Comparative study of toxicological responses of chlorophyll-related parameters in the marine diatom *Skeletonema* sp. exposed to tributyltin and Irgarol

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**ABSTRACT**

We investigated the growth and chlorophyll-related parameters of the marine diatom *Skeletonema marinoi-dohrnii* complex (NIES-324) exposed to tributyltin (TBT) or photosystem (PS) II inhibitor Irgarol as a reference chemical for elucidating the effect of TBT on photosynthetic activity. Fifty percent effective concentrations for growth (EC₅₀) were 2.14 µg/L in TBT and 1.14 µg/L in Irgarol. Chlorophyll *a* content per one cell (pg/cell) was significantly decreased at the highest Irgarol concentration (1.0 µg/L) but chlorophyll *c* content was not affected. Following TBT treatment, however, both chlorophyll *a* and *c* content per cell increased significantly at the highest concentration (2.0 µg/L). The ratio of chlorophyll *a* and *c* content (chl. *a/c* ratio) decreased significantly and the minimum level of chlorophyll fluorescence (F₀) per µg/mL of chlorophyll *a* (F₀/chl. *a*) increased significantly in all Irgarol treatment groups, but TBT had no effect on either parameter. The present results suggest that TBT has no direct effect on electron flow in the photosystem and that the chl. *a/c* ratio and F₀/chl. *a* are sensitive bio-markers for detecting the effect of the PSII inhibitor in diatoms.

**Key words:** tributyltin; Irgarol; diatom; chlorophyll; photosynthesis

1. **INTRODUCTION**

Antifouling paints are essential for suppressing the adherence of organisms to various marine materials such as ship's bottoms, fishnets and power facilities. Tributyltin (TBT) compounds were used as major antifouling agents in the marine environment from 1960s (Huggett et al. 1992) but the use of TBT was banned from 2008 by signatories of the International Maritime Organization because of its high toxicity and persistency in aquatic ecosystems. Even after its worldwide ban, however, TBT has been detected in sea water and sediment (Yozukmaz et al. 2011, Undap et al. 2013, Kim et al. 2014) and marine ecosystems are still at risk from TBT pollution.

In general, TBT is known to inhibit ATPase activity in a broad spectrum of organisms such as unicellular green algae (Catt et al. 1984), mussels (Pagliarani et al. 2008) and fish (Pinkney et al. 1989). On the other hand, another study
showed the suppression of photosynthetic parameters in microalgae exposed to TBT. Peterson and Kusk (2000) reported that TBT decreased the carbon fixation rate in the cryptophyte Rhodomonas salina and diatom Skeletonema costatum. Guanzon and Nakahara (2002) also showed the inhibition of oxygen production in some phytoplankton species. In addition Far-gašová (1997) reported a decrease in chlorophyll content in Scenedesmus quadricauda following exposure to TBT. However, they measured each photosynthesis-related parameter as a surrogate of biomass and did not evaluate physiological conditions at a cellular level. The photosynthetic pigment content per one cell was also analyzed in a previous study for evaluating physiological condition of marine dinoflagellates Cochlodinium polykrikoides exposed to CuSO₄ and NaOCl (Ebenezer et al. 2014). Photosynthesis is an activity of great significance to aquatic primary production, and toxic mechanisms to photosynthesis can provide information that is useful in predicting the effects of mixed chemicals (Cedergreen 2014). It is therefore crucial to investigate the effect of TBT on photosynthetic function.

Chlorophyll content and fluorescence have traditionally been measured to evaluate the condition of the light-harvesting complex or photosynthetic reaction center in photosynthesis function. The present study investigated the effect of TBT on growth, chlorophyll a and c content per one cell, chlorophyll fluorescence and related parameters of a marine diatom that is an important primary producer in marine ecosystems, to evaluate the integrity of the antenna complex or photosynthetic reaction center. We also selected Irgarol, an alternative antifouling agent known as a photosystem II (PSII) inhibitor (Hall et al. 1999), to compare the toxicological response of chlorophyll-related parameters between TBT and Irgarol.

2. MATERIALS AND METHODS

2.1. Test organism and culture conditions

An axenic strain of the marine diatom Skeletonema marinoi-dohrnii complex (NIES-324) was obtained from the National Institute for Environmental Studies (NIES), Japan. Cultures were maintained in glass vials with screw caps (35 mm × 78 mm; Maruemu Corporation, Osaka, Japan) containing 10 mL of modified SWM-3 medium (pH 7.9, salinity of 30) (Yamasaki et al., 2007) at 25°C under 100 µmol/m²/s of light-emitting diode (LED) (LDA7N, Toshiba, Kanagawa, Japan) on a 12/12-h light/dark cycle in a growth chamber (Eyelatron FLI-160, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). Seawater used in the medium was collected from an area around Oki Island (Tsushima Warm Current) and aged in the laboratory for more than 1 year. Irradiance in the growth chamber was measured with a irradiance sensor (QSL-2101, Biospherical Instruments, San Diego, CA, USA). Unless otherwise indicated, the temperature and light conditions in subsequent culture tests were as described here.

2.2. Test chemicals

Irgarol (98.4% purity, Sigma-Aldrich, St. Louis, MO, USA) and TBT chloride (95% purity, Tokyo Kasei Kogyo, Tokyo, Japan) were used for growth inhibition tests. Each chemical was dissolved in dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Osaka, Japan) as stock solution (1 mg/mL DMSO).

2.3. Growth inhibition test

High-concentration solution of TBT or Irgar-
rol (0.5 mg/L) was prepared by adding 50 µL of each stock solution to 100 mL of sterile filtered (0.45 µm) sea water and serially diluted with modified SWM-3 medium to give Irgarol concentrations of 0 (control), 0.05, 0.1, 0.5 and 1 µg/L; and TBT concentrations of 0 (control), 0.2, 0.5, 1 and 2 µg/L. Final DMSO concentrations were 0.0001% for Irgarol and 0.0002% for the TBT group including control. Test glass tubes (13 mm × 100 mm, with screw caps, Maruemu Corporation, Osaka, Japan) containing 5 mL of test medium (n=4 in each treatment group except n=5 in Irgarol control) were inoculated with a Skeletonema cell suspension in early stationary phase at an initial density of 2 × 10⁴ cells/mL. During culture, each tube was shaken twice a day, and the growth of Skeletonema was monitored by in vivo fluorescence with a Turner Designs Model 10-AU fluorometer (Sunnyvale, CA, USA) at 0, 24, 48, 72 and 96 h of exposure. The wave length range of half bandwidth is 340 - 480 nm in the excitation filter and more than 665 nm in the emission filter. Before measuring in vivo fluorescence, cell suspension was kept in the dark for 40 min. The algal growth was also monitored by direct cell counting with microscopic observation at 0 and 96 h of exposure. The cells which have chloroplast inside the cells were judged as live cells. The growth rate (divisions/day) in each treatment group was calculated by the method of Guillard (1973) using cell counting data of 0 and 96 h (Fig. 1) as following equations:

\[ \mu = \frac{(\ln N_t - \ln N_0)}{t} \quad (1) \]

Growth rate (divisions/day) = \( \mu / \ln 2 \) \quad (2)

where \( \mu \) is the specific growth rate, \( t \) is the days, \( N_t \) is the cell density (cells/mL) after \( t \) days from beginning of exposure, \( N_0 \) is the initial cell density in equation (1).

On the final day of the exposure test, chlorophyll \( a \) and \( c \) content in each test tube was measured by the method as described in section 2.4.

2.4. Measurement of chlorophyll \( a \) and \( c \) content

After the period of 96h exposure ended, Skeletonema cells in each test tube were precipitated by centrifugation (1500 × g for 5 min). The cell free supernatant was discarded and 5 mL of 90% acetone was added to each test tube. The intracellular chlorophyll was then extracted by sonication for 10 min, and the suspended matter was removed by centrifugation (1500 × g for 5 min). The acetone extracts obtained, including chlorophyll, was subjected to spectrophotometric analysis (U-2001, Hitachi, Tokyo, Japan). Absorbance was measured at 664, 647, 630 and 750 nm and chlorophyll \( a \) and \( c \) concentrations were calculated by the equation of Jeffrey and Humphrey (1975) as follows:

\[ \text{chlorophyll } a \ (\mu g/mL) = (11.85 \ E_{664} - 1.54 \ E_{647} - 0.08 \ E_{630}) \times N / (L \times V) \]

\[ \text{chlorophyll } c \ (\mu g/mL) = (-1.67 \ E_{664} - 7.60 \ E_{647} + 24.52 \ E_{630}) \times N / (L \times V) \]

where \( E_{664} \), \( E_{647} \) and \( E_{630} \) were the values that deducted absorbance at 750 nm from absorbances at 664, 647 and 630 nm, respectively. \( N \) was the volume (5 mL) of 90% acetone added, \( L \) was the light path length (1 cm) of the glass cell and \( V \) was the volume (5 mL) of each culture in the exposure test.

2.5. Confirmation of the linear relationships of cell density with chlorophyll content per one cell or fluorescence
Skeletonema cell suspension in early stationary phase (8×10⁵ cells/mL) was serially diluted with modified SWM-3 medium, then 5 mL of each diluted cell suspension (0, 1×10⁵, 2×10⁵, 4×10⁵ and 8×10⁵ cells/mL) was divided to four test tubes (13 mm × 100 mm, with screw caps, Maruemu Corporation, Osaka, Japan), respectively. After that, chlorophyll a or c content per one cell and in vivo fluorescence were measured by the methods described in sections 2.3 and 2.4. Finally, the linear relationships of cell density with chlorophyll a or c content per one cell or in vivo fluorescence were checked by simple linear regression analysis (Fig. 2) using the Statecel 3rd edition (OMS Publishing Inc. Saitama, Japan).

2.6. Calculation of chlorophyll-related parameters

Four chlorophyll-related parameters were calculated, as follows:
(1) Chlorophyll a content per one cell (pg chl. a/cell) as an indicator of the integrity of the antenna complex and photosynthetic reaction center (Fig. 3).
(2) Chlorophyll c content per one cell (pg chl. c/cell) as an indicator of the integrity of the antenna complex (Fig. 4).
(3) Chlorophyll a content and chlorophyll c content ratio (chl. a/c ratio) as an indicator of the ratio of the photosynthetic reaction center/antenna complex (Fig. 5).
(4) in vivo fluorescence (minimum level of fluorescence: Fₒ) per 1 µg/mL of chlorophyll a concentration (Fₒ /chl. a) as an indicator of the PSII activity (Fig. 6). After dark adaptation for 40 min, the minimum level of fluorescence was measured by excitation light at less than 1 µmol/m²/s of. The excitation or emission wavelength is described in section 2.3.

2.7. Statistical analysis

Effective concentrations for growth rate (EC₅₀, 20 and 50) in both exposure tests were estimated by probit analysis using Statistical Package for the Social Sciences software (SPSS 10.0; SPSS, Chicago, IL, USA).

Statistical differences in treatment groups compared with the control were analyzed by Dunnett’s test after assumptions of homogeneity of variance were tested by Bartlett’s test. If homogeneity among all groups was not observed (Figs. 3 and 6), the nonparametric Shirley–Williams test was used for statistical comparison. These statistical analyses were conducted using the Statecel 3rd edition (OMS Publishing Inc. Saitama, Japan).

3. RESULTS

Fig. 1 shows the growth rate of Skeletonema exposed to TBT (Fig. 1A) and Irgarol (Fig. 1B).
for 96 h. Growth rates were significantly (p<0.01) decreased by 1 and 2 µg/L of TBT treatment and 0.5 and 1 µg/L of Irgarol treatment compared with each control. Each toxicological parameter for growth rate is shown in Table 1. Effective concentration (EC) values in TBT were almost twice those of Irgarol. In both exposure tests, no observed effective concentrations (NOEC) were lower than EC10 values and the lowest observed effective concentrations (LOEC) were between EC10 and EC20 values.

The cell densities in the control group and treatment groups after 96 h were within the range of 1×10⁵ - 8×10⁵ cells/mL in both TBT and Irgarol exposure test. Thus we analyzed the

![Fig. 3](image1)

**Fig. 3** Chlorophyll a content (pg/cell) of *Skeletonema* sp. exposed to TBT (A) or Irgarol (B) after 96 h from beginning of exposure. Asterisks indicate significant differences in chl. a content compared with control (*:p<0.05, **:p<0.01; Shirley-Williams test).

![Fig. 4](image2)

**Fig. 4** Chlorophyll c content (pg/cell) of *Skeletonema* sp. exposed to TBT (A) or Irgarol (B) after 96 h from beginning of exposure. Asterisks indicate significant differences in chl. c content compared with control (**:p<0.01; Dunnett's test).
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...linear relationship of cell density with chlorophyll $a$ or $c$ content per one cell or *in vivo* fluorescence (Fig. 2). As a result, significant ($p<0.01$) linear relationships of cell density with chlorophyll $a$ (Fig. 2A) or $c$ (Fig. 2B) content per one cell or *in vivo* fluorescence (Fig. 2C) were confirmed within the range of $1\times10^5 - 8\times10^5$. Thus we investigated the toxicological responses of chlorophyll-related parameters (chlorophyll $a$ or $c$ content per one cell or $F_o$ per 1 µg/mL of chlorophyll $a$ concentration).

Fig. 3 shows the chlorophyll $a$ content (pg) per one cell in each treatment group. In the TBT exposure group, chlorophyll $a$ content was significantly increased at the highest TBT treatment ($p<0.05$; Fig. 3A). The values were about one-half (0.5 µg/L treatment) or less than one-half (1.0 µg/L treatment) of the control values in the Irgarol exposure group, although only the highest concentration group exhibited a significant decrease ($p<0.05$).

Fig. 4 shows the chlorophyll $c$ content (pg) per one cell in each treatment group. Chlorophyll $c$ content increased significantly ($p<0.01$) with the highest (2.0 µg/L) TBT treatment. No statistical difference was observed in any Irgarol treatment (Fig. 4B), unlike the chlorophyll $a$ content.

Fig. 5 shows the content ratio of chlorophyll $a$ and chlorophyll $c$ (chl. $a/c$ ratio). There was no statistical difference between the control and TBT treatment groups (Fig. 5A). In contrast, the chl. $a/c$ ratio decreased significantly in all Irgarol treatment groups compared with the control, showing a decrease of less than one-half the control by the highest Irgarol treatment (1.0 µg/L, Fig. 5B).

Fig. 6 shows $F_o$ per µg/mL of chlorophyll $a$ ($F_o$/chl. $a$). This parameter increased when elec-
electron transport activity in PSII was decreased by PSII inhibitor treatment. In the present study, TBT had no effect on this parameter in any treatment group (Fig. 6A) ; however, Irgarol treatment increased \( F_o/\text{chl. } \alpha \) (Fig. 6B) in a concentration-dependent manner.

At the end of the exposure test, pH was in the range of 8.1–8.4 in the TBT test group and 8.0–8.5 in the Irgarol test group.

4. DISCUSSION

The present study showed that toxicological responses of chlorophyll-related parameters in Skeletonema sp. exposed to TBT were clearly different from those exposed to the PSII inhibitor Irgarol. Especially the chlorophyll \( \alpha \) content (\( \mu g/\text{cell} \)) increased in the highest TBT treatment group but decreased in the highest Irgarol treatment group compared with each control (Fig. 3). These results strongly suggest that TBT inhibits the growth of Skeletonema sp. through a different mechanism with the PSII inhibitor, Irgarol.

In Irgarol treatment, chlorophyll \( \alpha \) content decreased significantly to less than one-half of the control value in the highest concentration (1.0 \( \mu g/L \)) groups (Fig. 3B), but chlorophyll \( c \) content was not affected (Fig. 4B). Chlorophyll \( \alpha \) is a component of the antenna pigment–protein complex and of the photosynthetic reaction center. Chlorophyll \( c \) is a component of the antenna pigment–protein complex only. Therefore, decrease in only the chlorophyll \( \alpha \) content strongly suggests that the activity of photosynthetic reaction center was inhibited by Irgarol treatment. This speculation is also supported by the increased level of \( F_o/\text{chl. } \alpha \) (Fig. 6B), because an increase in \( F_o \) is generally known to indicate the occurrence of photoinhibition (Maxwell and Johnson 2000).

The different response of chlorophyll-related parameters to Irgarol indicated that growth suppression of Skeletonema sp. by TBT is not due to direct effect to the photosystem. Neuwoehner et al. (2008) also observed that TBT did not affect quantum yield in PSII in the green alga Scenedesmus vacuolatus. From this and our results, the main causes of a decrease in the chlorophyll content (Fargašová 1997) and oxygen production (Guanzon and Nakahara 2002) by TBT exposure in previous studies are thought to decrease algal cell density but not inhibit photosynthesis. Some studies have reported that TBT has an inhibitory effect on ATPase activity in green algae (Catt et al. 1984), mussel (Pagliarani et al. 2008) and fish (Pinkney et al. 1989). ATPase is an essential enzyme for supporting life, which catalyzes the hydrolysis of the high-energy phosphate bond of ATP. Inhibition of ATPase by TBT might be

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<th>TBT</th>
<th>Irgarol</th>
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<tr>
<td>EC_{10}</td>
<td>0.68 ( \mu g/L ) (0.31–0.94)</td>
<td>0.29 ( \mu g/L ) (0.09–0.43)</td>
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<tr>
<td>EC_{20}</td>
<td>1.18 ( \mu g/L ) (0.92–1.46)</td>
<td>0.58 ( \mu g/L ) (0.44–0.74)</td>
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<tr>
<td>EC_{50}</td>
<td>2.14 ( \mu g/L ) (1.80–2.75)</td>
<td>1.14 ( \mu g/L ) (0.93–1.52)</td>
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<tr>
<td>NOEC</td>
<td>0.5 ( \mu g/L )</td>
<td>0.1 ( \mu g/L )</td>
</tr>
<tr>
<td>LOEC</td>
<td>1.0 ( \mu g/L )</td>
<td>0.5 ( \mu g/L )</td>
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involved in the present growth suppression of *Skeletonema* sp.

TBT significantly increased chlorophyll *a* and *c* content in the highest TBT treatment group (Figs. 3A and 4A). In general, the antenna size was changed by long-term light intensity variations (Melis 1991). However, increases in both chlorophyll *a* and *c* content in the present study were thought to indicate increases in each photosynthetic reaction center, as well as in the antenna complex, since no significant change in the chl. *a/c* ratio was observed when compared with the control (Fig. 5A). An increase in chlorophyll content by TBT was also observed in three Chlorophyta species, *Tetraselmis tetrahele*, *Nannochloropsis oculata* and *Dunaliella* sp. (Rumampuk et al. 2004). A possible reason for this is that interruption of ATP metabolism, a general mode of action of TBT (Catt et al. 1984), induces depletion of usable ATP. Thus the increase in chlorophyll *a* and *c* content suggests an increase in the number of both photosynthetic reaction centers and in the antenna complex. These may be adaptations to depletion of ATP by TBT because reduction power, obtained through photosystem function, is essential to produce ATP. Further study is needed to reveal the underlying mechanism.

Previous studies have indicated that EC<sub>50</sub> values for growth in the diatom *Skeletonema costatum* were in the range of 0.33–1.1 µg/L in TBT (Walsh et al. 1985, Bao et al. 2011) and 0.29–0.57 µg/L in Irgarol (Zhang et al. 2008, Bao et al. 2011). The present EC<sub>50</sub> values (2.14 µg/L in TBT; 1.14 µg/L in Irgarol) were slightly higher than those found in earlier data and these differences may be due to experimental culture conditions. The present study also indicated that the chl. *a/c* ratio (Fig. 5B) and F<sub>v</sub>/chl. *a* (Fig. 6B) of *Skeletonema* sp. exposed to Irgarol were significantly affected even at low concentrations but did not affect algal growth. These results suggest that the chl. *a/c* ratio and F<sub>v</sub>/chl. *a* are simple but sensitive parameters for detecting the PSII-inhibiting effect of the field water sample.

Knowledge of the toxic mechanism is useful in predicting the results of mixed chemical effects (Cedergreen 2014). We indicate that TBT had no adverse effects on the chlorophyll-related parameter at a cellular level in the diatom *Skeletonema* sp., and present study will provide important information relating to eco-toxicology about marine primary production.

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