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Acute toxicity of organic antifouling biocides to phytoplankton *Nitzschia pungens* and zooplankton *Artemia* larvae☆

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ABSTRACT

The toxicity of the antifouling biocides Irgarol 1051, Diuron, Chlorothalonil, Dichlofluanid, Sea-nine 211, Copper pyrithione, Zinc pyrithione, Ziram and Zineb were evaluated on *Nitzschia pungens* and *Artemia* larvae. Results showed that EC_{50} for Irgarol 1051 was 0.586 μg l^{-1} was the strongest effect on *N. pungens* following by Copper pyrithione (4.908 μg l^{-1}), Ziram (5.421 μg l^{-1}), Zinc pyrithione (5.513 μg l^{-1}), Diuron (6.640 μg l^{-1}), Zineb (232.249 μg l^{-1}), Sea-nine 211(267.368 μg l^{-1}), Chlorothalonil (360.963 μg l^{-1}) and Dichlofluanid (377.010 μg l^{-1}) in 96 h. In *Artemia* larvae, the biocides were evaluated the LC_{50} for larval survivals at 48 h. Sea-nine 211 and Copper pyrithione were 0.318 and 0.319 mg l^{-1}. Chlorothalonil, Zinc pyrithione and Ziram were 2.683, 3.147 and 4.778 mg l^{-1}. Irgarol 1051, Diuron, Zineb and Dichlofluanid were 9.734, 30.573, 41.170 and 154.944 mg l^{-1}. These results provide baseline data concerning the toxicity of antifouling biocides against marine environment.

1. Introduction

Generally, an antifouling agent is used to prevent the attachment and growth of marine organisms on submerged surfaces like ship hulls. Tributyltin (TBT) is an antifouling coating that has been shown capable of decreasing the surface resistance of ship and reducing fuel consumption (Bennett, 1996). For many years, TBT compounds were the most widely used active ingredients in antifouling paint formulations. However, since 1990, International Maritime Organization (IMO) adopted a resolution recommending governments to adopt measures to eliminate antifouling paints containing TBT. This is classified as an endocrine disruptors due to its induction of imposex (imposition of male sexual organs on females) in gastropod species (Oehlmann et al., 1996; Horiguchi et al., 1997). In 2001, IMO adopted a new International Convention on the Control of Harmful Anti-fouling Systems on Ships, which was prohibit the use of harmful TBTs in antifouling paints used on ships and was establish a mechanism to prevent the potential future use of other harmful substances in anti-fouling systems. The resolution called for a global prohibition on the application of organotin compounds, which act as biocides in anti-fouling systems on ships by 1 January 2003, and a complete prohibition by 1 January 2008. As a consequence, ships must either replace or overcoat their existing organotin-based antifouling systems (IMO, 2008). This ban has led to an increase in vessels using alternative TBT-free coatings containing copper combined with booster biocides. Unfortunately, these TBT alternatives are also toxic to non-target marine organisms (Omae, 2003). Their contamination of the marine environment has been increasingly recognized (Konstantinou and Albanis, 2004; Karlsson and Eklund, 2004). However, risk assessment of the significance of the TBT alternatives has been difficult to gauge since data on the use and concentration of biocides for continuous monitoring is scant.

Studies have shown that two of the most popular biocides in use, Irgarol 1051 and Diuron, persist in surface waters. In contrast, other biocides, such as Sea-nine 211, Dichlofluanid, Zinc pyrithione and Chlorothalonil tend to disappear quickly (Thomas, 2001; Thomas et al., 2002, 2003). But, many alternative antifouling agents still have been used without biological and marine ecological inspection. Although several toxic or harmful effects were reported, organism- and substance-related testing has not been consistent (Kim et al., 2014). Moreover, it has been demonstrated that some of the test biocides are also toxic to fouling organisms, which should be also protected since they are ecological components of the marine ecosystem (Gallo and Tosti, 2013; Gallo and Tosti, 2015).

In the United Kingdom, the use of booster biocides such as Diuron, Irgarol 1051, Sea-nine 211, Dichlofluanid, Zinc pyrithione, Chlorothalonil and 2-(thiocyanomethylthio) benzothiazole (TCMTB) has been restricted depending on vessel length (HSE, 2000). Other nations also have implemented or plans for biocide restriction legislation. To systemically manage antifouling biocides, many factors including the amount used; effects of biocide residue; and effect of biocide accumulation in seawater, sediment and marine organisms should be considered, as should the effects of biocide degradation products. Toxicity data of antifouling biocides are also very important. Marine toxicity affects not only the

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organisms in the marine ecosystem but can be detrimental to other creatures including humans.

In the present study, we assessed toxicity of nine organic antifouling biocides (Irgarol 1051, Diuron, Chlorothalonil, Dichlofluanid, Sea-nine 211, Zinc pyrithione, Copper pyrithione, Zineb, and Ziram) on a representative phytoplankton (*Nitzschia pungens*) and zooplankton (*Artemia* larvae). Moreover, *Artemia* spp. (brine shrimp) has gained popularity as a test organism due to its ease of culture, short generation time, cosmopolitan distribution and the commercial availability of its dormant eggs (cysts) (Koutsaftis and Aoyama, 2007). The plankton is the fundamental foodstuff for everything that lives in the ocean. So, this toxicity test using planktons has potentially significant importance in the monitoring and protecting of all marine ecosystems.

2. Materials and methods

2.1. Phytoplankton: Nitzschia pungens

The toxicity test was performed as recommended in the test guidelines of the OECD 201, with modification (OECD, 2006). The microalgae used in the toxicity test was *N. pungens*, which was obtained from the Korea Marine Microalgae Culture Center. The medium used was f/2 (pH 7.5 ± 0.1) at a salinity of 32 psu (Guillard and Ryther, 1962). The medium (20 ml in a 50 ml flask) make predetermined concentrations mixed with stock solution which DMSO and each biocide were combined. The each mixtures were concentrations of 0.1, 1, 5, 10, 100, 1000 and 2000 mg l^{-1}, and inoculated with 5×10^4 cells ml^{-1} and incubated at 25 °C (product indication but OECD recommending a temperature between 18–22 °C) statically using an alternating 12 h cycle of illumination (60 μE m^{-2} s^{-1}). Growth was monitored by optical density measurements at 680 nm using a Shimadzu UV-1601 spectrophotometer, and cell numbers were determined by counting of culture samples in a hemocytometer. Chlorophyll *a* was extracted with 90% acetone and chlorophyll *a* content was calculated by the following equation (Strickland and Parsons, 1972).

$$\text{Chlorophyll } a \left(\mu g \ ml^{-1}\right) = 11.47\,A_{664} - 0.4\,A_{647}$$

where A_{664} = Absorbance at 664 nm; A_{647} = Absorbance at 647 nm.

After culturing, measurements of microalgae growth were monitored 48 h and 96 h. For every treated concentrations, the tests were independently conducted in triplicate.

2.2. Zooplankton: Artemia larvae

The toxicity test performed as recommended in the test guideline of OECD 202, with modification (OECD, 2004). *Artemia* sp. eggs were kindly provided by Professor Ma (Department of Marine Biotechnology, Soonchunhyang University, Asan, South Korea). The eggs were allowed to hatch during incubation under aerated condition with constant illumination (60 μE m^{-2} s^{-1}) at 25 °C in filtered seawater (salinity of 32 psu) collected from the west coast of South Korea. Within approximately 24 h after hatching, *Artemia* larvae were used for toxicity testing as previously described (Castritsi-Catharios et al., 2007). Briefly, 2 ml of filtered seawater and 10 *Artemia* larvae were added to each well of a 24-well culture plate (25 °C and 60 μE m^{-2} s^{-1}). Six wells were prepared for each concentration of biocide which dissolved with DMSO. The mixture concentrations were 0.001, 0.001, 0.01, 1, 10 and 100 mg l^{-1}. After 48 h treatment of each biocide concentration, the number of living and dead *Artemia* larvae was ascertained by the presence or absence, respectively, of swimming when the larvae were examining microscopically. The dead individuals were determined if no movement of the appendages was observed within 10 s. For every treated concentration, the tests were conducted in triplicate.

2.3. Organic solvent test

Since the antifouling biocides examined all display low solubility in seawater (Table 1), each biocide was dissolved in dimethyl sulfoxide (DMSO) (Kobayashi and Okamura, 2002). To determine the appropriate DMSO concentration, concentration ranges of DMSO were applied to *N. pungens* (0– 40,000 mg l^{-1}) and *Artemia* larvae (0– 100,000 mg l^{-1}). The measurements were carried out in growth and survival rate, respectively.

2.4. Antifouling biocides

The organic antifouling biocides used for acute toxicity were 2-methylthio-4-tertiary-butylamino-6-cyclopropylamino-*s*-triazine (Irgarol 1051, 99%; Dr. Ehrenstorfer Co.), 2,4,5,6-tetrachloroisophtalonitrile (Chlorothalonil, 99.5%; Dr. Ehrenstorfer Co.), *N*-dimethyl-*N*-phenylsulfamide (Dichlofluanid, 99%; Dr. Ehrenstorfer Co.), 4,5-dichloro-2-*N*-octyl-3-(2H) isothiazolone (Sea-Nine 211; Rohm and Haas), 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Diuron, 98%; Sigma-Aldrich), zinc dimethyldithio carbamate (Ziram, 97%; Aldrich), zinc ethylenebis (Zineb, 92%; Dr. Ehrenstorfer Co.), bis(2-pyridylthio)zinc 1,1′-dioxide (Zinc pyrithione, 98%; TCI Co.), and Bis(1-hydroxy-1H-pyridine-2-thionato-O,S)copper (Copper pyrithione, 98%, Wako) (Table 1). All biocides were dissolved in 99.9% pure DMSO (Junsei Chemical) to make stock solutions. Previous studies demonstrates that the most effective concentration against microalgae showed >1,000 μg l^{-1}, while the LC_{50} of *Artemia* sp. ranges 0.50– 12.61 mg l^{-1}. Therefore, these concentration ranges of each biocide were 0– 2000 μg l^{-1} for *N. pungens* and 0– 100 mg l^{-1} for *Artemia* larvae. The toxicity value were based on nominal concentrations of the antifouling biocides. Median effective concentration (EC_{50}) and no observed effective concentration (NOEC) values at 96 h for microalgae toxicity testing and median lethal concentration (LC_{50}) of *Artemia* larvae at 48 h were calculated using BioDatafit 1.02 software (http://www.changbioscience.com/stat/ec50.html) using cell growth rate and survival rate compared with each control.

2.5. Statistical analysis

The statistical significance of differences between means and groups ($p < 0.05$) was estimated based on the one-way ANOVA and Tukey-HSD test using SPSS ver. 17.

3. Results

3.1. Relationships between cell number, optical density and chlorophyll a content

Growth rate was evaluated in three ways; cell number, optical density and chlorophyll *a* determinations. In results, coefficient of determination (R squared, r^2) between cell number and OD was 0.968. The value of OD and chlorophyll *a* was 0.966. The Cell number and chlorophyll *a* was 0.963 (Fig. 1). Therefore, the values are displayed an obvious correlation from the three methods. The subsequent growth rate data was determined using cell counts.

3.2. Effect of DMSO on N. pungens and Artemia larvae

To establish that the DMSO concentration used as the solvent was not toxic to the test organisms, *N. pungens* and *Artemia* larvae were treated by various concentrations of DMSO and their growth rate were assessed. *N. pungens* samples received 0, 1, 10, 100, 1000, 10,000, 20,000, or 40,000 mg l^{-1} DMSO and growth was determined after 96 h (Fig. 2). DMSO concentrations up to 1000 mg l^{-1} did not impede growth, as compared to the untreated control. But, growth was inhibited at DMSO concentrations over 20,000 mg l^{-1}, with growth being inhibited over 90% by 40,000 mg l^{-1} DMSO (*t*-test, $p < 0.05$)

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