Korean Journal of Microbiology (2016) Vol. 52, No. 3, pp. 306-312
DOI http://dx.doi.org/10.7845/kjm.2016.6050

pISSN 0440-2413
eISSN 2383-9902

Article

Toxic effects of Aroclor 1016 and bisphenol A on marine green algae *Tetraselmis suecica*, diatom *Ditylum brightwellii* and dinoflagellate *Prorocentrum minimum*

Vinitha Ebenezer and Jang-Seu Ki*

Department of Life Science, Sangmyung University, Seoul 03016, Republic of Korea

해양 녹조류 *Tetraselmis suecica*, 규조류 *Ditylum brightwellii*, 와편모조류 *Prorocentrum minimum*에 대한 Aroclor 1016과 비스페놀 A의 독성 효과

비니타 에베네저 · 기장서*

상명대학교 생명과학과

(Received August 29, 2016; Revised September 23, 2016; Accepted September 26, 2016)

ABSTRACT: Microalgae are the potential bioindicators of environmental changes, for the environmental risk assessment as well as to set limits for toxic chemical release in the aquatic environment. Here, we evaluated the effects of two endocrine disrupting chemicals (EDCs), namely bisphenol A (BPA) and Aroclor 1016, on the green algae *Tetraselmis suecica*, diatom *Ditylum brightwellii*, and dinoflagellate *Prorocentrum minimum*. Each species showed wide different sensitivity ranges when exposed to these two EDCs; the 72 h effective concentration (EC_{50}) for these test species showed that Aroclor 1016 was more toxic than BPA. EC_{50} values for the diatom *D. birghtwellii* were calculated at 0.037 mg/L for BPA and 0.002 mg/L for Aroclor 1016, representing it was the most sensitive when compared to the other species. In addition, these results suggest that these EDC discharge beyond these concentrations into the aquatic environments may cause harmful effect to these marine species.

Key words: Aroclor 1016, bisphenol A, EC_{50}, ecotoxicity assessment, marine microalgae

Industrialization and demand for novel products have resulted in the release of diverse class of synthetic and organic compounds including the endocrine disrupting chemicals (EDCs) into the aquatic ecosystem. These result in changes in the nature of the pollutant burden (Liu *et al*., 2010). EDCs are commonly used in the manufacture of pesticides, plastics, fire retardants, etc. There is a considerable concern over the environmental occurrence of EDCs, because they have a potential to modulate or disrupt the synthesis, secretion, transport, binding action, or elimination of hormones in the body, thereby affecting homeostasis, development, reproduction, and the behavioral changes in aquatic organisms (Tarrant, 2005).

Bisphenol A (BPA) is a possible EDC, and it is widely used to produce adhesives, coatings, paints and building materials (Li *et al*., 2009). BPA has potential estrogenic activity, at lower concentration (1.0 μg/L). It is clear that the effects of BPA to the aquatic organisms at environmentally relevant concentrations could produce deleterious impact to the aquatic ecosystem. In addition, polychlorinated biphenyls (PCBs) are common pollutants, and their manufacture, use and subsequent release have resulted in global contamination (Jensen, 1966). The lipophilic property of these organic compounds can lead to their accumulation in the lipid of the microalgae and can be transferred to higher organisms (Sleiderink *et al*., 1995).

Microalgae based tests are mostly employed in environmental risk assessment for evaluating the toxicity of heavy metals and

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***For correspondence.** E-mail: kijs@smu.ac.kr;
Tel.: +82-2-2287-5449; Fax: +82-2-2287-0070

other emerging contaminants, and in forming regulatory guidelines (Stauber and Davies, 2000; Ebenezer and Ki, 2012, 2013a). The toxicity tests are carried out by measuring growth rate, cell densities, and/or chlorophyll content of the tested species (OECD, 2011). These bioassays are reliable, reproducible, rapid, and inexpensive compared with fish testing schemes (Monteiro *et al*., 2011). To date, algae based toxicity assessments have been mainly carried out by using freshwater algae (e.g., *Chlorella vulgaris*, *Pseudokirchneriella subcapitata*, and *Scenedesmus subspicatus*) (OECD, 2011); relatively little emphasis has been laid to marine algae. Marine microalgae play a significant role in maintaining the balance of the aquatic ecosystem, by being the key players in marine energy production and transfer. They are a diverse assemblage of both autotrophs and heterotrophs, and have substantial biomass and abundance in the marine ecosystem (Shi *et al*., 2011). Therefore, there is a need for more data on the effects of toxic substances on marine microalgae to better assess the risk of organic and inorganic substances discharged into the marine environment. In addition, more studies involving microalgae of different classes in ecotoxicology assessment for a particular chemical of interest is mandatory.

In the present study, the marine green algae *Tetraselmis suecica* was used due to its rapid growth rate, thus it has been used as an aquaculture feed, a nutritional supplement (Muller-Feuga, 2000) and in toxicity tests (Fabregas *et al*., 1984). In addition, the diatom *Ditylum brightwellii* is used as a model organism for aquatic toxicity and heavy metal bioaccumulation studies (Gerringa *et al*., 1995). The dinoflagellate *Prorocentrum minimum* is very important, because of its potentially harmful effects on marine animals and humans (Heil *et al*., 2005), and it is commonly used in genomic, toxicology, and evolutionary studies (Guo and Ki, 2011). The main objective of the present study was to determine the effect of EDCs (e.g., BPA and Aroclor 1016) on growth rate of these marine species.

Materials and Methods

Test organisms

Strains of the three microalgae *T. suecica* (P-039), *D. brightwellii* (B-326), and *P. minimum* (D-127) were obtained from the Korea Marine Microalgae Culture Center (Pukyung National University, Korea). The cells were cultured in f/2 medium, at 20°C, and 12:12-h light:dark cycle with a photon flux density of approximately 65 mmol photons/m^2/sec.

Test chemicals and experimental design

Bisphenol A (BPA) and Aroclor 1016 (a commercial type of PCBs) were used in the present study. BPA (Cat. No. A133027, Sigma) was dissolved in 10% dimethyl sulfoxide (DMSO) to obtain a stock solution of 10,000 mg/L. Working solutions were obtained by diluting the stock in DMSO, and the concentrations used here were as follows: 0.05, 0.10, 0.25, 1.0, 2.5, 5.0, 10, and 20 mg/L for *D. brightwellii*, 0.01, 0.10, 0.5, 1.0, 2.5, 5.0, 10 mg/L for *P. minimum*, and 0.5, 1.0, 2.5, 7.5, 15, 25, 50, 75, and 100 mg/L for *T. suecica*, respectively. A stock solution of Aroclor 1016 (Cat. No. 48701, Sigma) with concentration of 100,000 mg/L was commercially obtained, the stock was further diluted to obtain the following working concentration: 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2.5, and 5 mg/L for *D. brightwellii*, 0.001, 0.005, 0.025, 0.05, 0.1, 0.5, 1, 5, and 10 mg/L for *P. minimum*, and 0.001, 0.01, 0.05, 0.1, 0.5, 1, 2.5, 5, 10, 25, and 50 mg/L for *T. suecica*.

Fifty milliliter of the cell culture at exponential phase were transferred into sterile tubes. The toxicants at each respective nominal concentration were dosed into the tubes in duplicate. The initial cell concentration was $5.50 \pm 0.1\times10^5$ cells/ml for *T. suecica*, whereas $3.25 \pm 0.5\times10^5$ cells/ml for *D. brightwellii,* and $3.75 \pm 0.1\times10^5$ cells/ml for *P. minimum*. The samples were drawn at 0, 12, 24, 48, and 72 h for cell count and chlorophyll *a* (Chl *a*) estimation.

Cell counting and chlorophyll *a* estimation

Based on standardized OECD assays (OECD, 2011), toxicity tests were carried out in the present study. Cell counts and Chl *a* levels were chosen as the endpoints to determine the effective concentration. Cell counts in each test flask were determined using a hemocytometer (Marienfeld GmbH). Cell counts were plotted against time using $\log_{10}$ values of cell numbers.

Chl *a* was estimated using 10 ml of the culture at specific times, as mentioned above. The pigment was extracted with 90% acetone after overnight incubating in the dark. Optical density of the extracted pigments was measured using a DU730 Life Science UV-Vis spectrophotometer (Beckman Coulter, Inc.). The Chl *a* concentration was estimated following Parsons *et al.* (1984).

Median effective concentration (EC_{50}) and bioavailability of the chemicals

EC_{10} and EC_{20} values and EC_{50}-72 h values were estimated by using a sigmoidal dose-response curve, and were plotted using Origin ver. 8.5 (MicroCal Software) based on the following equation: Sigmoidal (Log EC_{50}) = a + (b - a)/[1 + $10^{(x - c)}$] (Mensink *et al.*, 2008). Furthermore, bioavailability and effective range of BPA and Aroclor 1016 in each culture were evaluated by calculating the maximum concentration (C_{max}) and minimum concentration (C_{min}) values for the test chemicals. These were determined by using the EC_{50}-72 h values and dose-response curves, followed by Craig *et al*. (2010).

Data analysis

Cell count and Chl *a* levels were selected as the endpoints for evaluating toxicity response in this study. Results were expressed as means and standard deviation (SD ±) of duplicates. The data between different treated groups in each measurement were compared statistically by one-way ANOVA using InStat GraphPad (GraphPad Software Inc.). The significance was set at $P < 0.05$. Pearson's correlation and two-tailed T tests were carried out using InStat GraphPad to identify a possible correlation between cell counts and Chl *a* levels.

Results and Discussion

In the present study, we counted the cell numbers and measured the Chl *a* levels of the test species to assess the effect of short-term exposure (72 h) to EDCs. Through these analyses, we found that the Pearson's correlation coefficient between cell count and Chl *a* was positively correlated in all the tested chemicals (Table 1). EC_{50} was calculated by using cell counts, based on a sigmoidal dose-response curve for the test microalgae. In the diatom *D. brightwellii*, EC_{50} was calculated by using Chl *a* content due to clumping of cells.

Toxicity of Aroclor 1016 to marine microalgae

Among the two EDCs evaluated in the present study, Aroclor 1016 (a type of PCBs) was the more toxic to all the tested species, of which EC_{50} values ranged from 0.002 to 3.96 mg/L. The variation in the cell numbers and Chl *a* content are shown in Fig. 1. Our EC_{50} results were compared with the previous reports (Mayer *et al*., 1998; Millán de Kuhn *et al*., 2006; Debelius *et al*., 2009). According to Mayer *et al*. (1998), the EC_{50} values of the freshwater green algae *Pseudokirchneriella subcapitata* after exposes to different PCB compounds (31, 48, and 105) were ranging from 0.004–0.062 mg/L. Similarly, the EC_{50} value of the marine dinoflagellate *Lingulodinium polyedrum* when exposed to the PCB compound Aroclor 1254 was 0.122 mg/L (Leitão *et al*., 2003). Compared to these data, our test species *D. brightwellii* and *P. minimum* showed almost similar or less sensitivity patterns. However, the green algae *T. suecica* was exceptional, showing that it was more tolerant to Aroclor 1016 toxicity. In general, marine *Tetraselmis* spp. was found to be tolerant to chemical stress, such as copper, lead, and cadmium (Liu *et al*., 2011). However, *Tetraselmis* spp. responses to heavy metal exposures were well documented (Pérez-Rama *et al*., 2002; Millán de Kuhn *et al*., 2006; Debelius *et al*., 2009) and they reported that this species is highly tolerance and has the ability to bioaccumulate higher when compared to other species. The environmental discharge limit for PCBs in coastal waters as set by USEPA was 0.02 µg/L (Nagpal *et al*., 2006). Since, PCB compounds were reported to affect various physiological functions in algae at very lower concentrations, such as phosphate uptake and respiration (Larsson and Tillberg, 1975), possibly leading to disturbances in the primary productivity in the

Table 1. Pearson's correlation and two-tailed T test between cell count and chlorophyll *a* level in microalgae after exposing to BPA and Aroclor 1016

Species	Chemicals	Pearson's correlation coefficient (r)	*P* value	95% confidence limits
T. suecica	BPA	0.9770	<0.0001	0.9076–0.9944
	Aroclor 1016	0.7234	0.0078	0.2554–0.9167
P. minimum	BPA	0.7188	0.1075[a]	0.4244–2.9020
	Aroclor 1016	0.8931	<0.0001	0.6546–0.9699

[a] Not significant ($P > 0.05$)

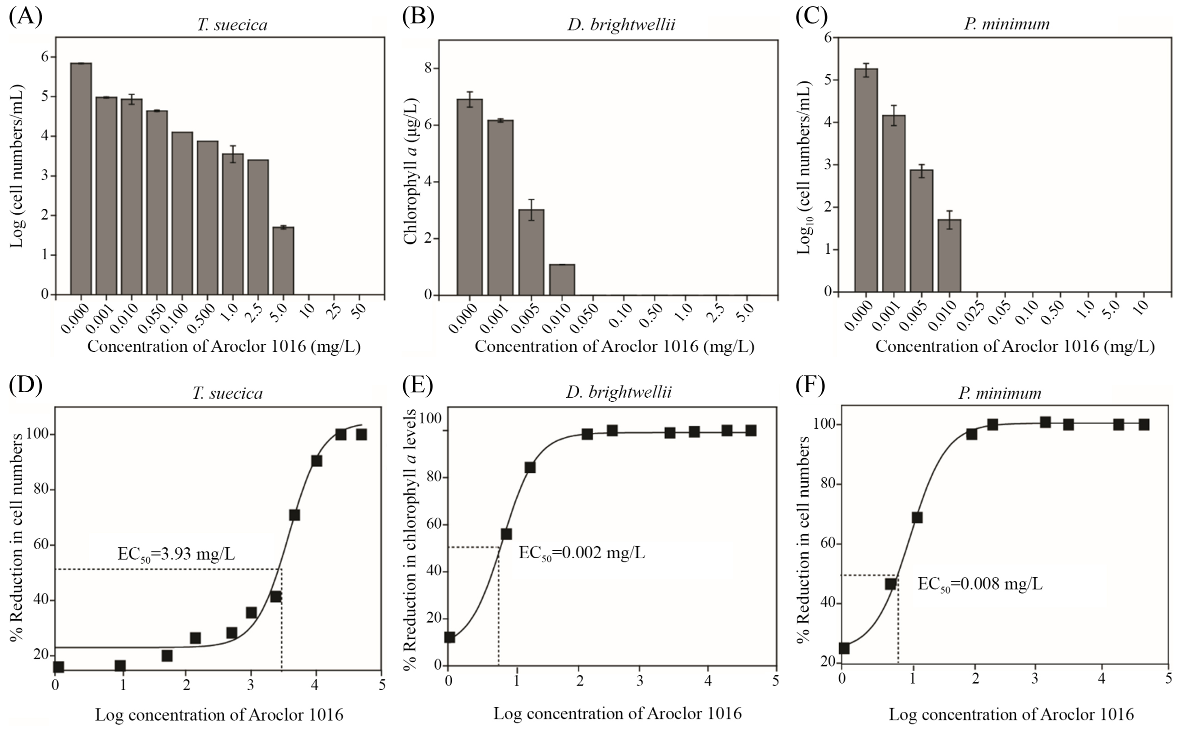

Fig. 1. Effects of Aroclor 1016 on microalgae, *Tetraselmis suecica*, *Ditylum brightwellii*, and *Prorocentrum minimum* based on cell numbers and Chl *a* after 72 h (A, B, C) and dose response curves (D, E, F).

marine environment. These previous reports and our result clearly suggest that EDCs toxicity shows a heterogenous response among the different marine species; of them, diatoms and dinoflagellates may be more sensitive than green algae. This finding was supported by the result of Ebenezer and Ki (2013b).

Toxicity of BPA to marine microalgae

In addition, BPA toxicity to three species was carried out with a wide range of concentrations (Fig. 2). Overall, we found that BPA exerted a dose-dependent decrease in cell numbers and Chl *a* level of the test species (Fig. 2). Of them, *D. brightwellii* and *P. minimum* cells were significantly affected at the lower BPA concentrations (ca. 0.05–0.1 mg/L). However, *T. suecica* was much tolerant at lower concentrations (~2.5 mg/L) when compared to other species. Higher concentrations of BPA (>7.5 mg/L) caused significant decrease in cell counts (80–100%). In addition, the values of 72-h EC_{50}, were estimated using a sigmoidal dose-response curves drawn by cell count data or Chl *a* concentration. The EC_{50} values of BPA were 15.55 mg/L for *T. suecica*, 0.037 mg/L for *D. brightwellii*, and 1.506 mg/L for *P. minimum* (Fig. 2). Upon comparison of EC_{50} values, BPA was less toxic and tolerant when compared to Aroclor 1016. This was in accordance with previous results (Alexander *et al*., 1988; Staples *et al*., 1998; Li *et al*., 2009). For example, Staples *et al.* (1998) reported that BPA was a weak toxicant to aquatic organisms. The EC_{50} reported for other microalgae was more or less within the same range as observed in the present study. The reported values were as follows: for examples, 1.0–3.1 mg/L for the green algae *Pseudokirchneriella subcapitata* (Staples *et al*., 1998), 1.0 mg/L for the marine diatom *Skeletonema costatum* (Alexander *et al*., 1988), 8.65 mg/L for the freshwater diatom *Stephanodiscus hantzschii* (Li *et al*., 2009), and 3.73 mg/L for the freshwater diatom *Navicula incerta* (Liu *et al*., 2010). However, BPA can cause harmful chronic effects in the Japanese medaka (*Oryzia latipes*), after exposed over 14 days, and they found to produce ovotestis at concentration as less as 0.01 mg/L (Metcalfe *et al*., 2001). It is obvious that BPA is not a potential toxicant for short-term exposure; however, its effects can be more pronounced for long-term exposures.

Threshold effects and bioavailability of tested chemicals

As for the threshold effect parameter, we calculated additional EC_{10} and EC_{20} values, which represented the initial concentration of the test chemical that enhances an effect on our tested algae (Table 2). For example, 10% effective concentration (EC_{10}) value for tested species were as follows: *T. suecica*, BPA (2.5 ± 0.001 mg/L) and Aroclor 1016 (zero mg/L); *D. brightwellii*, both BPA and Aroclor 1016 (zero mg/L); *P. minimum*, (BPA 0.2 ± 0.01 mg/L) and Aroclor 1016 (zero mg/L). These suggested that Aroclor 1016 might be much more toxic than BPA at extremely lower concentrations in all the tested species. In addition, the EC_{50} values showed that the sensitivity pattern of the three species against both EDCs were as follows *D. brightwellii* > *P. minimum* > *T. suecica.*

In toxicity tests, total doses administered need not necessarily be correlated to the total doses available to tested organisms (Monro, 1992). Thus, the bioavailability of the added chemicals has to be considered in toxicity tests. This can help us in determining an approximate value for both bioavailability and effective range of a particular chemical to the test organism (Saghir *et al.*, 2006). In the present study, we calculated the

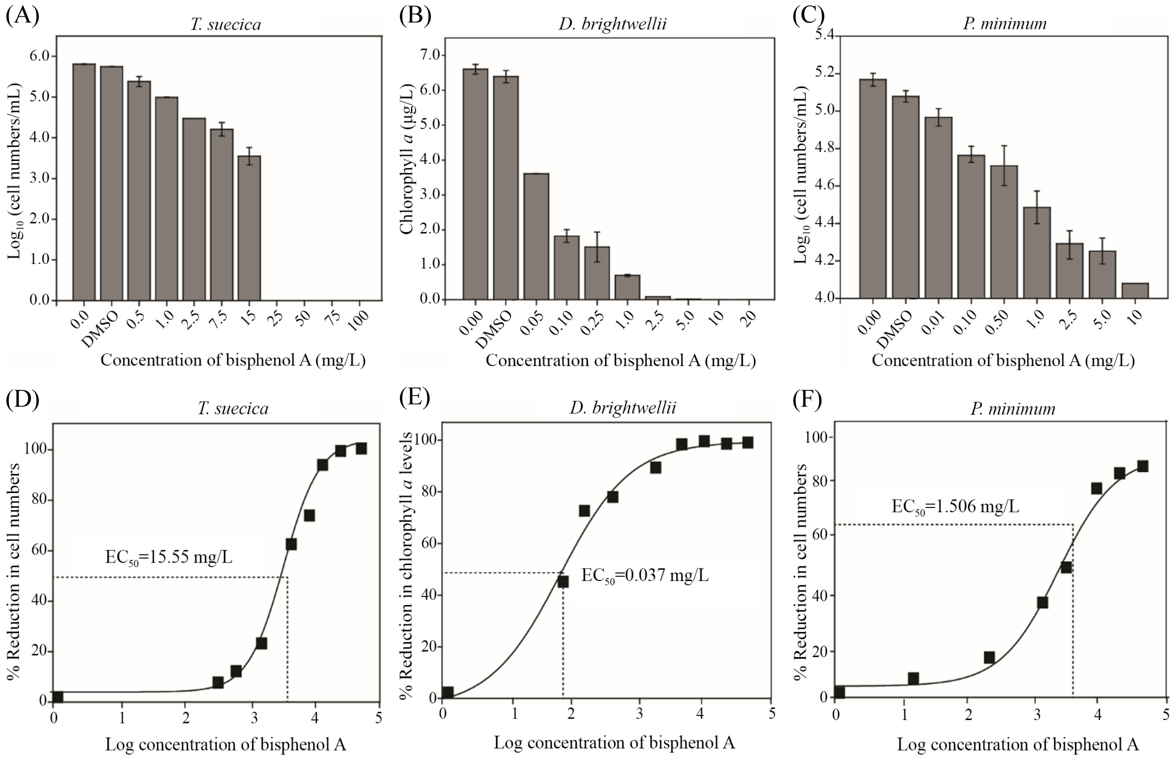

Fig. 2. Effects of Bisphenol A on microalgae, *Tetraselmis suecica*, *Ditylum brightwellii*, and *Prorocentrum minimum* based on cell numbers and Chl *a* after 72 h (A, B, C) and dose response curves (D, E, F).

Table 2. The median effective concentration (EC_{50}) after 72-h exposure of BPA and Aroclor 1016) to microalgae

Species	Chemicals	EC_{10} (mg/L)	EC_{20} (mg/L)	EC_{50} (mg/L)	95% confidence limits
T. suecica	BPA	2.5 ± 0.001	6.3 ± 0.23	15.5 ± 0.28	14.56–16.27
	Aroclor 1016	0	1.6 ± 0.60	3.9 ± 0.18	3.22–4.86
D. brightwellii	BPA	0	0.009 ± ND[a]	0.03 ± 0.004	0.022–0.059
	Aroclor 1016	0	0.00098 ± ND	0.002 ± 0.0003	0.0017–0.0032
P. minimum	BPA	0.2 ± 0.01	0.7 ± 0.38	1.5 ± 0.11	1.25–1.89
	Aroclor 1016	0	0.003 ± 0.0002	0.008 ± 0.0006	0.0075–0.0098

[a] ND, not determined

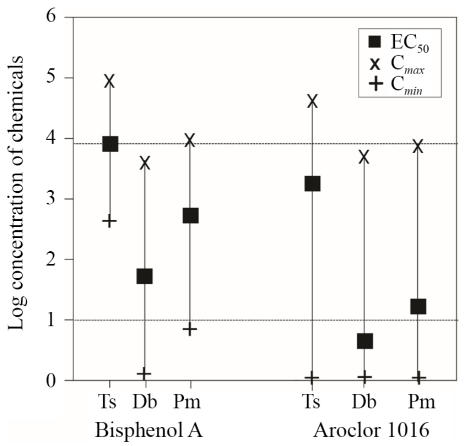

Fig. 3. Range of EC_{50} and bioavailability concentrations of BPA and Aroclor 1016 to microalgae. Abbreviations: Ts, *Tetraselmis suecica*; Db, *Ditylum brightwellii*; Pm, *Prorocentrum minimum*.

minimum concentration (C_{min}) and maximum concentration (C_{max}) values of BPA and Aroclor 1016 for the tested species (Fig. 3), by using the EC_{50}-72 h and dose response curves. Overall our results represented that EC_{50} was positioned at the center between C_{min} and C_{max}, suggesting a sigmoidal response pattern (or dose-dependent decrease) in cell counts. In addition, the cell counts were dramatically decreased at higher dose of test chemicals, suggesting that test EDCs were very toxic to these tested species.

In summary, this study evaluated the sub-lethal effects of two EDCs to different class of species. The EC_{50} value obtained in this study showed different sensitivities, indicating that exposure of EDCs was species specific. Of two EDCs, we found that Aroclor 1016 was much more toxic than BPA in the aquatic organisms, as judged by ECx data (see Table 2). In addition, we found that diatom was the most sensitive species when compared to other green algae and dinoflagellate. These findings suggest that the diatoms *Ditylum brightwellii* may be used for the ecotoxicology assessment for continuous monitoring of EDCs from the marine environments.

적 요

미세조류는 수환경으로 유입되는 독성물질의 배출기준을 설정하거나 환경영향을 평가하기 위한 환경변화의 잠재적 생물지표이다. 본 논문에서 해양 미세조류인 녹조류 *Tetraselmis suecica*, 규조류 *Ditylum brightwellii*, 와편모조류 *Prorocentrum minimum*에 대한 내분비 교란물질(EDCs) 비스페놀 A (BPA)와 Aroclor 1016의 영향을 평가하였다. 처리한 EDCs에 대하여 각각의 종은 매우 다른 민감도 차이를 보였다. 각 종에 대한 50% 영향농도(EC_{50})는 Aroclor 1016가 BPA보다 더 유해하였다. 실험에 사용한 미세조류중에서 규조류 *D. birghtwellii* (0.037 mg/L BPA과 0.002 mg/L Aroclor 1016)가 다른 종보다 매우 민감하게 반응하는 것으로 조사되었다. 본 연구 결과는 수서생태계에로 배출되는 기준 농도 이상의 EDCs가 해양 생물에게 유해 효과가 있다는 것을 제시해 준다.

Acknowledgements

We thank to Dr. R. Sathasivam for English editing and critical comments on the early version of manuscript. This work was supported by a research grant from Sangmyung University.

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