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Quantification of the Sub-lethal Toxicity of Metals and Endocrine-disrupting Chemicals to the Marine Green Microalga *Tetraselmis suecica*

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Abstract

Microalgae are sensitive indicators of environmental changes, and hence they are widely used in environmental risk assessments and for the development of discharge guidelines. Here we evaluated the toxicity of metals and endocrine-disrupting chemicals (EDCs) to the marine green microalga, *Tetraselmis suecica*. The toxicants investigated included the metals, Cu, Ni, and Pb; and the EDCs, bisphenol A (BPA), endosulfan (ES), and polychlorinated biphenyl (PCB). The endpoints were variations in cell counts and chlorophyll *a* levels. *T. suecica* displayed a varied pattern of sensitivity to the toxicants. Based on the 72-h median effective concentration (EC_{50}), ES (0.045 mg/L) was most toxic to *T. suecica*, followed by PCB (3.96 mg/L) and Pb (9.62 mg/L). Interestingly, *T. suecica* was relatively tolerant to Cu (43.03 mg/L). The 72-h EC_{50} values of Ni and BPA were approximately 16 mg/L. Our data suggest that this species may be relatively tolerant to most of the chemicals within their permissible limits in the environment.

Key words: *Tetraselmis suecica*, Ecotoxicity assessment, 72-h EC_{50}, Metals, EDCs

Introduction

Microalgae are an important component of the aquatic environment (Elser et al., 2007), and are key players in primary productivity and biogeochemical cycles (Sarthou et al., 2005). They are rich in nutrients and organic compounds, and hence are used as aquaculture feeds, health supplements and alternative energy sources (Becker, 2007). Moreover, microalgae are a diverse assemblage of both autotrophs and heterotrophs, have substantial biomass, and are abundant in the marine ecosystem (Shi et al., 2011). They are especially useful as bioindicators of environmental changes, for both short and long-term environmental monitoring as well as ecotoxicology assessments (Franklin et al., 2002). Owing to the involvement of microalgae in the global cycling of toxic chemicals in the aquatic environment, monitoring the effects of chemicals, such as metals and endocrine disruptors, on microalgae is of considerable importance (Torres et al., 2008).

Algae-based bioassays are commonly employed in environmental risk assessments to assess the toxicity of metals, novel chemicals and other emerging contaminants and to establish regulatory guidelines (Stauber and Davies, 2000). Algal toxicity tests routinely use freshwater green algae (*e.g.*, *Chlamydomonas* sp., *Chlorella vulgaris, Pseudokirchneriella subcapitata*, and *Scenedesmus subspicatus*) and the diatom *Navicula pelliculosa* (Franklin et al., 2007). However, in aquatic ecosystems, algae comprise diverse taxa, including green algae, cyanobacteria, diatoms, and dinoflagellates (Shi et al., 2011). Each of these taxa responds differently to chemical toxicants; therefore, it is mandatory to conduct tests on a wide range of species representing different classes to determine safe discharge guidelines. Moreover, the available algae-based toxicity data were determined using freshwater algae (Nalewajko and Olaveson, 1998; Sverdrup et al., 2001); relatively little

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emphasis has been placed on marine algae, hence the need to assess the responses of marine species to various toxicants.

In the present study, we quantified the sub-lethal effects of metals and endocrine-disrupting chemicals (EDCs) on the marine green alga *Tetraselmis suecica*. The test toxicants included Cu, Ni, Pb, bisphenol A (BPA), endosulfan (ES), and polychlorinated biphenyl (PCB). Finally, we calculated the median effective concentrations (EC_{50}) of the chemicals tested and discussed their significance in terms of an environmental risk assessment. *T. suecica* is a marine green alga belonging to Chlorodendrophyceae, Chlorophyta. It has a high growth rate, and it has been used commercially as an aquaculture feed and as a nutritional supplement (Brown, 2002). In addition, *T. suecica* is considered a potential candidate for biodiesel production (Montero et al., 2011). Thus determining its potential as a model for toxicity assays could be advantageous.

Materials and Methods

Test species and culture conditions

T. suecica (P009) was obtained from the Korea Marine Microalgae Culture Center (Pukyung National University, Busan, Korea). The cells were cultured in f/2 medium, using filtered seawater with additional macronutrients, vitamins and trace metals ($CuSO_4$, $ZnSO_4$, $CoCl_2$, $MnCl_2$, and $NaMoO_4$) according to Guillard and Ryther (1962). The cells were maintained at 20°C, under a 12:12-h light: dark cycle with a photon flux density of ca. 65 μmol photons/m^2/s.

Toxic chemicals

In this study, three metals (Cu, Ni, and Pb) and three EDCs (BPA, ES, and PCB) were selected as test toxicants. The concentrations of each were chosen based on EC_{50} values reported for other aquatic organisms (Millán de Kuhn et al., 2006; Soto-Jiménez et al., 2011). Accordingly, a range of concentrations of each chemical was prepared, as described below.

For Cu (as $CuSO_4$; Cat. No. C1297, Sigma, St. Louis, MO, USA), the concentrations used were 0.5, 1.5, 3, 6, 12, 25, 75, 150, 500, and 750 mg/L. For Pb (as $PbCl_2$; Cat. No. 268690, Sigma), the chosen concentrations were 0.5, 1.5, 5, 10, 25, 50, 100, 150, 250, 500 and 750 mg/L. For Ni (as $NiCl_2$; Cat. No. 339350, Sigma), the concentrations used were 0.1, 0.5, 1, 5, 10, 50, 100, 250, and 500 mg/L. All test concentrations were higher than that added to the f/2 medium. For example, amongst the four test metals, only 0.0068 mg/L $CuSO_4$ was added to the f/2 medium, and thus the medium-containing metals or EDCs contained negligible endpoint concentrations.

For BPA (Cat. No. A133027, Sigma), concentrations of 0.5, 1, 2.5, 7.5, 15, 25, 50, 75, and 100 mg/L were prepared from a stock solution. BPA was dissolved in 10% dimethyl sulfoxide. For ES (Cat. No. 36676, Sigma), the concentrations used were 0.001, 0.01, 0.1, 0.5, 1 and 10 mg/L. PCBs were prepared from Aroclor 1016 (Cat. No. 48701, Sigma) at 0.001, 0.01, 0.05, 0.1, 0.5, 1, 10, 20, and 50 mg/L. All dilutions were from standard stock solutions.

Toxicity bioassay

Fifty-milliliter aliquots of the algal culture, comprising cells in the exponential phase of growth, were transferred into sterile tubes. The chemicals, at the concentrations mentioned above, were transferred into test tubes in duplicate. The initial cell concentration was $5.5 \pm 0.1 \times 10^5$/mL. and samples were withdrawn for cell counts and chlorophyll *a* (Chl *a*) estimation at 0, 12, 24, 48, and 72 h.

Cells were enumerated using a hemocytometer (Marienfeld GmbH, Lauda, Germany). Cell counts were plotted against exposed time as $\log_{10}$ values. In addition, Chl *a* was measured by concentrating a 10 mL sample of the culture at various time points. The pigments were extracted with 90% acetone after incubation overnight in the dark. The supernatants extracted were measured using a DU730 Life Science UV/Vis spectrophotometer (Beckman Coulter, Fullerton, CA, USA). The Chl *a* concentrations were estimated following Parsons et al. (1984).

EC_{50} and statistical tests

The median effective concentration (EC_{50}) and the percentile growth inhibition were calculated as recommended in the Organisation for Economic Cooperation and Development (OECD) test guidelines (OECD, 2011). The 72-h EC_{50} values were estimated using a sigmoidal dose-response curve plotted in Origin 8.5 (MicroCal Software Inc., Northampton, MA, USA) based on the sigmoidal four parameter equation (Teisseyre and Mozrzymas, 2006): $\text{Log } EC_{50} = a + (b - a)/[1 + 10(x - c)^d]$, where a is the response value at zero or minimum asymptote, b is the response value for an infinite concentration or maximum asymptote c is the mid-range point, d is the steepness of the curve or the Hill slope and x is the dilution coefficient.

All data presented are the mean values of duplicate determinations. A one-way analysis of variance (ANOVA) with post hoc Student's Newmann Keul's test in Graphpad InStat (Graphpad Software, Inc., La Jolla, CA, USA) was used for comparisons between non-treated and treated cultures. $P < 0.05$ was accepted as significant. In addition, correlations between cell counts and Chl *a* using Pearson's correlation coefficient (r^2) were calculated using an Excel spreadsheet (Microsoft Corporation, Redmond, WA, USA).

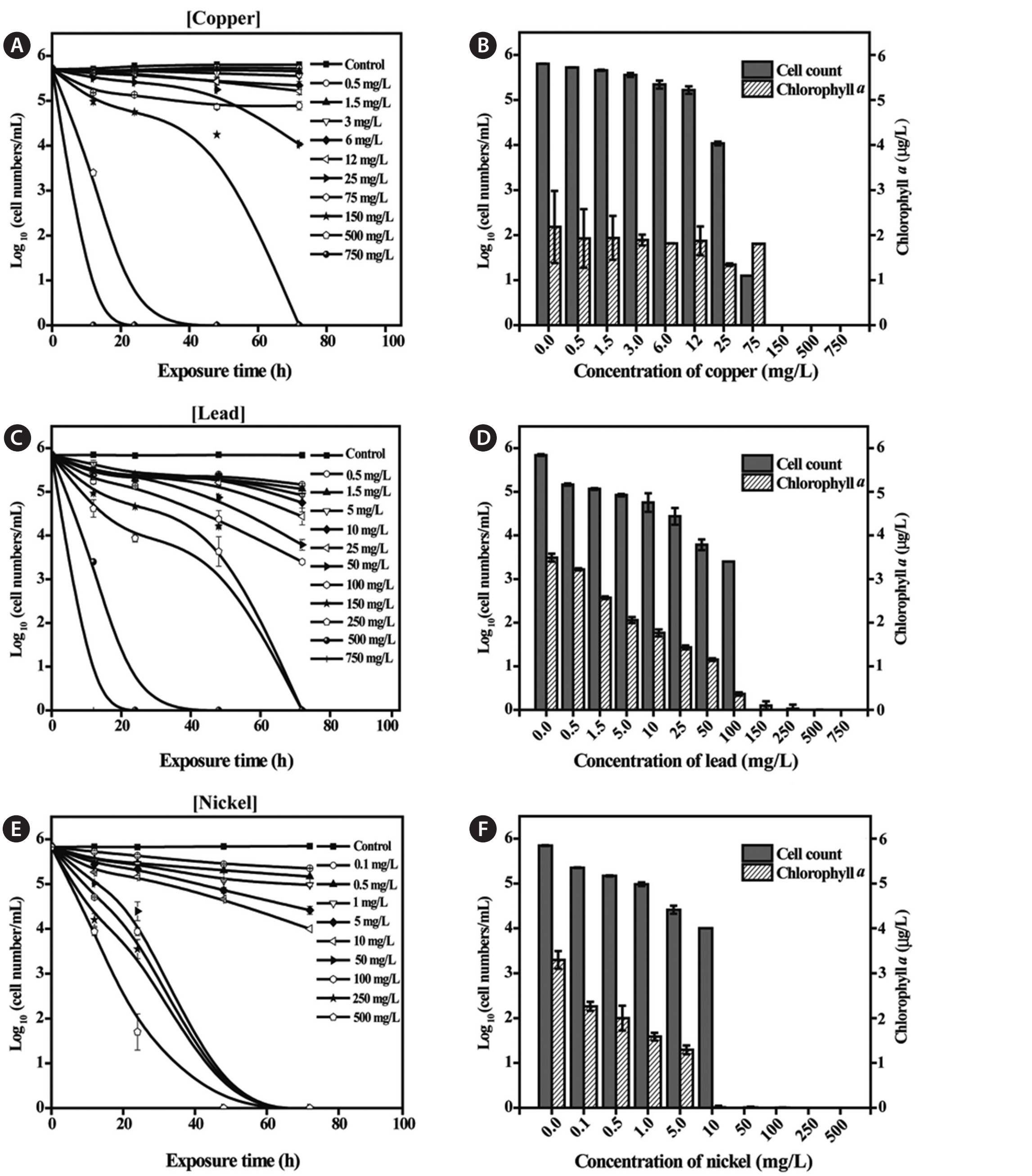

Fig. 1. Variation in cell count of *Tetraselmis suecica* following exposure to metals. (A, C, E) Variation in cell numbers following exposure to metals. (B, D, F) Variation in cell count and chlorophyll *a* following 72-h exposure to metals.

Results

Toxicity of metals to *Tetraselmis suecica*

Exposure of *T. suecica* to metals (Cu, Ni, and Pb) induced a wide range of responses, depending on the toxicant concentrations (Fig. 1). In all cases, the initial experimental concentrations used (*i.e.*, 0.1-10 mg/L) of the metals had very little or no effect on the cell counts or Chl *a* levels. However, when cells were exposed to higher concentrations of metals (50-750 mg/L), significant reductions ($P < 0.001$) were recorded. We observed no significant changes in *T. suecica* cells exposed to levels up to 150 mg/L Cu and up to 10 mg/L Pb and Ni (Fig. 1A, 1C, and 1E). However, above these concentrations, significant reductions ($P < 0.001$) in cell count and Chl *a* level were observed for all of the tested metals (Fig. 1B, 1D, and 1E).

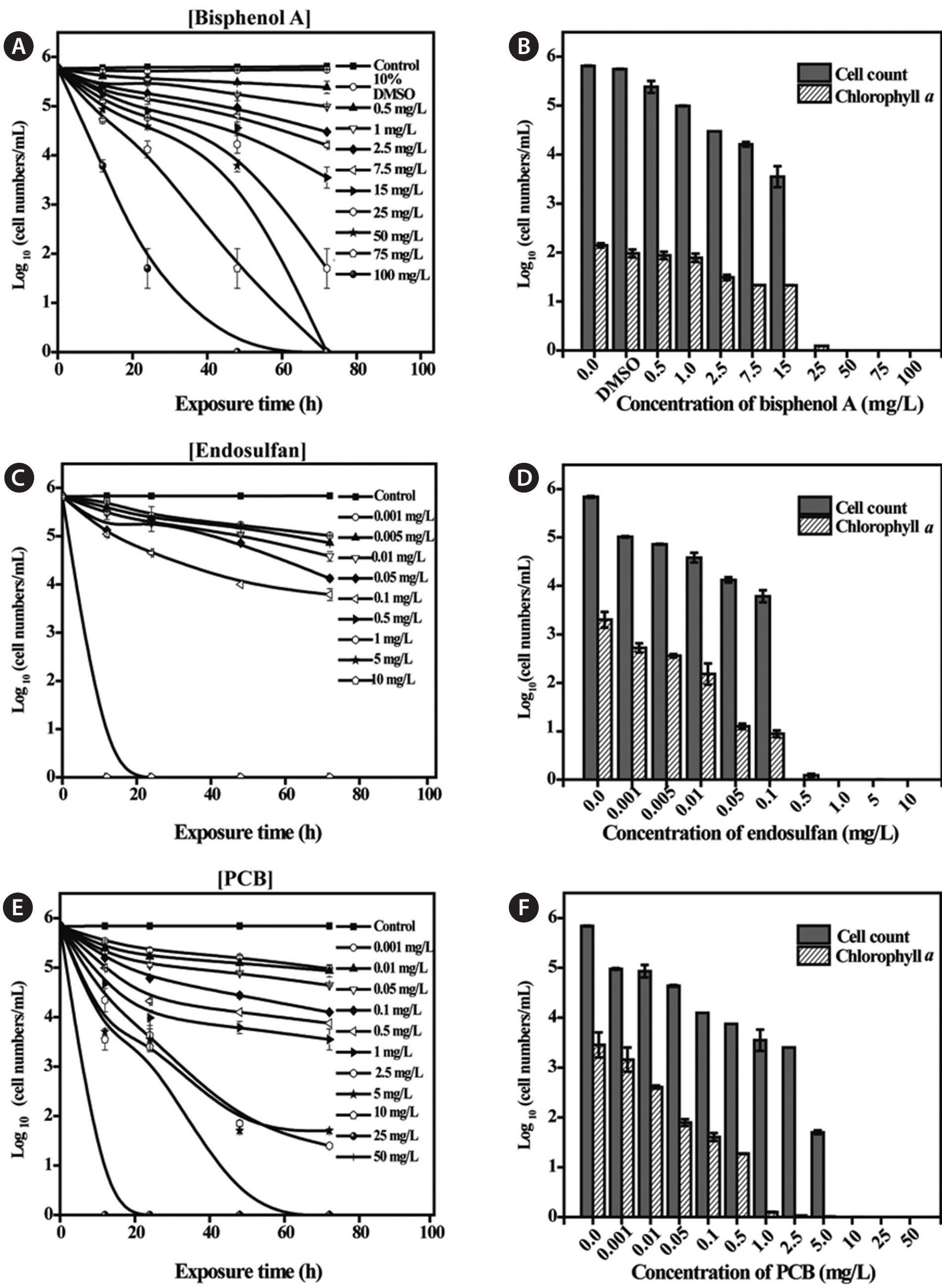

Fig. 2. Variation in cell count of *Tetraselmis suecica* following exposure to endocrine-disrupting chemicals (EDCs). (A, C, E) Variation in cell numbers following exposure to EDCs. (B, D, F) Variation in cell count and chlorophyll *a* following 72-h exposure to EDCs. PCB, polychlorinated biphenyl.

Toxicity of EDCs to *Tetraselmis suecica*

Additional toxicity tests for the three EDCs (BPA, ES, and PCB) were performed over a wide range of concentrations (Fig. 2). BPA was administered at concentrations of 0.5-100 mg/L. *T. suecica* cells were not significantly affected ($P > 0.05$) at lower BPA concentrations (~25 mg/L). However, higher concentrations of BPA (>50 mg/L) caused a significant decrease in the cell count (79-100%). Exposure of *T. suecica* to the lower concentrations of ES (0.001-0.05 mg/L) led to a 14-30% reduction in cell count, but there was a highly significant ($P < 0.0001$) reduction at higher concentrations (0.1-10 mg/L). PCB exposure followed a similar trend, with concentrations greater than 1 mg/L significantly decreasing both cell numbers and Chl *a* levels.

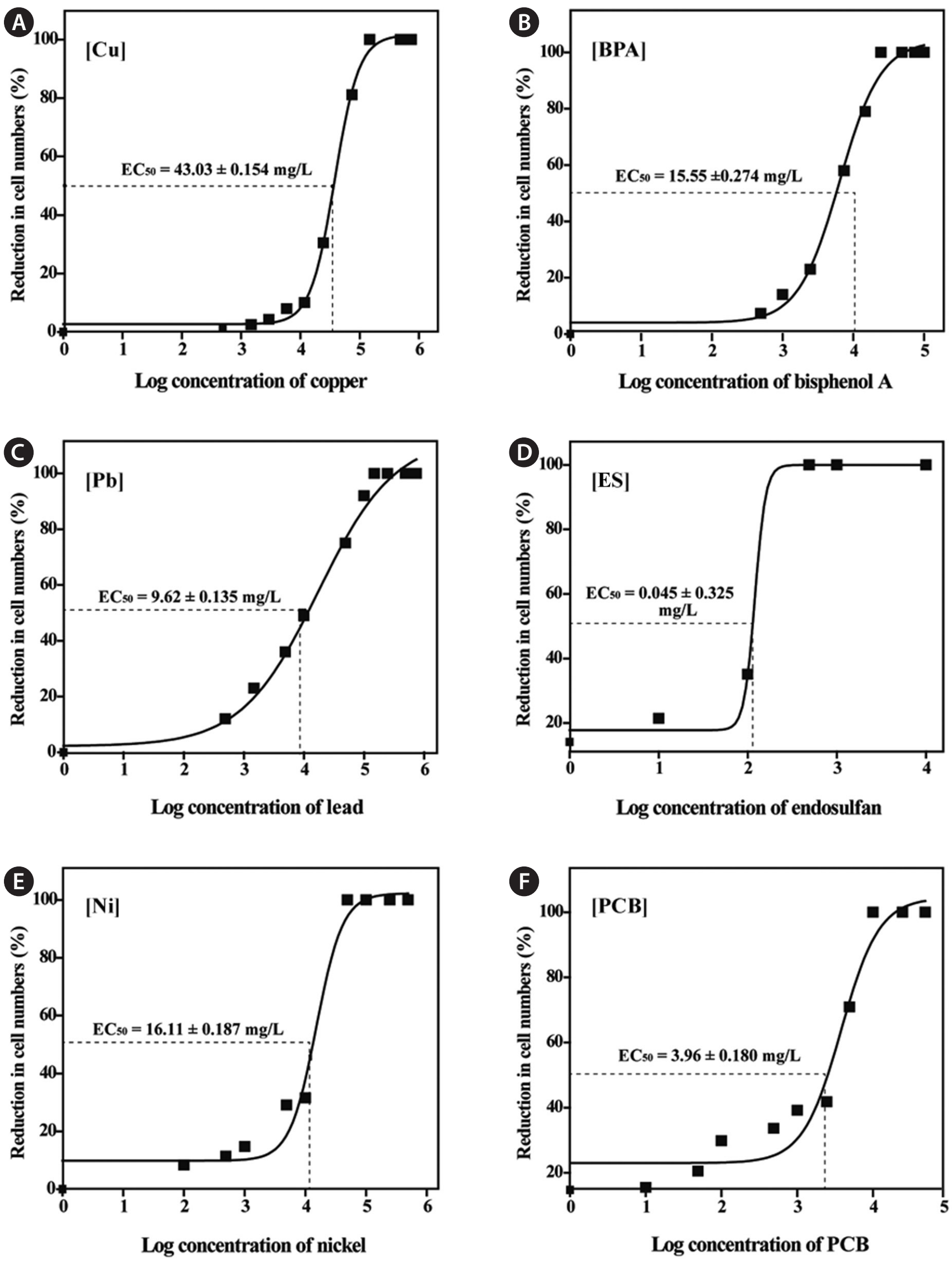

Fig. 3. Dose response curve of *Tetraselmis suecica* following 72-h exposure to metals and endocrine-disrupting chemicals. (A) Copper, (B) bisphenol A (BPA), (C) lead, (D) endosulfan (ES), (E) nickel, and (F) polychlorinated biphenyl (PCB).

Dose-response curves and 72-h EC_{50} values

Table 1 shows the Pearson's correlations between cell count and Chl *a* level; the data for both endpoints were valid and correlated in most experiments. In this study, we calculated 72-h EC_{50} values (Table 2) using the cell count data and plotted sigmoidal dose-response curves for *T. suecica* exposed to metals and EDCs (Fig. 3). The EC_{50} values for the metals were as follows: EC_{50} (Cu) = 43.03 ± 0.154 mg/L, EC_{50} (Pb) = 9.62 ± 0.135 mg/L, and EC_{50} (Ni) = 16.11 ± 0.187 mg/L. The EC_{50} values of the EDCs were as follows: EC_{50} (BPA) = 15.55 ± 0.274 mg/L, EC_{50} (ES) = 0.045 ± 0.032 mg/L, and EC_{50} (PCB) = 3.96 ± 0.180 mg/L.

Discussion

Toxicity assessments using marine species can be challenging compared with assessments using freshwater species. This is because the marine environment can have a more profound influence on toxicity evaluation than freshwater ecosystems. In addition, the higher ionic strength and buffering capacity of seawater can alter the bioavailability of discharged chemicals due to complex chemical reactions and the subsequent formation of by-products (Moffett and Zika, 1987). As noted previously, relatively few species can be described as "standard" for marine algae, although several guidelines have been published and some marine species have been recommended as model species (Nalewajko and Olaveson, 1998; Sverdrup et al., 2001). In this study, we present additional toxicity data for various metals and EDCs to the marine green alga *T. suecica*.

Discharge guidelines for metals in the marine environment are as follows: Cu, 0.5 mg/L; Pb and Ni, 0.1 mg/L (United States Environmental Protection Agency, 1996). Individual discharges of Cu, Pb, or Ni confined to the stipulated levels should have very little, if any, effects on cell counts or Chl *a* levels in *T. suecica* (see Figs. 1 and 3). In addition, compared to most of the frequently used freshwater green algae, such as *Chlorella vulgaris*, *Selenastrum capricornutum, Pseudokirchnereiella subcapitata*, etc., *T. suecica* is highly resistant to these commonly used metal pollutants. For example, the 72-h EC_{50} value for Cu was 0.016 mg/L in *S. capricornutum* at a similar cell density (Franklin et al., 2002) to that used in this study. Vasseur et al. (1988) reported that the EC_{50} values for Cu and Zn in *S. capricornutum* were 10 µg/L and 90 µg/L, respectively. However, Millán de Kuhn et al. (2006) reported markedly higher EC_{50} values for Cu in various marine algae than freshwater algae. The authors recorded EC_{50} values of Cu of 220 mg/L for the green alga *Dunaliella salina*, 34.0 mg/L for the flagellate *Euglena gracilis*, 13.5 mg/L for the dinoflagellate *Heterocapsa triquetra*, and 7.0 mg/L for the dinoflagellate *Prorocentrum minimum*, respectively. Our previous studies also showed high EC_{50} values for Cu (12.74 mg/L) and Pb (46.70 mg/L) in the marine dinoflagellate *Cochlodinium polykrikoides* (Ebenezer and Ki, 2012). These data suggest that marine algae may be more tolerant to metal exposure than freshwater species, particularly at the level of the safe discharge standards recommended by the United States Environmental Protection Agency (US EPA) (US EPA, 1996).

In addition, comparisons of available EC_{50} data revealed that our test species was generally more tolerant than other freshwater algae, including *Desmodesmus subspicatus* and *Chlorella kessleri* (Pavlić et al., 2006). The high tolerance of *T. suecica* is generally in accordance with previous reports stating that *T. suecica* was more tolerant and bioaccumulative upon exposure to metals (Pèrez-Rama et al., 2002) than other algae. Millán de Kuhn et al. (2006) reported an EC_{50} (Cu) value of 40 mg/L in *T. suecica*. Moreover, Debelius et al. (2009) found that *Tetraselmis chuii* [EC_{50} (Cu) = 0.33 mg/L and EC_{50} (Pb) = 2.6 mg/L] was more tolerant in toxicity assessments than the other marine microalgae tested, including the diatom *Chaetoceros* sp. and green algae *Rhodomonas salina*, *Isochrysis galbana*, and *Nannochloropsis gaditana*. Overall, previous reports and our findings suggest that the marine algae genus *Tetraselmis* is more tolerant to metals than other algae.

To date, the available data regarding the toxicity of EDCs to algae is limited compared to metals. One reason for this is that algae do not have an endocrine system, and thus may show limited effects of exposure to EDCs. However, recent studies have shown that most EDCs, such as BPA, ES, PCB or metolachlor, do have toxic effects on algae (Liu et

Table 1. Pearson's correlation between cell count and Chl *a* level in *Tetraselmis suecica* cells following exposure to toxic chemicals

Chemicals	Correlation coefficient (r^2)	*P*-value (significance)	95% Confidence interval
Copper	0.9958	<0.001	0.9833-0.9989
Lead	0.8931	<0.001	0.7546-0.9699
Nickel	0.7650	0.0099	0.6610-0.8413
Bisphenol A	0.9770	<0.0001	0.9676-0.9944
Endosulfan	0.9101	0.0003	0.8567-0.9788
PCB	0.7234	0.0078	0.6554-0.8167

Chl *a*, chlorophyll *a*; PCB, polychlorinated biphenyl.

Table 2. 72-h EC_{50} values of chemicals exposed to *Tetraselmis suecica*

Chemicals	EC_{50} value* (mg/L)	*P*-value (significance)	r^2	95% Confidence interval	
				Lower	Higher
Copper	43.03 ± 0.154	<0.0001	0.9711	42.234	44.750
Lead	9.62 ± 0.135	<0.0001	0.9640	7.681	10.062
Nickel	16.11 ± 0.187	0.0033	0.9675	15.699	17.532
Bisphenol A	15.55 ± 0.274	<0.0001	0.9277	14.801	16.983
Endosulfan	0.045 ± 0.032	<0.0001	0.9048	0.0326	0.0576
PCB	3.96 ± 0.180	<0.0001	0.9569	3.143	5.077

PCB, polychlorinated biphenyl.
*EC_{50} values are mean ± SD (n = 2).

al., 2010; Ebenezer and Ki, 2012), in particular by damaging photo system II energy fluxes in chloroplasts (Perron and Juneau, 2011). In contrast, the data from this study indicated that *T. suecica* was relatively tolerant to BPA and PCB (Table 2), although not to ES, when compared with other algae. For example, the EC_{50} values of BPA were recorded as 3.73 mg/L and 7.96 mg/L for the diatoms *Navicula incerta* and *Cyclotella caspia*, respectively (Li et al., 2009; Liu et al., 2010). The 96-h EC_{50} value of PCBs for the dinoflagellate *Lingulodinium polyedrum* was 0.122 mg/L (Leitão et al., 2003). In the marine dinoflagellate *Cochlodinium polykrikoides*, the 72-h EC_{50} of PCB was 1.07 mg/L (Ebenezer and Ki, 2012). Alternatively, the EC_{50} value of ES for the green alga *Pseudokirchneriella subcapitata* was 0.427 mg/L (De Lorenzo et al., 2002), thus *T. suecica* was more sensitive [EC_{50} (ES) = 0.045 mg/L]. EC_{50} values for the EDCs tested were mostly higher than the environmental discharge limits set by the US EPA for coastal waters, which would have had no effect on the marine algae. The US EPA guidelines set safe discharge levels of 0.09 µg/L for BPA (Kolpin et al., 2002), 0.034 µg/L for ES (Agency for Toxic Substances and Disease Registry, 2000) and 0.02 µg/L for PCBs (Nagpal et al., 2006). Although these compounds do not pose a risk as acute toxicants, their chronic effects can be deleterious. The high tolerance of *T. suecica* to the EDCs is in accordance with previous metal toxicity data.

In conclusion, *T. suecica* exhibited a dose-dependent response upon exposure to selected metals and EDCs. This species was generally tolerant to most of the chemicals at their permissible concentrations. According to the EC_{50} values obtained, in terms of the metals tested, *T. suecica* was most sensitive to Pb (9.62 mg/L) and most tolerant to Cu (43.03 mg/L). Of the three tested EDCs, *T. suecica* was most sensitive to ES (0.045 mg/L) and most tolerant to BPA (15.55 mg/L). These data indicate that *T. suecica* may be relatively tolerant of toxic chemicals compared with other marine algae, and has a markedly higher tolerance than freshwater algal species.

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