Remarkable Synergistic Effects in Antifouling Chemicals against *Vibrio fischeri* in a Bioluminescent Assay

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A bioluminescent assay using *Vibrio fischeri* Deutsche Sammlung von Mikroorganismen (DSM) 7151 was applied to evaluate the toxicities of some new antifouling chemicals. First, the efficient concentration, led to 50% inhibition of bioluminescence (EC_{50}) values of the single antifouling chemicals zinc 2-pyridinethiol-1-oxide (Zn-pt), copper 2-pyridinethiol-1-oxide (Cu-pt), $CuSO_4$, zinc bis(N,N′-dimethyl)-dithiocarbamate (Ziram), 4,5-dichloro-2-(n-octyl)-3(2H)-isothiazolone (SeaNine 211), pyridine triphenylboron (PTPB), 3-iodo-2-propynyl butylcarbamate (IPBC), 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Diuron), dichlofluanid (N-dichlorofluoromthylthio-N′,N′-dimethyl-N-phynylsulfamide) (DCF), and 2-methylthio-4-*tert*-butylamino-6-cyclopropylamino-s-triazine (Irgarol 1051) were determined to be 0.08, 0.12, 0.22, 0.31, 0.35, 0.75, 8.49, 12.74, 39 and > 40 mg/l at 30 min of incubation, respectively. Then, 45 different combinations composed of two antifoulants each were evaluated. Based on the EC_{50} values at 30 min of incubation, typical patterns of interaction for the combinations were classified into three groups based on the comparison of inhibition difference between single chemicals and their mixtures. Mixture toxicity indices were also introduced to examine the interaction effect of each combination. The results showed that most combinations were partially additive, and there was no antagonistic effect among the present combinations of chemicals. Additive effects were observed in the case of Diuron, PTPB, or SeaNine 211 when mixed with IPBC. Marked synergistic effects were observed for Irgarol 1051, Ziram, Zn-pt, and Cu-pt when mixed with Cu^{2+}, which will make these chemicals more toxic against organisms in marine environments.

Key words —— antifoulant, synergistic effect, bioluminescent assay, *Vibrio fischeri*

INTRODUCTION

Many types of chemicals, which may be toxic to organisms and communities, have been discharged into various environments through human activities. Numerous antifouling chemicals used for controlling the growth of marine organisms on submerged structures such as hulls of ships have been released into marine environments, while more vessels are sailing around the world.[1)] Under the International Maritime Organization, since 2003 organotin-based antifoulants, which have been widely used for many years in antifouling paints, have been strictly regulated and their use prohibited because of their severe negative impacts on marine organisms.[1–3)] The ecotoxicologic behaviors of new antifoulants used in place of organotin compounds are poorly understood. In particular, their toxicities when used as mixtures have not been evaluated preciously regardless of the high possibility of serious impacts on the environment.[4,5)] Estimation of the toxicity of mixtures of these chemicals is important due to various antifoulants that are mixed in paint products.

Bioluminescence occurs mainly (although not exclusively) in species living in marine environments.[6)] Importantly, light-emitting bacteria are the most abundant and widespread among luminescent organisms.[7)] *Vibrio fischeri* (*V. fischeri*) is a symbiotic bacterium living in the light organs of fish of the family Monocentridae, as well as the cephalopods *Sepiola* and *Euprymna*, and its bioluminescence intensity (BLI) is sensitive to environmental changes.[8,9)] The BLI of cells in certain phases is strictly related to cell activities, which ensures that

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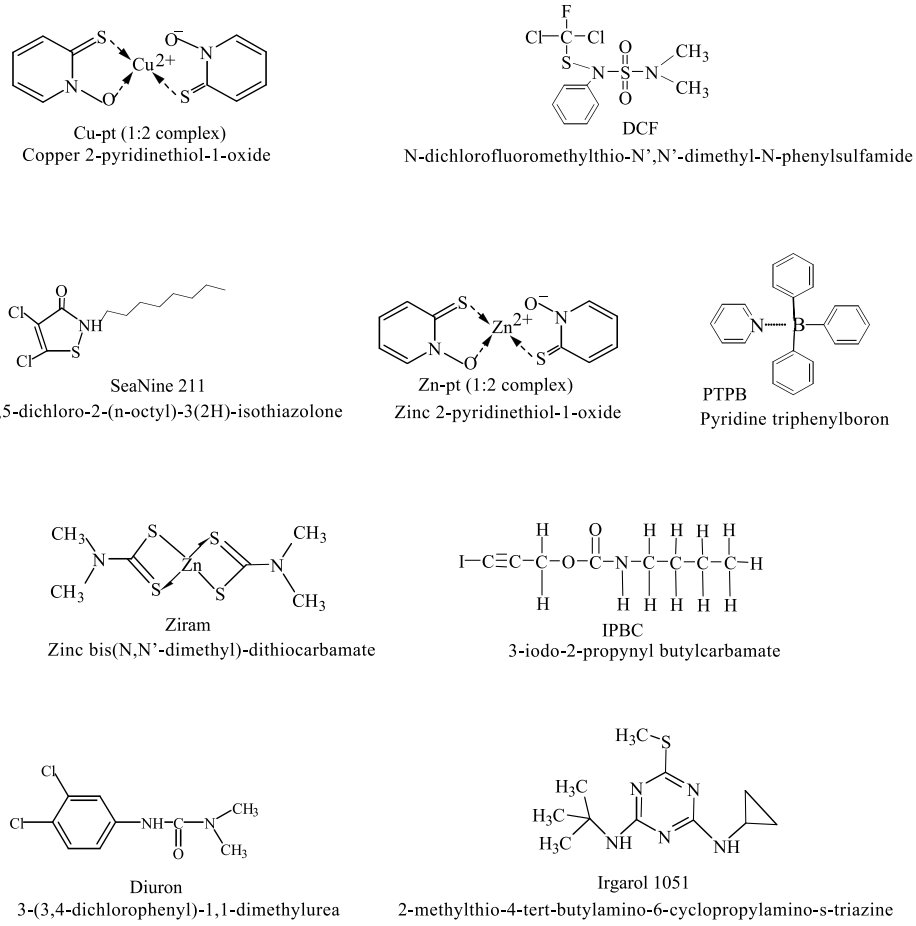

Fig. 1. Molecular Structure of Antifouling Chemicals Examined in this Study

the changes in BLI reflect the impact of the surrounding environment.[10] The usage of bioluminescent bacteria to detect toxic chemicals was proposed as a relatively simple method based on the measurement of BLI reduction by toxic compounds. Such bioluminescent assays were confirmed to be simple and reliable in the detection of toxic substances.[11–13] Our previous study proposed an assay system using freshly incubated *V. fischeri* cells, in which BLI reduction of the cells responded to the toxicity of test samples.[2,3,13]

In this study, 10 types of single antifouling chemicals as well as 45 combinations composed of two each were evaluated in our assay system. In addition, the interactions of these chemicals were examined based on efficient concentration, led to 50% inhibition of bioluminescence (EC_{50}) values as well as the percentage of inhibition efficiency [INH (%)] for single and mixed chemicals, the latter of which were also analyzed through calculation of the mixture toxicity index (MTI).

MATERIALS AND METHODS

Culture Conditions —— *V. fischeri* Deutsche Sammlung von Mikroorganismen (DSM) 7151 used in this study was grown in luminescence (LM) medium, in which 0.5% yeast extract (Difco Laboratories, Detroit, MI, U.S.A.), 0.5% tryptone (Difco), 0.1% $CaCO_3$, and 0.3% glycerol were mixed in artificial seawater (ASW, JIS K-2510). The pH of the medium was adjusted to 7.0 with NaOH. The cells were incubated with a rotary shaker (120 rpm) at 30°C by adding 1%(v/v) of precultures incubated in LM media for 24 hr.

Tested Chemicals —— Except for $CuSO_4$ and $CuCl_2$, all antifouling compounds tested are shown in Fig. 1, and all had purity > 95%. Among them, 2-methylthio-4-*tert*-butylamino-6-cyclopropylamino-s-triazine (Irgarol 1051) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Diuron) are algaecidal and inhibit photosystem-II by interfering with the electron transport chain of photosynthesis in chloroplasts.[14,15]

Dichlofluanid (N-dichlorofluoromthylthio-N′,N′-dimethyl-N-phynylsulfamide) (DCF) was formerly used as a fungicide in agriculture and acts against a wide range of organisms. High activity was observed for 4,5-dichloro-2-(n-octyl)-3(2H)-isothiazolone (SeaNine 211) against a wide spectrum of bacteria, diatoms, fungi, and algae. 3-iodo-2-propynyl butylcarbamate (IPBC) is an inhibitor of acetylcholinesterase in animals in addition to being a highly effective fungicide and bactericide.[15] Zinc 2-pyridinethiol-1-oxide (Zn-pt) and copper 2-pyridinethiol-1-oxide (Cu-pt) are known to be effective biocidal agents and widely used in personal-care products such as anti-dandruff shampoos and a particularly desirable biocide against soft-fouling, respectively.[16] Zinc bis(N,N′-dimethyl)-dithiocarbamate (Ziram) and pyridine triphenylboron (PTPB) are used as a fungicide and antifouling biocide, respectively. Different concentrations of chemicals dissolved in dimethyl sulfoxide (DMSO) were added to ASW by 1%(v/v). To determine the EC_{50} value of a single chemical as well as their combinations precisely, concentrations of chemicals were increased by 1.5-fold between adjacent concentrations. The concentration ratio of two chemicals in a mixture was at the same ratio of each EC_{50} value detected singly. The controls for all samples were ASW containing 1% DMSO.

Measurement of BLI —— As described in our previous paper, cells thus manipulated were suspended in ASW.[13] 0.2-ml cell suspension of *V. fischeri* was added to each well of a 96-well microplate. Then BLI changes in samples in each well were detected using a Multi-detection Micro-plate Reader (Powerscan HT, Dainippon Pharmaceutical, Osaka, Japan) at 30 min of incubation.

Calculation of Inhibition Efficiency and EC_{50} —— To determine the toxicity of chemicals against *V. fischeri*, INH (%) was used in this study, the values of which were calculated from BLI changes between samples and controls as follows:

$$INH(\%) = \left[1 - \left(\frac{IT_t \times IC_0}{IT_0 \times IC_t}\right)\right] \times 100$$

where *INH (%)* is the percentage of inhibition efficiency; intensity of test samples at time t (IT_t) is the BLI of the sample after contact time (t) with chemicals; intensity of test samples at time 0 (IT_0) is the initial BLI of the sample; intensity of control samples at time t (IC_t) is the BLI of controls after contact time (t) with chemicals; and intensity of control samples at time 0 (IC_0) is the initial BLI of controls.

Table 1. EC_{50} Values of Single Chemicals (mg/l)[a]

Chemical	EC_{50} value
Zn-pt	0.08 ± 0.01
Cu-pt	0.12 ± 0.01
$CuSO_4$	0.22 ± 0.02
Ziram	0.31 ± 0.02
SeaNine 211	0.35 ± 0.02
PTPB	0.75 ± 0.05
IPBC	8.49 ± 0.79
Diuron	12.74 ± 1.21
DCF	39.02 ± 2.18
Irgarol 1051	> 40.00[b]

a) EC_{50} values were calculated at 30 min of incubation from triplicate experiments. *b*) The EC_{50} value of Irgarol 1051 was undetectable due to its solubility limitation (about 40 mg/l).

The INH (%) values in this paper are the averages of at least three independent measurements. EC_{50} values are the concentrations corresponding to INH (%) = 50 in the relationship between INH (%) and concentration of chemicals.

Calculation of MTI —— MTIs were calculated using the following equation of Könemann[17]

$$MTI = 1 - (\log M / \log M_0)$$

where M is $\Sigma f(i) = \Sigma C(i)/LC_{50}(i)$, $C(i)$ is the concentration of the ith component in the mixture; $LC_{50}(i)$ is the LC_{50} concentration of the ith component (in the present study, EC_{50} values were used instead of LC_{50}); and $M_0 = M/f_{Max}$. When MTI values were lower than 0, the mixture potency was defined as antagonism, and when it equaled to zero, there was no addition. When it was higher than zero but less than 1, the mixture potency was defined as partially additive. When it equaled to 1 or greater than 1, the mixture potency was defined as additive and synergistic, respectively.

RESULTS AND DISCUSSION

Toxicity Evaluation of Single Chemicals

The BLI of *V. fischeri* was reduced as incubation time increased when antifouling chemicals were added. From the different reduction rates of BLI between samples and controls, we calculated the EC_{50} values. Based on EC_{50} values at 30 min of incubation (Table 1), we divided these chemicals into three groups. The first group is highly toxic with low EC_{50} values of less than 0.5 mg/l, such as Cu-pt, Zn-pt, SeaNine 211, $CuSO_4$ and Ziram. The sec-

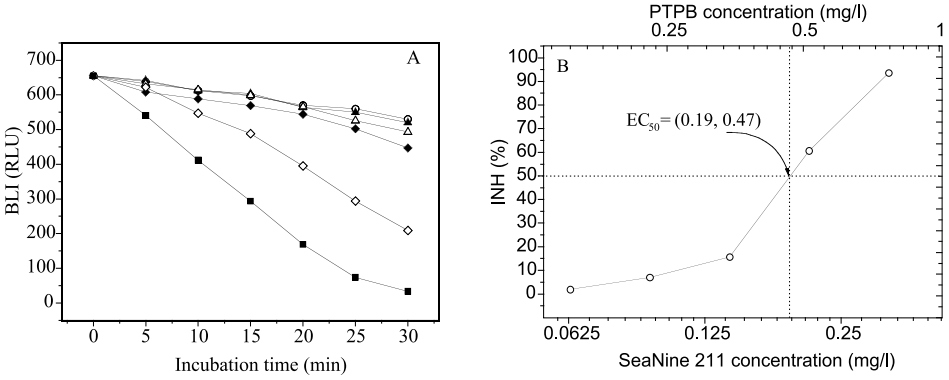

Fig. 2. Inhibition of Bioluminescence Intensity of *V. fischeri* by Combined Chemicals (PTPB and SeaNine 211)

BLI changes in *V. fischeri* were followed using a luminescence reader as a function of the incubation time when combined PTPB and SeaNine 211 were added (A). Concentrations of PTPB and SeaNine 211 were 0.16 and 0.06 (closed triangles), 0.25 and 0.09 (open triangles), 0.38 and 0.14 (closed diamonds), 0.56 and 0.21 (open diamonds), and 0.84 and 0.32 mg/l (closed squares), respectively. Control samples containing ASW with 1% DMSO are shown by open circles. The INH (%) of BLI for this mixture was calculated at 30 min of incubation (B). EC_{50} values of combinations were obtained as the concentrations corresponding to INH (%) = 50. All the data are expressed as the mean of triplicate experiments.

ond group has medium-level toxicity with EC_{50} values ranging from 0.75–10 mg/l for PTPB and IPBC. The third group consists of DCF, Irgarol 1051, and Diuron, which showed low toxicity, that is, EC_{50} values greater than 10 mg/l. Among the highly toxic chemicals, it is interesting that they all have metal elements in their molecular structures, except for SeaNine 211, which indicates that this bioluminescent assay is sufficiently sensitive to detect the toxicity of chemicals containing heavy metals as well as organic molecules like SeaNine 211.[2,3] It is meaningful to note that the toxicity ranking of these chemicals detected in the present assay is consistent with published data obtained from various bioassay systems.[1,11,15]

Toxicity Evaluations of Chemical Combinations

Detection of EC_{50} Values of Chemical Combinations: To clarify whether the toxicities of combined chemicals were enhanced or reduced, each combination of two chemicals was evaluated, an example of which is shown in Fig. 2. The BLI of *V. fischeri* was reduced in the presence of combined chemicals (PTPB and SeaNine 211) with an increase in the concentrations of chemicals as well as longer incubation time (Fig. 2A). At 30 min of incubation, the inhibitory effects at different concentrations became clear. As shown in Fig. 2B, INH (%) was enhanced by the increased concentration of both PTPB and SeaNine 211 in combination. From the cross point of 50%, we determined the EC_{50} values for this mixture to be 0.47 mg/l of PTPB and 0.19 mg/l of SeaNine 211. As shown in Table 2, EC_{50} values for all other mixtures, (A, B), were calculated in the same way at 30 min of incubation.

The toxicity of samples can be described based on EC_{50} values, in which a low value reflects high toxicity since 50% of the BLI of cells is inhibited by a relatively low concentration of test sample. The EC_{50} values in Tables 1 and 2 show the toxicities of each single chemical and combined chemicals, respectively. To determine the interactions of each combination of two chemicals, their toxicity was compared with that of each single chemical.

Comparison of EC_{50} Values of Single and Combined Chemicals: In comparison with each EC_{50} value of Ziram (0.31 mg/l) or $CuSO_4$ (0.22 mg/l) alone in Table 1, the EC_{50} values of their combination were markedly low (Table 2), both of which were less than 1/5 of those for single chemicals. From the comparison of EC_{50} values for single and combined chemicals in Tables 1 and 2, respectively, it becomes clear that EC_{50} values for such combinations as $CuSO_4$ with Cu-pt, Zn-pt, or Irgarol 1051 are also extremely low. All EC_{50} values of these three combinations were reduced to about 1/4 of those for single chemicals. On the contrary, most EC_{50} values of other combinations were about half those of their single chemicals. In comparison with the respective single chemicals, the same concentrations of their combinations showed much higher inhibitory activities, which suggests that toxicity was enhanced because of the synergistic effect of their combination.[18,19]

Typical Patterns of Interaction of Chemicals: Further evidence of toxicity enhancement by com-

Table 2. EC_{50} Values for Combined Chemicals (mg/l)[a)]

Chemical 1	Chemical 2	EC_{50} value	Chemical 1	Chemical 2	EC_{50} value
Zn-pt	Cu-pt	0.06, 0.07[c)]	$CuSO_4$	Irgarol 1051	0.10, 16.08
Zn-pt	$CuSO_4$	0.03, 0.05	Ziram	SeaNine 211	0.21, 0.24
Zn-pt	Ziram	0.04, 0.11	Ziram	PTPB	0.22, 0.59
Zn-pt	SeaNine 211	0.06, 0.17	Ziram	IPBC	0.22, 3.47
Zn-pt	PTPB	0.06, 0.44	Ziram	Diuron	0.28, 6.43
Zn-pt	IPBC	0.07, 2.67	Ziram	DCF	0.21, 25.75
Zn-pt	Diuron	0.07, 4.09	Ziram	Irgarol 1051	0.27, 19.05
Zn-pt	DCF	0.06, 18.50	SeaNine 211	PTPB	0.19, 0.47
Zn-pt	Irgarol 1051	0.06, 17.16	SeaNine 211	IPBC	0.22, 3.00
Cu-pt	$CuSO_4$	0.03, 0.05	SeaNine 211	Diuron	0.27, 5.44
Cu-pt	Ziram	0.07, 0.15	SeaNine 211	DCF	0.25, 26.42
Cu-pt	SeaNine 211	0.09, 0.23	SeaNine 211	Irgarol 1051	0.31, 19.29
Cu-pt	PTPB	0.09, 0.55	PTPB	IPBC	0.51, 2.90
Cu-pt	IPBC	0.09, 3.16	PTPB	Diuron	0.71, 5.98
Cu-pt	Diuron	0.12, 6.03	PTPB	DCF	0.48, 21.05
Cu-pt	DCF	0.08, 21.86	PTPB	Irgarol 1051	UD[b)]
Cu-pt	Irgarol 1051	0.10, 14.86	IPBC	Diuron	4.39, 6.43
$CuSO_4$	Ziram	0.04, 0.05	IPBC	DCF	4.39, 33.92
$CuSO_4$	SeaNine 211	0.09, 0.16	IPBC	Irgarol 1051	UD[b)]
$CuSO_4$	PTPB	0.11, 0.45	Diuron	DCF	6.10, 32.18
$CuSO_4$	IPBC	0.19, 4.39	Diuron	Irgarol 1051	UD[b)]
$CuSO_4$	Diuron	0.12, 4.22	DCF	Irgarol 1051	22.80, 13.44
$CuSO_4$	DCF	0.12, 21.59			

a) EC_{50} values were means at 30 min of incubation from triplicate experiments. Standard deviations are not shown because each was less than 1%. *b*) UD: undetectable due to the low toxicity of Irgarol 1051. *c*) EC_{50} values are shown in terms of (A, B). 50% inhibition of BLI of *V. fischeri* occurred with the combination of chemical 1 and chemical 2, the concentrations of which are denoted by A and B, respectively.

bining chemicals was obtained from typical combinations of Ziram + $CuSO_4$, Zn-pt + $CuSO_4$, SeaNine 211 + PTPB, and Cu-pt + Diuron. Ziram (0.08 mg/l) and $CuSO_4$ (0.06 mg/l) had low INH (%) values when added singly, but their combination showed INH (%) as high as 76% at the same incubation time, as shown in Fig. 3A. Toxicity enhancement with combination was also observed for Zn-pt (< 10%) and its mixture with $CuSO_4$ (83%). The enhanced toxicity provided further evidence of the occurrence of synergistic effects with their combination. The relationship between the toxicities of single chemicals and their combinations shown in Fig. 3B and 3C is different from those in Fig. 3A. In Fig. 3B, SeaNine 211 (0.19 mg/l) and PTPB (0.45 mg/l) showed approximately 25% inhibition of BLI at 30 min of incubation, while their combination resulted in 44% inhibition. Toxicity enhancement with combination, on the other hand, was not observed in the combination of SeaNine 211 and PTPB. In Fig. 3C, however, the toxicity of Diuron or Cu-pt was not increased as markedly in combination, since

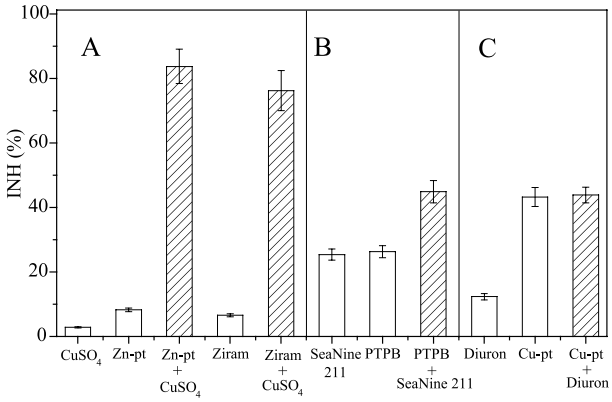

Fig. 3. Typical Patterns of Interactions of Antifouling Chemicals

The INH (%) values of BLI for single chemicals as well as their combinations were calculated based on the reductions of BLI at 30 min of incubation. As typical patterns, the combinations of Zn-pt or Ziram and $CuSO_4$, SeaNine 211 and PTPB, and Diuron and Cu-pt are shown in A, B, and C, respectively. The concentrations of $CuSO_4$, Zn-pt, Ziram, SeaNine 211, PTPB, Diuron, and Cu-pt were fixed at 0.06, 0.04, 0.08, 0.19, 0.45, 5.00, and 0.10 mg/l, respectively. The INH (%) values of single and combined chemicals are shown by open and shaded bars, respectively. All the data are expressed as mean ± standard deviation from triplicate experiments.

Table 3. MTIs for Combinations of Chemicals[a)]

Chemical 1	Chemical 2	MTI	Chemical 1	Chemical 2	MTI
Zn-pt	Cu-pt	0.57	$CuSO_4$	Irgarol 1051	> 1.73[c)]
Zn-pt	$CuSO_4$	2.20	Ziram	SeaNine 211	0.56
Zn-pt	Ziram	1.30	Ziram	PTPB	0.38
Zn-pt	SeaNine 211	0.58	Ziram	IPBC	0.76
Zn-pt	PTPB	0.40	Ziram	Diuron	0.22
Zn-pt	IPBC	0.54	Ziram	DCF	0.56
Zn-pt	Diuron	0.40	Ziram	Irgarol 1051	> 0.33[c)]
Zn-pt	DCF	0.57	SeaNine 211	PTPB	0.75
Zn-pt	Irgarol 1051	> 0.79[c)]	SeaNine 211	IPBC	1.08
Cu-pt	$CuSO_4$	2.33	SeaNine 211	Diuron	0.59
Cu-pt	Ziram	0.86	SeaNine 211	DCF	0.51
Cu-pt	SeaNine 211	0.44	SeaNine 211	Irgarol 1051	> 0.29[c)]
Cu-pt	PTPB	0.37	PTPB	IPBC	0.99
Cu-pt	IPBC	0.37	PTPB	Diuron	0.13
Cu-pt	Diuron	−0.01	PTPB	DCF	0.74
Cu-pt	DCF	0.60	PTPB	Irgarol 1051	UD[b)]
Cu-pt	Irgarol 1051	> 0.56[c)]	IPBC	Diuron	1.00
$CuSO_4$	Ziram	2.60	IPBC	DCF	0.31
$CuSO_4$	SeaNine 211	1.20	IPBC	Irgarol 1051	UD[b)]
$CuSO_4$	PTPB	0.84	Diuron	DCF	0.42
$CuSO_4$	IPBC	0.36	Diuron	Irgarol 1051	UD[b)]
$CuSO_4$	Diuron	1.27	DCF	Irgarol 1051	> 1.18[c)]
$CuSO_4$	DCF	0.87			

a) MTIs were calculated based on the EC_{50} values of single chemicals and their combinations at 30 min of incubation. *b*) UD: undetectable due to low toxicity of both Irgarol 1051 and its combinations. *c*) Since the EC_{50} value of Irgarol 1051 was > 40, the resulting MTI was greater than the values shown.

the toxicity of Diuron and Cu-pt was almost the same as that of Cu-pt alone. In comparison with the two examples of combination shown in Fig. 3B and 3C, Ziram or Zn-pt with $CuSO_4$ in Fig. 3A showed markedly increased toxicity in combination, which was classified as synergistic.

In the same way, toxicity analyses for $CuSO_4$ in combination with Cu-pt or Irgarol 1051 were performed, and marked synergistic effects were seen (data not shown). The interactions of the combinations in Fig. 3B and 3C obviously are other types rather than synergism, as classified in the subsequent section.

MTI Analyses of Interactions

To classify the types of all interactions in the combinations examined, MTIs were calculated from the EC_{50} values of single chemicals and their combinations at 30 min of incubation. As shown in Table 3, most MTIs were positive except for that of the combination of Cu-pt and Diuron, –0.01. The toxicity of Cu-pt did not change with the addition of Diuron, as shown in Fig. 3C. Since this MTI was almost zero, we can conclude that the interaction of this combination has no additive effect.[17)] Except for this combination, we obtained positive data for all the combinations examined. Therefore we can conclude that no antagonistic effect (MTI < 0) occurred in all combinations examined.

Among positive MTI values, the mixtures of $CuSO_4$ with Irgarol 1051, Ziram, Cu-pt, or Zn-pt were > 1.73, 2.60, 2.33, and 2.20, respectively. The MTIs of these combinations were in agreement with their markedly synergistic interactions, as indicated by the toxicity comparisons of single and combined chemicals described above. For the combinations of DCF and Irgarol 1051, $CuSO_4$ and Diuron, Zn-pt and Ziram, and $CuSO_4$ and SeaNine 211, MTIs ranged from 1.2 to 1.3. For these combinations, there were some synergistic effects, since their MTIs were greater than 1. The degrees of toxicity enhancement, however, were lower than those of $CuSO_4$ with Irgarol 1051, Ziram, Cu-pt, or Zn-pt, which had much higher MTI values as mentioned above.[17)] Some MTIs in combinations including IPBC were nearly 1, that is, IPBC in combination with Diuron,

PTPB, or SeaNine 211 were approximately 1. Thus their interactions were classified as concentration additive. Other MTIs ranged from 0 to 1, which include the majority in Table 3 and correspond to partially additive. A typical example of partially additive is shown in Fig. 3B, in which the toxicity of combinations was greater than either SeaNine 211 or PTPB alone, but less than their sum.

Among four chemicals showing marked synergistic effects with $CuSO_4$, three of them, excluding Irgarol 1051, have similar molecular structures that include metal, sulfur, and nitrogen (see Fig. 1). In addition, metals in all these chemicals are loosely bonded with a sulfide linkage through electron donation. These molecular characteristics will be considered in future investigations to clarify the mechanism of these interactions.

Therefore, based on MTI analyses, there was no antagonistic effect among the present combinations examined. Additive effects were observed in the case of Diuron, PTPB, or SeaNine 211 with IPBC. Certain synergistic effects were observed in the combination of DCF with Irgarol 1051, $CuSO_4$ with Diuron, Zn-pt with Ziram, and $CuSO_4$ with SeaNine 211. Due to the synergistic effects, marked toxicity enhancement occurred when Irgarol 1051, Ziram, Zn-pt, or Cu-pt were combined with $CuSO_4$. The other combinations were found to be partially additive.

Toxicity Enhancement with Cu^{2+}

It is strange and interesting that all the marked synergistic effects found were due to the presence of $CuSO_4$. Copper ions have generally been used in the form of CuO or Cu_2O in the antifouling industry due to their poor solubility in water, which leads to a constant release of copper ions from the surface of a ship hull.[20] However, their poor solubility makes it difficult to prepare samples with the concentrations used in this study, and therefore $CuSO_4$ was used in place of CuO or Cu_2O. Since our medium used ASW, which contains a high concentration of SO_4^{2-} (2700 mg/l) but no Cu^{2+}, the chemical toxicity of Irgarol 1051, Ziram, Cu-pt, or Zn-pt alone was evaluated under conditions including a high concentration of SO_4^{2-}. Their toxicities were much lower in comparison with those detected in the presence of Cu^{2+}. Thus increased toxicities of these chemicals in combination with $CuSO_4$ would be due to the presence of Cu^{2+} rather than SO_4^{2-}. To confirm this assumption, $CuCl_2$ was used in place of $CuSO_4$ and combined with Zn-pt, Irgarol 1051, Ziram, and Cu-pt to detect INH (%) in the absence of SO_4^{2-}. As

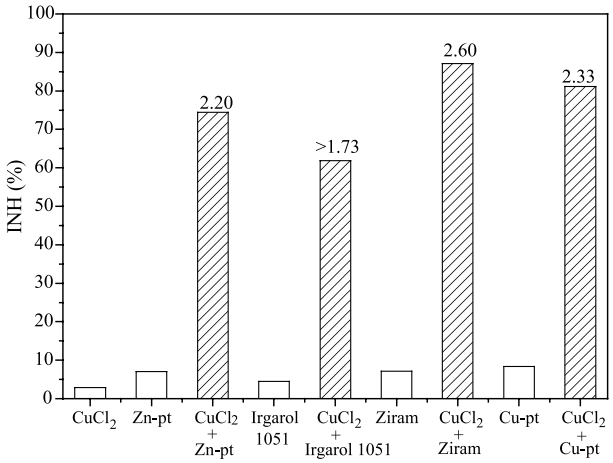

Fig. 4. Effects of Cu^{2+} on Toxicity Enhancement

The INH (%) values of BLI for single chemicals and their combinations were calculated based on reductions of BLI at 30 min of incubation in the presence of 0.3 M NaCl. As single agents or in combination, the concentrations of $CuCl_2$, Zn-pt, Irgarol 1051, Ziram, and Cu-pt were fixed at 0.05, 0.04, 9.0, 0.08, and 0.05 mg/l, respectively. Single and combined chemicals are shown by open and shaded symbols, respectively. The numbers above the bars are MTI values calculated based on Zn-pt, Irgarol 1051, Ziram, and Cu-pt in combination with $CuSO_4$ at 30 min of incubation. All data are expressed as mean obtained from triplicate experiments.

shown in Fig. 4, INH (%) of 0.05 mg/l of $CuCl_2$ was found to be less than 10% at 30 min of incubation. The inhibitory activities of Zn-pt, Irgarol 1051, Ziram, and Cu-pt at concentrations of 0.04, 9.0, 0.08, and 0.05 mg/l, respectively, were less than 10%. When each of them was combined with 0.05 mg/l of $CuCl_2$, the INH (%) was greater than 50% at 30 min of incubation. The degrees of toxicity enhancement were in accordance with MTIs calculated for their combinations with $CuSO_4$. These results strongly suggest that the marked synergistic effects of these chemicals in combination with $CuSO_4$ or $CuCl_2$ are due to the presence of Cu^{2+}.

Cu^{2+} commonly occurs in the environment as an essential element for the normal growth of all plants and animals.[21,22] Little accumulates in organisms due to its nonlipophilicity, but suppression of mitosis through glutathione reduction and breakage of cellular defense against oxygen-free radicals occur due to the passive diffusion of Cu^{2+} into cells.[22] The most bioavailable and toxic form of unbound Cu^{2+} is thought to be the free hydrated ion form, $Cu(H_2O)_6^{2+}$. Speciation of ionic copper is governed by pH, salinity, and the presence of dissolved organic matter.[22,23] Thus the toxicity of Cu^{2+} to bacteria also depends on the individual species and physiologic and environmental conditions. Synergistic effects were ob-

served among combinations examined in this study, which indicates that the toxicity of copper ions was enhanced rather than decreased. The mechanism of toxicity enhancement is that Cu^{2+} and some antifouling chemicals form lipophilic organic copper complexes that diffuse across the plasma membrane more easily in comparison with inorganic Cu^{2+} itself. Copper complexes in the cell cytosol are thought to dissociate and exchange transport ligands with intracellular complexes. This mechanism was also reported for the toxicity against microalgae by the combination of dithiocarbamate and some heavy metals.[23] Our results on copper ions as well as copper-containing chemiclas are consitent with previous results,[23] suggesting that the present assay system is applicable for a wide range of chemicals. On the basis of the present results showing that synergistic effects occurred in combinations with Cu^{2+}, we suggest that these antifouling chemicals enhance the uptake of a variety of toxic heavy metals through the formation of complexes from aquatic environments to biota.

In the literature, much attention has been paid to the toxicity of single chemicals rather than to complex systems. Interactions of combinations were rarely studied owing to the amount of work involved in determining the actual status of chemicals existing in nature. In marine environments, concentrations of Cu^{2+} as well as other ions and chemicals are present.[21] The present results showed that the toxicities of these antifoulants markedly increase upon combination when compared with their toxicity as single agents. Furthermore, these antifouling chemicals have a more serious impact on marine environments and organisms than previously recognized. Thus related studies of toxicity enhancement by various combinations of chemicals as well as their detailed mechanisms of action are now in progress.

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