Research Article

Evaluation of the sub-lethal toxicity of Cu, Pb, bisphenol A and polychlorinated biphenyl to the marine dinoflagellate *Cochlodinium polykrikoides*

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Algae are sensitive to a wide range of pollutants, and are effective bioindicators in ecotoxicity assessments. Here, we evaluated the sub-lethal sensitivity of the marine dinoflagellate *Cochlodinium polykrikoides* upon exposure to copper (Cu), lead (Pb), bisphenol A (BPA), and Aroclor 1016 (polychlorinated biphenyl, PCB). Toxic effects were assessed by observations of the reduction in cell counts and chlorophyll *a* levels after exposure to each toxicant. *C. polykrikoides* displayed dose-dependent, sigmoidal responses when exposed to the tested chemicals. EC_{50}-72 h values for Cu, Pb, BPA, and PCB were 12.74, 46.70, 68.15, and 1.07 mg L^{-1}, respectively. PCB, which is an endocrine-disrupting chemical, was the most sensitive, proving its toxic effect on the dinoflagellate. This study provides baseline data on the toxic effects of commonly used heavy metals and endocrine-disrupting chemicals to a marine dinoflagellate.

Key Words: *Cochlodinium polykrikoides*; ecotoxicity assessment; EC_{50}; endocrine disrupting chemicals; heavy metals; marine dinoflagellate

INTRODUCTION

Tons of toxic chemicals are released into water bodies, and their high concentrations have an enormous impact on living organisms in the aquatic ecosystem. Heavy metals are considered a serious threat to aquatic organisms in particular, and these metals have the ability to accumulate in the biota and natural environment (Levy et al. 2008). Cadmium (Cd), lead (Pb), and nickel (Ni) are among the heavy metals that are toxic to organisms in the aquatic ecosystem. In addition, a new class of toxic chemicals, endocrine disrupting chemicals (EDCs), that are commonly used in the manufacture of pesticides, plastics, and fire retardants, have resulted in changes in the nature of the pollutant burden on the aquatic ecosystem. Hence, there is considerable concern over the environmental occurrence of EDCs, because they have the 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 behavior of aquatic organisms (Tarrant 2005).

Dinoflagellate algae are a large group of freshwater and marine protists. About half of all dinoflagellates are photosynthetic and, therefore, they play a crucial role in the aquatic ecosystem (Taylor 1987). Alterations in the algal population due to external environmental factors, such as variations in water temperature and toxic chemical discharges, can have serious implications for water quality and on the community structures of higher trophic organisms, because algae are an important source of energy (Imhoff et al. 2004). Hence, algae based bioassays are

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commonly employed in environmental risk assessment for evaluating the toxicity of heavy metals and novel class environmental contaminants, and in forming regulatory guidelines (Stauber and Davies 2000). Toxicity tests are carried out by measuring growth rate or cell densities of tested algal species (OECD 2006).

Green algae and diatoms are widely-used for toxicity assessments (Moreno-Garrido et al. 2000). These include *Chlorella vulgaris, Closterium ehrenbergii, Ditylum brightwellii, Navicula pelliculosa, Nitzschia closterium, Scenedesmus subspicatus,* and *Skeletonema costatum*. Particularly, algae toxicity tests are carried out mainly using green algae and diatoms such as *C. ehrenbergii* and *N. pelliculosa* (Sverdrup et al. 2001), with relatively few dinoflagellates included in the toxicity screening experiments. For example, metal toxicity (e.g., Cd, Cu, and Zn) to the marine dinoflagellate *Prorocentrum minimum* has been examined (Miao et al. 2005, Millán de Kuhn et al. 2006). In addition, toxicity data on newly emerging contaminants such as EDCs have been generated from extremely few algal species, particularly marine species. One reason is that algae do not have an endocrine system, and so perhaps may be only marginally affected by exposure to EDCs. Recent studies, however, showed that EDCs affected photo system II energy fluxes of green algae and cyanobacteria (Perron and Juneau 2011). Thus, more analyses of the responses of toxicants to dinoflagellates and marine species are required.

In the present study, we used the marine dinoflagellate *Cochlodinium polykrikoides* to assess its response and sub-lethal effects upon exposure to selected heavy metals and EDCs. *C. polykrikoides* is a naked, marine, planktonic, harmful dinoflagellate that is responsible for most frequent fish kills (Ahn et al. 2006). *C. polykrikoides* are widely-distributed in the tropical and warm-temperate waters around the world (Kudela et al. 2008, Richlen et al. 2010). Due to its detrimental ecological and economical impacts, several genomic and evolutionary studies on *C. polykrikoides* have been done (Ki and Han 2008, Guo and Ki 2011). The sub-lethal response of this organism to toxic chemicals was noted.

MATERIALS AND METHODS

Microalgal culture

C. polykrikoides (CP-1) was obtained from the National Fisheries Research and Development Institute (NFRDI). For microalgal culture, f/2 medium was prepared with fil-
tered seawater supplemented with macronutrients, vitamins, and trace metals (e.g., $CuSO_4$, $ZnSO_4$, $CoCl_2$, $MnCl_2$, and $NaMoO_4$), according to Guillard and Ryther (1962). The cells were cultured at 20°C using a 12 : 12-h light : dark cycle with a photon flux density of approximately 65 μmol photons^{-1} m^{-2} s^{-1}.

Toxic chemicals

As test toxicants, we used two heavy metals (Cu and Pb) and two EDCs: bisphenol A (BPA) and Aroclor 1016 (a polychlorinated biphenyl, PCB). Test concentrations of each toxicant were chosen by considering the median effective concentration (EC_{50}) values reported from other aquatic organisms, such as algae, copepods, and fishes (Millán de Kuhn et al. 2006, Monteiro et al. 2011). A range of nominal chemical concentrations was prepared for Cu ($CuSO_4$, Cat. No. C1297; Sigma-Aldrich, St. Louis, MO; 0.05, 0.2, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 200, and 500 mg L^{-1}) and Pb ($PbCl_2$, Cat. No. 268690; Sigma-Aldrich; 0.05, 0.2, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 200, 500, and 750 mg L^{-1}).

For BPA (Cat. No. A133027; Sigma-Aldrich), the concentrations used were 0.1, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 250, and 500 mg L^{-1}, by using a stock solution. This was prepared by dissolving the chemical in 10% dimethyl sulfoxide (Cat. No. D4540; Sigma-Aldrich), and subsequent working solutions were prepared from this stock. The concentrations of PCB (Aroclor 1016, Cat. No. 48701; Sigma-Aldrich) were 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 25, 50, and 100 mg L^{-1}; all dilutions were made from standard stock solutions.

Experimental design

Fifty milliliter aliquots of cell culture recovered 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 $2.25 \times 10^4 \pm 0.1$ cells mL^{-1} as per Organization for Economic Co-operation and Development (OECD) guidelines (OECD 2006), and the samples were drawn for cell count and chlorophyll *a* (chl *a*) estimation at 0, 12, 24, 48 and 72 h.

Cell counting and chl *a* estimation

Cell counts and estimation of chl *a* levels were chosen as the endpoints to determine the effective concentration based on standardized OECD tests (OECD 2006). Cell counts in each test flask were determined using a

Sedgwick-Rafter counting chamber (Matsunami Glass Industry Co., Ltd., Osaka, Japan). Cell counts were plotted against time using $\log_{10}$ of the cell counts. Chl *a* levels were similarly estimated using 10 mL of the culture at specific times. The pigment was extracted after incubating the culture in the dark with 90% acetone. Optical density of the extracted pigments was measured using a DU730 Life Science UV-Vis Spectrophotometer (Beckman Coulter, Fullerton, CA, USA). The chl *a* concentration was estimated following Parsons et al. (1984).

EC$_{50}$ determination

The EC$_{50}$-72 h and the percentile inhibition were calculated as recommended in OECD guidelines (OECD 2006). The concentration of the chemical that evoked the 50% reduction of the *C. polykrikoides* biomass after 72 h exposure to the heavy metals and EDCs were calculated and compared based on the reduction in the cell density and chl *a* levels as compared to the control population.

EC$_{50}$-72 h values were estimated by using a sigmoidal dose-response curve, and were plotted using Origin ver. 8.5 (MicroCal Software, Northampton, MA, USA) based on the following equation: Sigmoidal (Log EC$_{50}$) = a + (b - a) · (1 + 10$^{(x - c)}$)$^{-1}$ (Mensink et al. 2008). In addition, EC$_5$, EC$_{10}$ and EC$_{20}$ values were calculated from a dose-response curve derived using Origin ver. 8.5.

Bioavailability of the chemicals

Bioavailability of the test chemicals was calculated based on the concentration maximum (C$_{max}$) and concentration minimum (C$_{min}$) using recommended equations (Craig et al. 2010) as listed below:

$$C_{max} = C_{pk} \, (e^{(-Ke \cdot T)})^{-1}$$

where C$_{max}$ = concentration maximum, C$_{pk}$ = peak concentration, Ke = exponential volume distribution, and T = time interval, and

$$C_{min} = C_{ave} \, (T \cdot e^{(-Ke \cdot T)})^{-1}$$

where C$_{min}$ = concentration minimum, C$_{ave}$ = average concentration, Ke = exponential volume distribution, and T = time interval.

Data analysis

Decrease in cell counts and chl *a* levels were chosen as the endpoints for evaluating the sub-lethal effects of toxic chemicals. All the data are presented as mean ± standard deviation. Statistical analysis was carried out by one-way ANOVA test by GraphPad InStat (GraphPad Software,

LaJolla, CA, USA) to compare the differences among the treated samples and different time intervals. Significance was indicated at 0.05 levels.

RESULTS AND DISCUSSION

Experimental setup and measured endpoints

The evaluation of toxicity of metals and metal-conjugated compounds to algae is important from an ecological point of view. Although trace metals such as Cu are essential for the growth of these organisms, at high concentration they prove to be fatal (Monteiro et al. 2011). Moreover, in natural environments, the physicochemical form of the metal determines the bioavailability of the metal (Franklin et al. 2000). Bioavailability of heavy metals is controlled by several factors, including pH, redox potentials, salinity, and the presence of chelators (Campbell et al. 2002). Therefore, to establish an organism as a suitable bioindicator, it is important to standardize the culture conditions and experimental endpoints. In the present study, we cultured the test dinoflagellate *C. polykrikoides* in f/2 medium, in which the nutrient concentrations were almost similar to OECD and algal assay procedure (AAP) medium (OECD 2006), and the concentrations of the trace metals used in the media were very low. For instance, in the case of tests involving Cu exposure, the concentration of CuSO$_4$ added in f/2 medium was only 0.0068 mg L^{-1}. Thus, the medium-containing metals might be negligible to calculate available metal concentrations. This was supported by bioavailability analyses of test chemicals (discussed later).

Two endpoints (cell counts and chl *a* levels) were used to assess the effect of short-term exposure (72 h) of *C. polykrikoides* to the test metals and EDCs. The correlation (Pearson's correlation coefficient) between these two parameters was positively correlated in all the test chemicals (Table 1). Thus, the median effective concentration (EC$_{50}$) was calculated based on cell counts.

Table 1. Correlation between cell count and chlorophyll *a* levels in *Cochlodinium polykrikoides* cells exposed to chemical toxicants

Chemicals	Pearson's correlation coefficient (r)	p-value
Cu	0.8669	0.0001
Pb	0.8645	<0.0001
BPA	0.8873	0.0003
PCB	0.7580	0.0027

BPA, bisphenol A; PCB, polychlorinated biphenyl.

Metal toxicity on *C. polykrikoides*

The concentrations of Cu tested ranged from 0.05-500 mg L^{-1}, and the initial concentrations (0.05-10 mg L^{-1}) did not show a significant change in biomass, but concentrations over 25 mg L^{-1} showed a significant reduction (p <

0.0001) in cell counts and chl *a* levels, as compared to the control (Fig. 1A & B). After a 72-h-exposure to Cu concentrations of 0.05-5 mg L^{-1}, the percentage of reduction in cell counts ranged from 5-30%; cultures exposed to 10 and 25 mg L^{-1} showed a decrease in cell counts by 45 and 75%, respectively, as compared to the control. The cul-
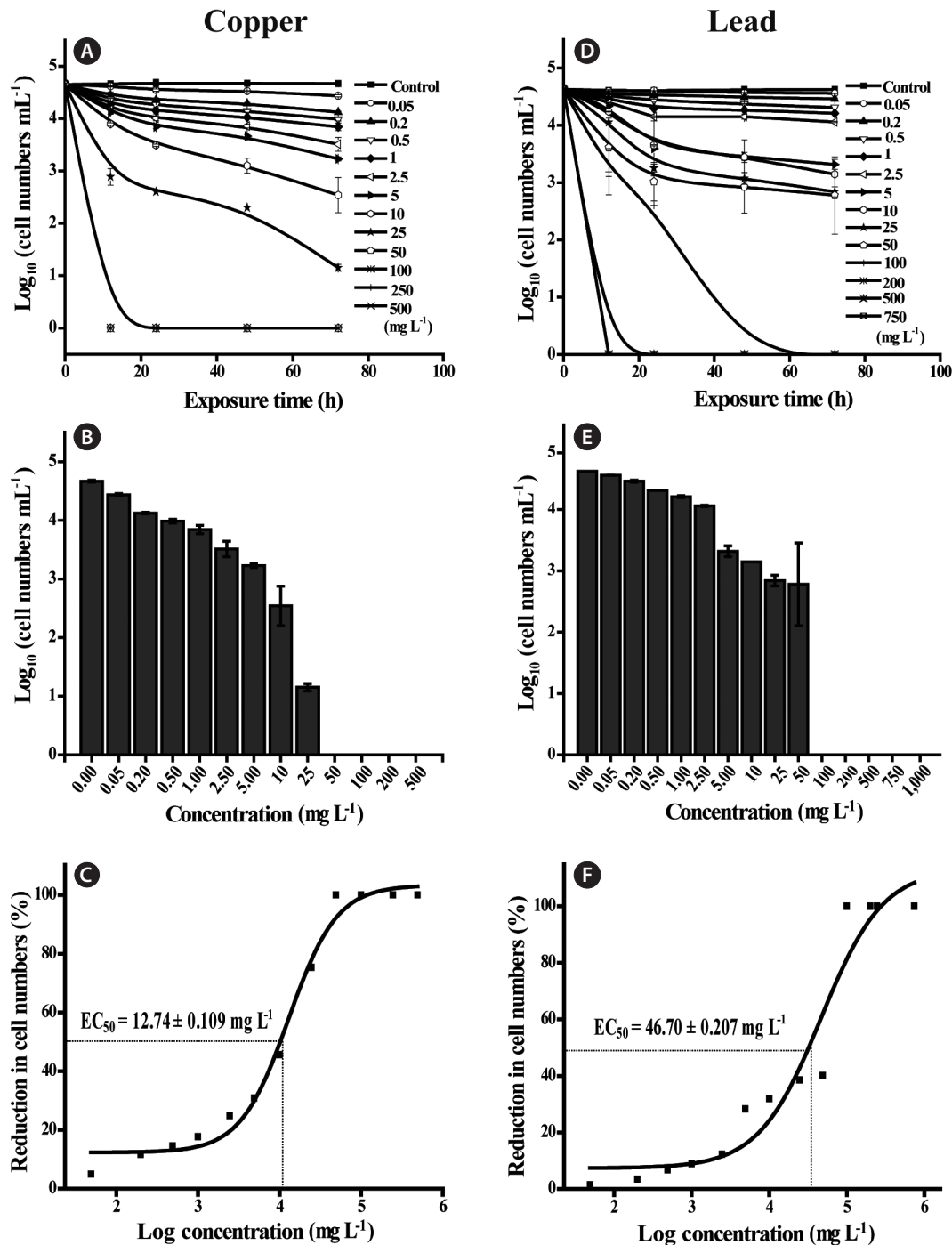
Fig. 1. Effect of heavy metals Cu (A-C) and Pb (D-F) to the cell counts of *Cochlodinium polykrikoides*. (A & D) Different time intervals. (B & E) Cell count after 72 h. (C & F) Dose response curve.

tures exposed to Pb also followed a similar pattern (Fig. 1D & E). The initial concentrations (0.05-25 mg L^{-1}) did not show a significant change, but the higher concentrations of 50-750 mg L^{-1} showed a significant reduction (p < 0.0001).

In addition, EC$_{50}$ values were calculated by using sigmoidal dose-response curves that were estimated from cell counts (Fig. 1C & F). As for threshold effect parameter, we calculated additional EC$_5$, EC$_{10}$ and EC$_{20}$ values, which represented the initial concentration of the test chemical that affected the dinoflagellates (Table 2). EC$_{50}$ values of Cu and Pb in *C. polykrikoides* were 12.75 ± 0.109 and 46.71 ± 0.207 mg L^{-1}, respectively (Table 2). *C. polykrikoides* seemed to be similarly tolerant to Cu compared to those of another marine dinoflagellate, *P. minimum* (Millán de Kuhn et al. 2006). The latter authors reported that the EC$_{50}$ values for two strains (Lissabon and Kattegat) of *P. minimum* were 13.5 and 7.5 mg L^{-1}. On the other hand, *C. polykrikoides* was less tolerant to Cu compared those of the green algae *Isochrysis galbana* and *Tetraselmis chui* (Liu et al. 2011), of which the half maximal inhibitory concentration (IC$_{50}$) values were 31.4 and 37.8 mg L^{-1}, respectively.

EDC toxicity on *C. polykrikoides*

Additionally, we assessed EDC toxicity on the dinoflagellate *C. polykrikoides* with wide-ranging concentrations of BPA and PCB, and found that overall patterns were similar to those observed in the previous experiments on heavy metals (Fig. 2). BPA experiments at the lower concentrations (0.1-10 mg L^{-1}) showed very little or no significant change in terms of cell counts (Fig. 2A & B). However, from 25-500 mg L^{-1}, there was a very significant (p < 0.0001) decrease in the cell counts. In case of PCB, *C. polykrikoides* was comparably sensitive, because the cell counts were markedly reduced in the presence of 0.005 mg L^{-1} PCB, with no survivors remaining in the presence

of PCB concentrations exceeding 5.00 mg L^{-1} (Fig. 2C & D). As noted previously, since algae do not possess endocrine organs or specific systems, they may be little affected by exposure to EDCs, compared with the adverse abnormal effects of EDCs on higher organisms (Vazquez-Duhalt et al. 2006). Recently, Perron and Juneau (2011) reported that the photosystem II energy flow in green algae such as *Chlamydomonas reinhardtii* and *Pseudokirchneriella subcapitata* was affected when exposed to EDCs, including nonylphenol, octylphenol, and estradiol. In the present study, *C. polykrikoides* was also very sensitive to PCB at concentrations <0.05 mg L^{-1}, but relatively tolerant of exposure to BPA. These observations are entirely consistent with the lack of an endocrine system in dinoflagellate, but their susceptibility to endocrine-disrupting chemicals; for example, photo system II energy fluxes (Perron and Juneau 2011).

In addition, we calculated the EC$_{50}$ of PCB and BPA based on the cell counts (Fig. 1C & F), of which values were 68.15 ± 0.257 mg L^{-1} for BPA and 1.07 ± 0.164 mg L^{-1} for PCB, respectively. According to previous studies (Li et al. 2009, Liu et al. 2010), the EC$_{50}$ values of BPA for the diatoms *Navicula incerta* and *Cyclotella caspia* were 3.73 and 7.96 mg L^{-1}, respectively. The 96-h EC$_{50}$ value of PCBs for the dinoflagellate *Lingulodinium polyedrum* was 0.122 mg L^{-1} (Leitão et al. 2003) and 0.210 mg L^{-1} for the freshwater crustacean *Daphnia magna* (Tonkopii et al. 2008). EC$_{50}$ comparison indicates that *C. polykrikoides* was more tolerant than other algae, even the dinoflagellate *L. polyedrum*.

Bioavailability of tested chemicals

In toxicity tests, the total dose administrated need not necessarily be correlated to the total dose available to the organism (Monro 1992). Especially, it should be considered in toxicity assays by using metals and metal-conjugated compounds, as described previously. Moreover,

Table 2. EC$_5$, EC$_{10}$, EC$_{20}$, and EC$_{50}$ values of heavy metals and EDCs exposed to *Cochlodinium polykrikoides*

Chemicals	EC$_5$ (mg L^{-1})	EC$_{10}$ (mg L^{-1})	EC$_{20}$ (mg L^{-1})	EC$_{50}$ (mg L^{-1})	p-value (significance)	95% confidence interval
Cu	[a]	[a]	5.03 ± 0.24	12.74 ± 0.25	<0.0001	4.515-4.819
Pb	2.43 ± 0.05	6.04 ± 0.18	12.93 ± 0.42	46.70 ± 0.20	<0.0001	3.986-4.566
BPA	1.48 ± 0.03	3.47 ± 0.04	47.91 ± 0.09	68.15 ± 0.25	<0.0001	3.204-4.568
PCB	[a]	[a]	0.32 ± 0.13	1.07 ± 0.16	0.0003	2.859-4.463

EDCs, endocrine disrupting chemicals; BPA, bisphenol A; PCB, polychlorinated biphenyl.
[a]Similar to control and no effect.

toxicity assays performed with marine organisms and in the marine environment may be more complicated, because many complex chemical reactions occur (Jenner et al. 1997). Hence, it becomes necessary to determine the bioavailability of the tested chemicals. In the present study, we calculated the maximum concentration (C_{max}) and minimum concentration (C_{min}) values to the dinoflagellate *C. polykrikoides* (Fig. 3) using the EC_{50}-72 h and dose response curves (Craig et al. 2010). In this case, the C_{max} represents the maximum, or peak, dose of the test chemical that the organism receives, whereas the C_{min} is the minimum concentration of the chemical to which the
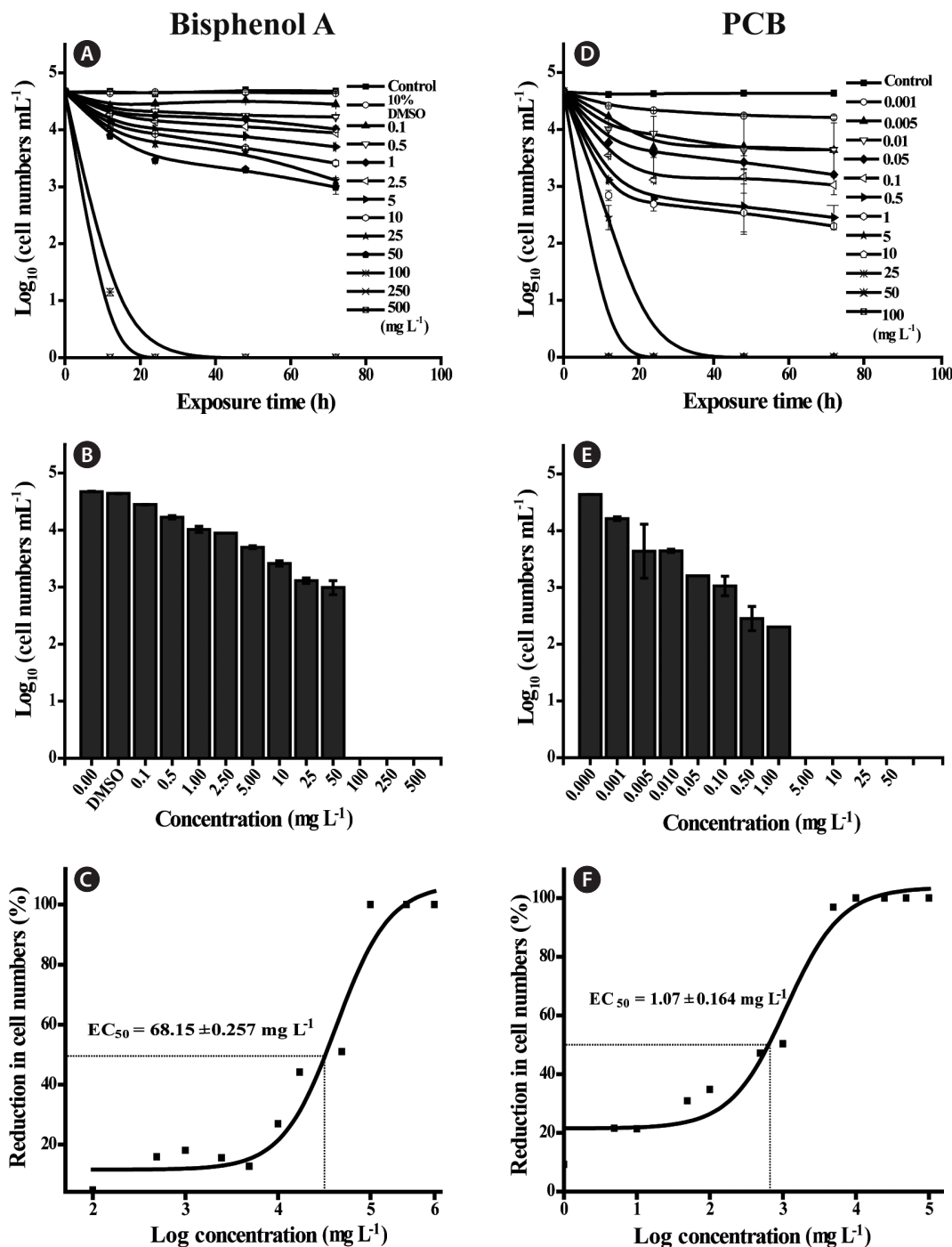
Fig. 2. Effect of endocrine disrupting chemicals bisphenol A (BPA) (A-C) and polychlorinated biphenyl (PCB) (D-F) to the cell count of *Cochlodinium polykrikoides*. (A & D) Different time intervals. (B & E) Cell count after 72 h. (C & F) Dose response curve.
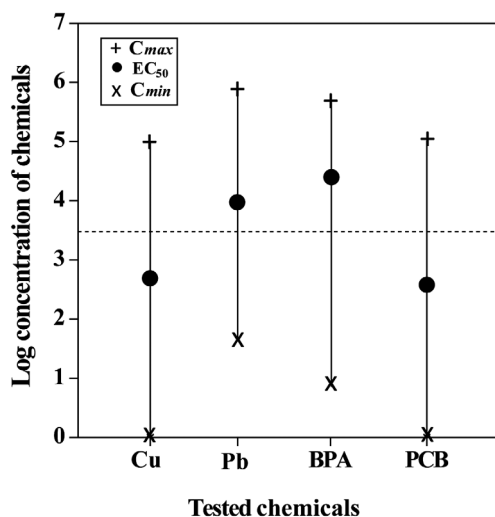
Fig. 3. Range of EC$_{50}$ and approximate bioavailable concentrations according to toxic contaminants. The dotted line represents the mean EC$_{50}$ of four chemicals. C$_{max}$, maximum concentration; C$_{min}$, minimum concentration; BPA, bisphenol A; PCB, polychlorinated biphenyl.

test species is actually exposed (Marzo et al. 2004). This could help us in determining an approximate value for both bioavailability and effective range of a particular chemical to the test organism, as pointed by Saghir et al. (2006). The present data positioned the *C. polykrikoides* EC$_{50}$ at the center between the C$_{min}$ and C$_{max}$ scores, showing a dose-dependent decrease (or sigmoidal response pattern) in cell counts. In addition, the cell counts were not dramatically decreased at higher concentrations of the tested chemicals. This provided a range of bioavailability of each tested chemical, with the added chemicals being correlated to the total dose available to the tested *C. polykrokoides*.

In summary, the marine dinoflagellate *C. polykrokoides* exhibited dose-dependent responses when exposed to two heavy metals and two EDCs. According to the EC$_{50}$ values obtained, *C. polykrokoides* was most sensitive to PCB (1.07 mg L^{-1}) and most tolerant to BPA (68.15 mg L^{-1}). As a unicellular eukaryote, *C. polykrokoides* should be affected by EDCs. However, we observed that this species was generally tolerant of most of the tested chemicals at their permissible limits in aquatic environments (U.S. Environmental Protection Agency 1996).

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