-Sea nine 211 was the only combination showing an antagonistic effect, and all of the other combinations displayed synergistic toxicities in the present study. The fact that these synergistic effects were found not only in the toxicological data of the present study but also in the monitoring data of certain literature, as well as the possible coexistence of biocides in the marine environment, indicate that a high environmental risk may be posed to the marine ecosystem by the presence of the target compounds, pointing out the importance of incorporating combined toxicity studies.

However, to predict the mixture toxicity of pollutants is a main goal from the environmental standpoint. Although we tried to establish two models of CA and IA to predict the toxicity of the antifouling mixtures, they failed. Thus, a new model, based on log K_{OW}, was developed that successfully evaluated the combined toxicities of the chemicals, indicating meanwhile the significant importance of the log K_{OW} of each single substance for combined toxicities. Therefore, the log K_{OW}-based model should be a useful attempt to predict the joint effect of mixtures of antifouling agents. For this purpose, more research concerning the combined action on various marine organisms and more potent models to predict the mixture toxicities are also needed, because antifouling biocides are still being used in many countries all over the world.

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