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Differences in susceptibility of marine bacterial communities to metal pyrithiones, their degradation compounds and organotin antifouling biocides

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Abstract

The susceptibility of marine bacterial communities to copper pyrithione ($CuPT_2$), zinc pyrithione ($ZnPT_2$) and their degradation product is described and toxicities of these relatively new antifouling biocides compared with those of their harmful organotin (OT) predecessors, tributyltin (TBT) and triphenyltin (TPT). These biocides were added to agar at concentrations of 0, 0.01, 0.1, 1 and 10 mg l^{-1} and coastal seawater including indigenous bacteria added to each batch of agar solution. The number of bacterial colony forming units (CFU) was measured after 7 days culture. Relative CFU (as a percentage of control) was more than 80% at a concentration of 0.01 mg l^{-1} of each compound, except for TBT. Relative CFU decreased as a function of dose of each biocide, although concentration-dependent changes in rate of CFU were relatively low during exposure to degradation products of $CuPT_2$ and $ZnPT_2$, pyridine *N*-oxide (PO) and pyridine-2-sulphonic acid (PSA). Based on comparisons of EC_{50}, TBT was the most bacterio-toxic of the tested compounds (0.2 mg l^{-1}), marginally more so than $CuPT_2$ (0.3 mg l^{-1}). Interestingly, EC_{50} values of degradation products of $CuPT_2$ and $ZnPT_2$, 2-mercaptopyridine *N*-oxide (HPT) and 2,2′-dithio-bispyridine *N*-oxide (PT_2) were 0.8 and 0.5 mg l^{-1}, respectively, lower than that of the parent chemical, $ZnPT_2$ (1.4 mg l^{-1}). The EC_{50} of PT_2 was also lower than that of TPT (0.7 mg l^{-1}), implying higher toxicity. Given the overlapping toxicity ranges, these results suggest that marine bacterial communities experience comparably high susceptibility to metal PTs and OTs during their life history.

Introduction

Amongst the organotin (OT) compounds, trisubstituted organotins (R_3SnX), including tributyltin (TBT) and triphenyltin (TPT), have been extensively used as anti-fouling agents for the prevention of biofouling on ship hulls (Snoeij *et al.*, 1987; Blunden & Evans, 1989; Bosselmann, 1996). TBT and TPT pose deleterious hazards for non-target aquatic biota (Fent & Meier, 1994; Ohji *et al.*, 2002, 2003; Grzyb *et al.*, 2003). Despite regulation of their use in antifouling paints, high concentrations of TBT and TPT persist in the aquatic ecosystem (Harino *et al.*, 2002). In October 2001, the International Maritime Organization (IMO) adopted the International Convention on the Control of Harmful Antifouling Systems (AFS Convention), which prohibited the use of OTs as active ingredients in antifouling systems for ships. Following final ratification of the international restrictions on the use of OT-based antifouling compounds (in 2008 mostly), paint manufacturers have subsequently developed and deployed a variety of alternative products (Harino *et al.*, 2005).

In Japan the new antifouling biocides substituted for OTs frequently include metal pyrithiones (PTs), such as copper pyrithione ($CuPT_2$; 2-mercaptopyridine *N*-oxide copper salt) and zinc pyrithione ($ZnPT_2$; 2-mercaptopyridine *N*-oxide zinc salt). Marinas and harbours represent high-risk areas for the accumulation of these PTs due to the high densities of boats on long-term moorings, as well as shore-based activities such as high-pressure hosing and washing of boats (Maraldo & Dahllöf, 2004). There are several studies which address the acute toxicity of $CuPT_2$ and $ZnPT_2$ to aquatic organisms, including algae, amphipods (Karlsson & Eklund, 2004; Eriksson Wiklund *et al.*, 2006; Mochida *et al.*, 2006), sea urchins, mussels (Bellas *et al.*, 2005) and marine fish (Mochida *et al.*, 2006, 2008), and these findings suggest that PTs can be toxic at environmentally relevant concentrations. Furthermore, six of the degradation products produced from $CuPT_2$ and/or $ZnPT_2$ by solar irradiation, namely 2,2′-dithio-bispyridine *N*-oxide (PT_2), 2,2′-dipyridyl disulphide (PS_2), 2-mercaptopyridine *N*-oxide (HPT), 2-mercaptopyridine (HPS), pyridine-2-sulphonic acid (PSA) and pyridine *N*-oxide (PO) (Sakkas *et al.*, 2007), are as likely to be toxic to aquatic organisms as their parent compounds. However, there is little information regarding the toxicity of those compounds to aquatic organisms.

Marine bacteria are key elements of the biomass in coastal ecosystems and are vital for the recycling of nutrients. Indeed bacteria constitute the primary agents for the early transformation of organic matter and regeneration of nutrients and also serve as a food source for

organisms at higher trophic levels. As such, they have an important ecological niche as decomposers and/or primary producers in coastal ecosystems worldwide. Consequently, monitoring microbial responses has been recommended as an early warning indicator of ecosystem stress, since microbes respond promptly to environmental perturbations (Griffiths, 1983; Kim *et al.*, 1994). However, there have been few studies to date regarding the susceptibility of marine bacterial communities to pollutants (Konstantinou & Albanis, 2003; Petersen *et al.*, 2004; Milenkovski *et al.*, 2010), especially to antifouling agents (Blanck & Dahl, 1996).

The pollution-induced community tolerance (PICT) approach has been used to assess minor effects of toxicants in biotic communities, and this method can establish causal linkages between contaminants and effects (Blanck, 2002). The PICT concept builds on the induced inter- and intra-specific selection of the most tolerant organisms to a toxicant, and on the establishment of mechanisms for detoxification (Corcoll *et al.*, 2014). The entire community may be restructured, present physiological alterations, and finally display an overall increase in tolerance to the toxicant, compared with a reference community (Tlili & Montuelle, 2011; Corcoll *et al.*, 2014). Defining PICT, as proposed for microbes here, therefore enables investigation of the cause-effect relationships between toxicant exposure and community response (Corcoll *et al.*, 2014) and is potentially a useful tool to assess the sensitivity of coastal microbial communities. R2A agar is generally used for enumerating heterotrophic organisms and was developed by Reasoner & Geldreich (1985) for bacteriological plate counts of treated potable water. A low nutrient medium, such as R2A agar, in combination with a lower incubation temperature and long incubation time stimulates the growth of stressed and chlorine-tolerant bacteria (Reasoner & Geldreich, 1985). Nutritionally rich media support the growth of fast-growing bacteria but may suppress slow-growing or stressed bacteria found in treated water. In comparison, studies with nutritionally rich media (Standard Methods Agar) such as Tryptone Glucose Yeast Extract Agar or Plate Count Agar, R2A agar has been reported to improve the recovery of stressed and chlorine-tolerant bacteria from drinking water systems (Means *et al.*, 1981; Fiksdal *et al.*, 1982; Kelly *et al.*, 1983). This agar is recommended in *Standard Methods for the Examination of Water and Wastewater* and can be used in pour, spread plate and membrane filter methods (Franson, 1998). Like the bacteria in potable water, the bacteria inhabiting ambient seawater are also heterotrophic organisms. Therefore, in the present study, we considered this agar to be a suitable choice for elucidating the susceptibility of marine bacterial communities to antifouling biocides.

The objective was to compare the susceptibility of colonies of coastal microbes to $CuPT_2$ and $ZnPT_2$, over 7 days of culturing. This included comparison of toxicities between parental compounds and their degradation products, and also a comparison of toxicities between these new antifouling biocides with toxic organotin predecessors, TBT and TPT. The results form the basis of discussion on the fluctuation of abundance of native marine bacterial communities as well as on the biological impact of antifouling biocides.

Materials and methods

Site description

Seawater including marine bacteria was collected in sterilized bottles from the Imazu Coast, located in Hyogo Prefecture, central Japan. The seawater samples were immediately brought back to the laboratory and stored at 4°C until they were used in the experiment testing the inhibitory effects of bacterial growth by antifouling chemicals.

Experimental solution

$CuPT_2$ and $ZnPT_2$ were provided by Yoshitomi Fine Chemicals (Osaka, Japan). PT_2 was purchased from Across Organics (Morris Plains, NJ, USA). PS_2, HPT, HPS, PO, TBTCl (tributyltin chloride) and TPTCl (triphenyltin chloride) were purchased from Tokyo Kasei Kogyo Company (Tokyo, Japan). PSA was purchased from Wako Pure Chemicals Industry (Osaka, Japan). The chemical structures of the metal pyrithiones, their degradation products, and TBT and TPT are shown in Figure 1. The R2A agar, purchased from Nihon Pharmaceutical Co., Ltd (Tokyo, Japan), contained yeast extract, 0.5 g, peptone, 0.5 g, casamino acids, 0.5 g, glucose, 0.5 g, soluble starch, 0.5 g, K_2HPO_4, 0.3 g, $MgSO_4$ $7H_2O$, 0.05 g, sodium pyruvate, 0.3 g, and agar, 15 g, in one litre of distilled water. NaCl was added to the agar media to match the culture salinity conditions of the marine bacteria with that of the ambient water.

Stock solutions of 1000 mg l^{-1} of $CuPT_2$, $ZnPT_2$, the other six compounds, TBT and TPT were made by dilution with a non-toxic organic dissolvent, dimethyl sulphoxide (DMSO), which was autoclaved. The stock solutions were further diluted with DMSO to make working solutions of 1, 10 and 100 mg l^{-1}, of which 1 ml of each was added to 100 ml of R2A agar media in a 200-ml Erlenmeyer flask and stirred to achieve dissolution before starting toxicity tests. Control solutions were also made using 1 ml DMSO in 100 ml of R2A agar media.

Each exposure experiment consisted of a control (0 mg l^{-1}) and four test concentrations of each compound (0.01, 0.1, 1 and 10 mg l^{-1}). Seawater including marine bacterial communities was mixed with autoclaved seawater to represent dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}.

Toxicity experiments

The comparative toxicity of antifouling compounds on bacteria was conducted using a modified form of the test used to assess the ecological effects in the risk assessment programme of the Organization for Economic Cooperation and Development (OECD, 1997).

One millilitre of seawater, or diluted seawater, containing marine bacterial communities was poured into a sterile Petri dish. R2A agar medium including each concentration of $CuPT_2$, $ZnPT_2$, PT_2, PS_2, HPT, HPS, PSA, PO, TBT and TPT was poured into the Petri dishes containing these seawater samples. These preparations were mixed thoroughly by rotating the plate several times. When the media had solidified, the plates were inverted and incubated at 25°C for 7 days in the dark in an incubator. The bacterial colonies were counted after 7 days of incubation. Each toxicity test was conducted in duplicate. The results were expressed as relative abundance (% of control). The CFU (colony forming unit) of each treatment was calculated relative to the control as follows:

$$\text{Relative CFU(\% of control)} = \frac{\text{Number of CFU of each compound}}{\text{Number of CFU of each control}} \times 100$$

The EC_{50} values were then determined from regressions of log transformation plots of the dose-response data (Nweke *et al.*, 2007).

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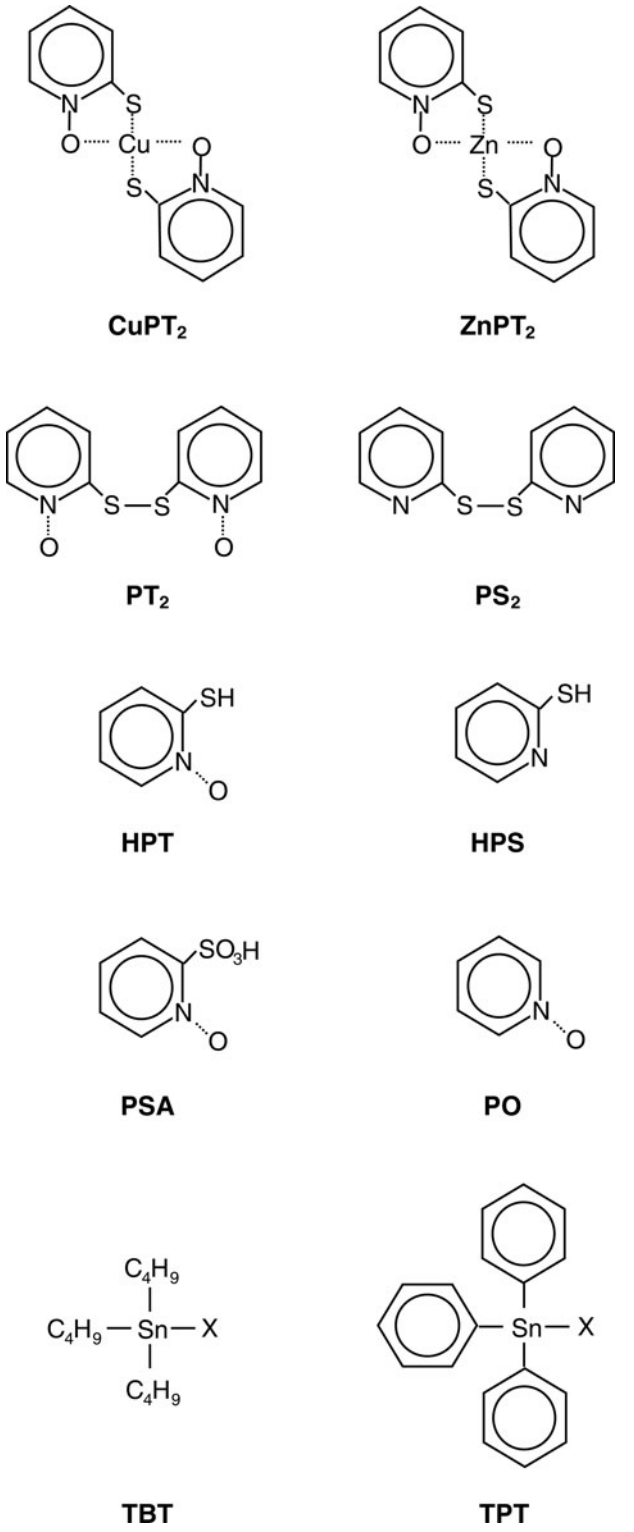

Fig. 1. Chemical structures of the metal pyrithiones, copper pyrithione ($CuPT_2$) and zinc pyirithione ($ZnPT_2$), and their degradation products, 2,2′-dithio-bispyridine *N*-oxide (PT_2), 2,2′-dipyridyl disulphide (PS_2), 2-mercaptopyridine *N*-oxide (HPT), 2-mercapto-pyridine (HPS), pyridine-2-sulphonic acid (PSA), and pyridine *N*-oxide (PO), and organotins, tributyltin (TBT) and triphenyltin (TPT).

Results

Toxicity of $CuPT_2$ and $ZnPT_2$ to marine bacteria

The relative CFU (% of control) of $CuPT_2$ at a concentration of less than 0.1 mg l^{-1} was 91.5–93.6%, and the rate of colony formation decreased dramatically at concentrations greater than 0.1 mg l^{-1} (Figure 2). The colony forming rate of samples exposed to $ZnPT_2$ was higher than controls (>100%) at 0.01 and 0.1 mg l^{-1} and decreased, progressively, at concentrations greater than 0.1 mg l^{-1} (1.0–10 mg l^{-1}). The EC_{50} value of $CuPT_2$ (0.3 mg l^{-1}), was lower than that of $ZnPT_2$ (1.4 mg l^{-1}). These results indicate that $CuPT_2$ was more toxic to marine bacterial communities than $ZnPT_2$.

Toxicity of the degradation products of PTs to marine bacteria

The relative CFU of four of the degradation products of $CuPT_2$ and $ZnPT_2$ decreased gradually with increasing concentration, except for PO and PSA when growth reductions were <20% across the exposure range (Figure 3). Hence the EC_{50} value of PO and PSA could not be calculated because the values of relative CFU of those chemicals were not below the required 50% reduction in growth needed for calculation. The EC_{50} value of HPS and PS_2 was 8.1 and 3.7 mg l^{-1}, respectively. The EC_{50} value of HPT and PT_2 was 0.8 and 0.5 mg l^{-1}, respectively, and these values were lower than those of their parent chemical, $ZnPT_2$. These findings suggest that the toxicities of HPT and PT_2 to marine bacteria were higher than that of $ZnPT_2$.

Toxicity of TBT and TPT to marine bacterial communities

The values of relative CFU decreased in a dose-dependent manner with increasing concentration of these two organotin compounds (Figure 4). The EC_{50} value of TBT was 0.2 mg l^{-1}, lower than that of TPT (0.7 mg l^{-1}), suggesting that the toxicity of TBT to marine bacteria was higher than that of TPT.

Discussion

The results in the present study of microbial communities indicate that the toxicities of alternative antifouling biocides, such as $CuPT_2$ and $ZnPT_2$, that have been used as substitutes for TBT and TPT, are almost the same as those of the triorganotins. This suggests that marine bacterial communities are equally susceptible to metal PTs in the environment as they are to TBT and TPT exposure during their life history. It is also noteworthy that the toxicities of some of the degradation products of PTs were the same as or higher than those of their parental compound $ZnPT_2$ and also TPT. Remarkably little is known about the toxicities of triorganotins to bacteria, other than the work of Avery *et al.* (1991) who showed that EC_{50} values (50% inhibition of growth after 7 days of incubation) ranged from 0.01 to 10 mg l^{-1}. The EC_{50} of marine bacterial communities examined in the present study was thus comparable to the range shown by the bacteria in the study of Avery *et al.* (1991).

$CuPT_2$ is readily converted from $ZnPT_2$ in the presence of Cu ions and is the most stable PT (Nakajima & Yasuda, 1999), and as such is recognized as a potentially hazardous compound to marine organisms. Currently, as most booster biocides are used as the combination of Cu-based antifoulants, the trans-chelation of the pyrithiones might be occuring in the ambient seawater. A few previous studies on toxicities of PTs to marine organisms such as bacteria have shown that the EC_{50} values (e.g. inhibition of bioluminescence) were of the order of 0.12 mg l^{-1} for $CuPT_2$ and 0.08 mg l^{-1} for $ZnPT_2$ (Zhou *et al.*, 2006). $CuPT_2$ was more toxic than $ZnPT_2$ to the sea bream *Pagrus major* and the toy shrimp *Heptacarpus futilirostris* (96-h LC_{50} of $CuPT_2$ and $ZnPT_2$: 9.3 and 98.2 µg l^{-1} for *P. major*, and 2.5 and 120 µg l^{-1} for *H. futilirostris* respectively) (Mochida *et al.*, 2006). $CuPT_2$ was also found to be more toxic to the copepod *Tigriopus japonicus* than $ZnPT_2$ (24-h LC_{50}: 41 µg l^{-1} and >500 µg l^{-1}, respectively) (Yamada, 2006). Furthermore, our previous study

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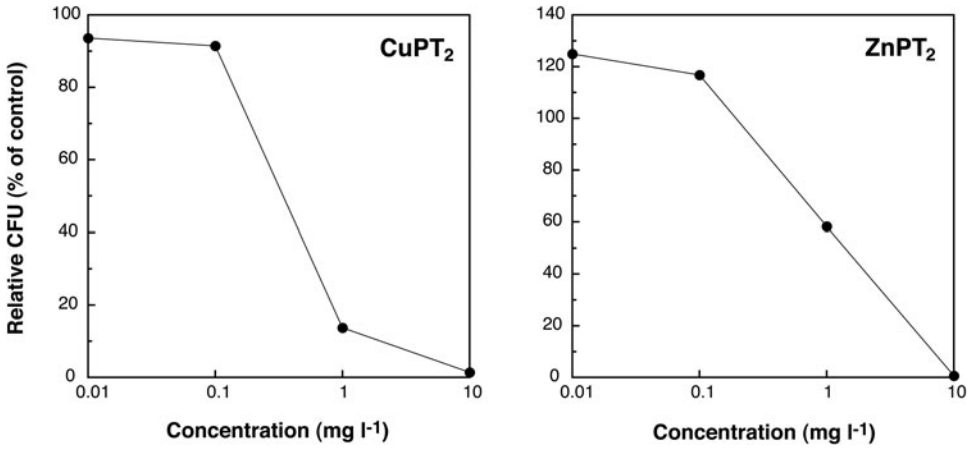

Fig. 2. Changes of relative colony forming units (CFU) (% of control) of individuals exposed to $CuPT_2$ and $ZnPT_2$ after 7 days of culturing.

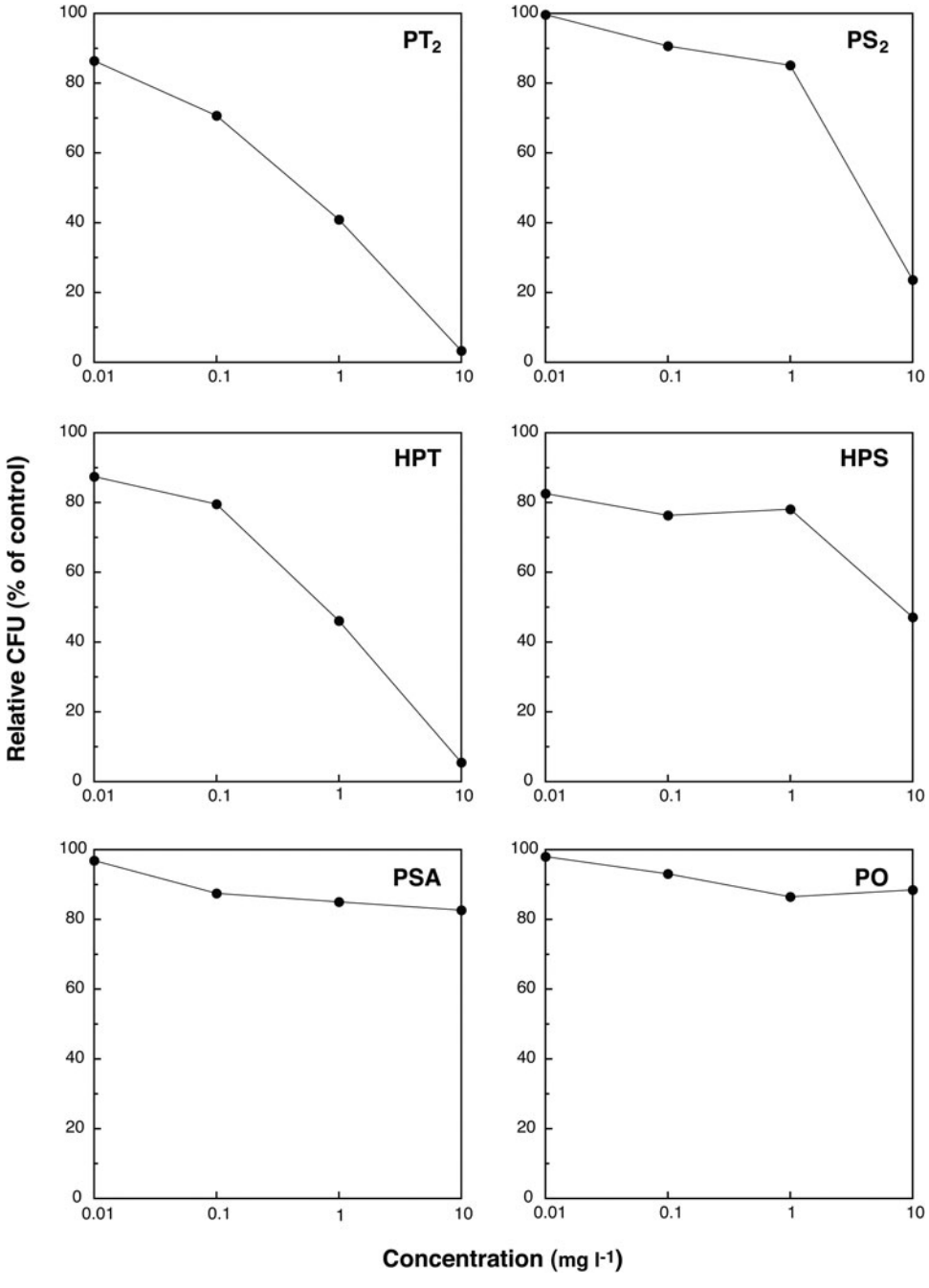

Fig. 3. Changes of relative colony forming units (CFU) (% of control) of individuals exposed to PT_2, PS_2, HPT, HPS, PSA, PO after 7 days of culturing.

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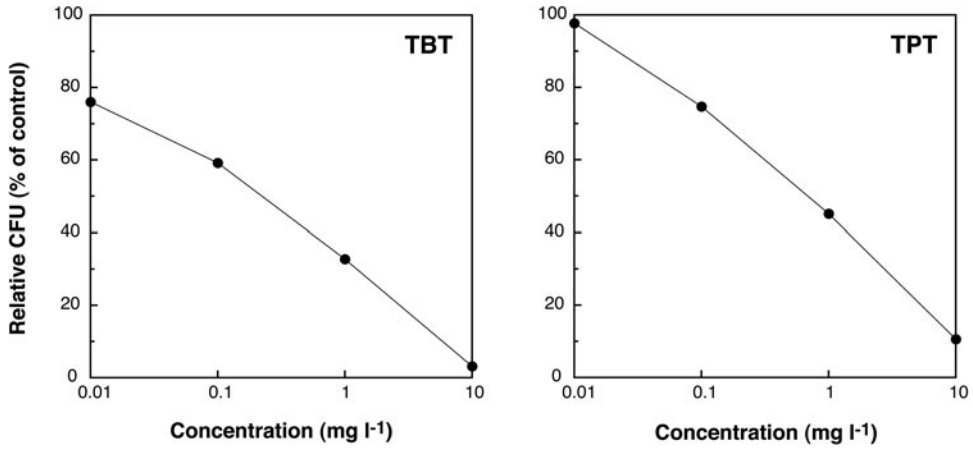

Fig. 4. Changes of relative colony forming units (CFU) (% of control) of individuals exposed to TBT and TPT after 7 days of culturing.

on Japanese killifish indicated that the toxicity levels of $CuPT_2$ were higher than those of $ZnPT_2$ (Ohji & Harino, 2017).

In the present study, the EC_{50} value of $CuPT_2$ was 0.3 mg l^{-1}, much lower than that of $ZnPT_2$ (1.4 mg l^{-1}), confirming that $CuPT_2$ is more toxic than $ZnPT_2$ to marine bacteria: The colony forming rate after exposure to $ZnPT_2$ was more than 100% of that of controls at concentrations of 0.01 and 0.1 mg l^{-1} of $ZnPT_2$. Only at a concentration of 10 mg l^{-1} $ZnPT_2$ was a significant decrease in growth observed.

In contrast to these bacterial trends, $CuPT_2$ has been reported to be less toxic to the marine diatom *Skeletonema costatum* (72-h EC_{50} was 28.4 µg l^{-1}) than $ZnPT_2$ (72-h EC_{50} was 2.1 µg l^{-1}) (Yamada, 2006). $CuPT_2$ appears to be less toxic to the algae *Chaetoceros gracilis* and *S. costatum*, because Cu is an essential element for their growth (Hall & Anderson, 1999). The stimulatory effect observed with marine bacterial populations exposed to $ZnPT_2$ at low concentrations may be attributed to the use of Zn as a trace element by marine bacterial communities. The same condition, perhaps a form of hormesis, was reported by Nweke *et al.* (2007), who a used dehydrogenase assay to assess the tolerance to Zn^{2+} by pure cultures of *Salmonella* species isolated from river sediment. The dehydrogenase activity was found to be slightly stimulated at 0.02 mM Zn^{2+} and progressively inhibited at concentrations greater than 0.2 mM (0.4–1.0 mM). Zinc is associated with a number of processes essential for growth and metabolism in bacteria (Choudhury & Srivastava, 2001). Although Zn is an essential element, it is also an inhibitor of respiratory electron transport systems of bacteria and mitochondria, where high concentrations of Zn are inhibitory, affecting many crucial functions (Kasahara & Anraku, 1974; Rogers & Li, 1985; Pérez-Garcia *et al.*, 1993; Beard *et al.*, 1995; Choudhury & Srivastava, 2001). Therefore, the inhibition of growth of bacterial populations observed in this study is considered to be consistent with the reported toxic effect of Zn at high concentrations (Ji & Silver, 1995).

Comparing toxicities of $CuPT_2$ and $ZnPT_2$ between marine and freshwater organisms indicates that freshwater species are more sensitive to the toxicity of metal pyrithione than are marine species (Madsen *et al.*, 2000; Yamada & Kakuno, 2002; Mochida *et al.*, 2006; Ohji & Harino, 2017). PTs are used as antifoulants not only in saline habitats but also in freshwater environments, such as lakes and rivers. Furthermore, $ZnPT_2$ has been widely used as a biocide in anti-dandruff shampoos in many countries for years (Shuster, 1984) and may enter aquatic habitats from domestic waste in addition to leachate from antifouling paints used on boats and ships. Therefore, it is important to consider the risk of $ZnPT_2$ to freshwater bacteria as well as that to marine bacteria.

The results of the present study confirm that PTs can affect marine bacterial communities. But to evaluate risk fully it is necessary to combine toxicity data with investigations into the ambient levels of metal pyrithione in marine ecosystems. However, there are few such studies on the occurrence of PTs in nature, and these have usually focused on $CuPT_2$ in sediment (Harino *et al.*, 2006). To understand the risk of these biocides to aquatic ecosystems more fully, further research on the use, occurrence and toxicity of these biocides in aquatic environments, both freshwater and marine, is needed. In the present study, the toxicity of a range of antifoulants to marine bacteria decreased in the sequence TBT>$CuPT_2$>PT_2>TPT>HPT>$ZnPT_2$>PS_2>HPS. In contrast, in our previous study comparing the toxicity of the same biocides to the freshwater fish *Oryzias latipes*, the metal pyrithiones $CuPT_2$, followed by $ZnPT_2$, were most deleterious whilst TBT, TPT and degradation compounds of PTs were less toxic (Ohji & Harino, 2017). The displayed differences in toxicity of these antifouling compounds may be habitat dependent and/or species specific, and this field clearly warrants further investigation.

Photolysis is a significant factor in the degradation of $CuPT_2$ and $ZnPT_2$ in natural environments (Armbrust, 2000; Turley *et al.*, 2000; Maraldo & Dahllöf, 2004; Harino *et al.*, 2005). It seems likely that degradation compounds will have a lesser impact on aquatic organisms than the parent compounds. However, Bellas *et al.* (2005) demonstrated that the toxicity of $ZnPT_2$ decreased but did not disappear entirely after exposure to direct sunlight. PTs may persist in the marine environment where the influence of the light is limited, such as shaded waters and sediments in marinas and harbours (Maraldo & Dahllöf, 2004). Here, for example, $ZnPT_2$ may accumulate in the sediment as manganese pyrithione and $CuPT_2$ (Galvin *et al.*, 1998), and impact on the aquatic ecosystem. Although to date there are only a few reports regarding the occurrence of degradation products of PTs, i.e. PS_2 in seawater (Mochida, personal communication), our previous study demonstrated that various sub-lethal biological effects on Japanese killifish (e.g. abnormalities in respiration and swimming behaviour and decreased hatchability) can be caused by exposure to degradation compounds of $CuPT_2$ and $ZnPT_2$ (Ohji & Harino, 2017). However, it is still not fully understood how these degradation products affect *O. latipes*, or their mode of action in marine bacteria.

In conclusion, our results indicate that in terms of the viability of marine bacterial communities, the toxicities of new antifouling biocides such as $CuPT_2$ and $ZnPT_2$ that are used as substitutes for TBT and TPT are almost the same as the banned chemicals they are replacing. These findings suggest that marine microbial

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communities may be at reasonably high ecological risk from the effects of $CuPT_2$ and $ZnPT_2$ exposure as well as those from TBT and TPT exposure. It was noteworthy that the toxicities of some of the degradation products of PTs are the same as, or higher than, those of their parental compound ($ZnPT_2$) and TPT. Thus, new pyrithione-based antifouling biocides cannot be assumed to be totally safe alternative biocides to OTs, as both types of compound appear equally capable of inducing disturbances in marine microbial communities.

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