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Pyrodinium bahamense var. *compressum* Böhm Survival in High and Low Cadmium Levels

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***Pyrodinium bahamense* var. *compressum* (*Pbc*) is a major public health concern particularly in the Southeast Asian region, and increasing threat brought by heavy metal pollution has greatly disturbed and altered the ecological balance of the region's marine waters. Herein, we report the effect of cadmium, a biotoxic metal, to cell cultures of *Pbc*. Within 72 h after treatment with high cadmium concentration (50 ppm Cd^{2+}), the cell density dramatically declined. Chlorophylls *a* and c_2 also decreased after 30-day exposure. However, the low Cd^{2+} (1 ppm)-treated cells had comparable response to the untreated cultures. Thus, the organism's ability to survive under low dose of cadmium implies a built-in stress response mechanism, but higher concentration is lethal to its survival and growth. The result of this study may lead to clearer insight on the role of metal ions in the growth and bloom dynamics of this important dinoflagellate.**

Key words: cadmium, cell density, chlorophyll, growth, *Pyrodinium bahamense*, uptake

INTRODUCTION

Uncontrolled generation and indiscriminate disposal of wastes can cause exponential accumulation of toxic metal ions in the marine environment. Such cumulative act has not only affected the ecological balance but also the homeostasis of marine microfauna and microflora. In practice, pollution in marine waters can be assessed by monitoring the growth and decline of phytoplankton, such as diatoms and dinoflagellates (Liu et al. 2012; Lavoie et al. 2014).

Pyrodinium bahamense var. *compressum* (*Pbc*), an important dinoflagellate due to its association with Paralytic Shellfish Poisoning (PSP), has been a major public health and economic problem in the Southeast Asian region including the Philippines (Azanza & Taylor 2001). It produces neosaxitoxin (NEO), gonyautoxin 5 (GTX5), gonyautoxin 6 (GTX6), decarbamoylsaxitoxin (dcSTX) and saxitoxin (STX) (Usup et al. 1994), which targets multi-class of neuro-receptors (Llewellyn 2006) and is supposed to be the major causative agent of PSP with the greatest outbreak reported in the Philippines (Fukuyo et al. 2011). *P. bahamense* var. *compressum* is the Indo-Pacific ribotype of *P. bahamense* widely distributed along the South Pacific and Indian coasts (Mertens et al. 2015). It forms spherical spiny cysts believed to be the organism's adaptive mechanism to harsh environmental conditions, and which can later fossilize to the hystrichosphere (fossilized dinoflagellate cyst) (Matsuoka et al. 1985)

Heavy metal ions, such as Fe, Zn, Mn, Ni, Cu and Co, are essential to the growth of phytoplankton but are lethal at high doses (Twining & Baines 2013). Originally believed to be a non-essential element, cadmium is considered as one of the most biotoxic metals (Sadiq 1992). This

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element, in its free ionic form (Cd^{2+}), may pose as a threat not only to aquatic and marine life but also to humans. It has also been observed to affect the growth of some important marine microalgae (Perez-Rama et al. 2001) and diatoms (Twining & Baines 2013). A decrease in cell density with increasing amount of Cd^{2+} was observed in *Tetraselmis suecica* (Kylin) Butch cell cultures (Perez-Rama et al. 2001). Increased amount of Cd to the population of Atlantic *Prochlorococcus* and Black Sea picoeukaryotes was found lethal to the organisms (LC_{50}=0.23 to 498.7 mg L^{-1}), but differences in Cd sensitivities were observed depending on the population size (Echeveste et al. 2012).

To our best knowledge, no study has been conducted yet on the effect of the heavy metals, particularly cadmium, on *Pyrodinium bahamense* var. *compressum*. In this work, the response of *Pbc* to 1 ppm (1 mg L^{-1}) and 50 ppm (50 mg L^{-1}) Cd^{2+} has been investigated by measuring its growth (cell density) and biomass (chlorophyll *a* and c_2). Intracellular uptake of cadmium by *Pbc* cell cultures was also examined. The implication of these results to the bloom dynamics, including the ecological and environmental significance and future prospects were also discussed.

MATERIALS AND METHODS

Existing *P. bahamense* var. *compressum* (PBCMz-RVA-061593) cell cultures collected from Bamban Bay, Masinloc, Zambales in 1993 and being maintained at the Marine Science Institute, University of the Philippines, Diliman, Quezon City, were used in this study. Culture conditions followed the protocol published by Corrales & Hall (1993). Briefly, 0.4 mL Guillard's "F" medium (full strength) was used to enrich filtered (0.45 µm pore size) and sterilized (1 h at 15 psi) seawater (~200 mL). The cultures were maintained in the growth chamber at an irradiance of 122-125 µE m^{-2} s^{-1}, with a dark-to-light cycle of 12:12 (h) at room temperature (24-28°C), and were acclimatized for three days before the introduction of Cd^{2+}. Low (1 ppm) and high (50 ppm) concentrations of Cd^{2+} were prepared by adding 0.02 mL and 1 mL of stock $CdCl_2$ solution (10,000 mg L^{-1}), respectively, to three replicate flasks containing the cultures. Control cultures without Cd^{2+} were also maintained.

There were two sets of cell density experiments: (a) a 72-hour counting period, wherein cell counting was done every 24 h, and (b) a 61-day counting period, wherein cell growth was monitored every three days. To examine the effect of nutrient depletion on growth, the cultures were starved on the 28th until 40th day (12 d). The number of cells per milliliter of culture was determined by taking 1 mL of culture and noting the number of live cells using a Sedgwick slide under a light microscope. Initial cell density per inoculum ranges from 450-650 cells mL^{-1}.

The effect of Cd^{2+} to the cell biomass of *Pbc* cell cultures was examined by monitoring chlorophylls *a* (A=430 nm) and c_2 (A=453 nm). Ten milliliters (10 mL) of the cultures were taken from each flask for zero (0)-, three (3)- and thirty (30)-day periods. Chlorophyll extraction and analysis were carried out accordingly (Sterman 1998). The number of cells before and after chlorophyll extractions was also recorded using Ultraspec UV-Vis spectrophotometer to calculate the actual chlorophylls (*a* and c_2) per cell.

For the uptake experiment, the cells were harvested two weeks after treatment (maximum growth: ~1,000 cells mL^{-1}) by filtration (0.45 µm pore membrane filter) and washing with 50-75 mL of 0.003 *M* EDTA. In order to differentiate between membrane- and intracellularly-bound cadmium, the filtered algal cells were re-suspended twice in 10 mL of 0.003 *M* EDTA for 10 min and were re-filtered with suction to remove all adsorbed cadmium on the cell membrane (Knauer et al. 1997). The cells on the filters were air-dried and weighed before ashing in the muffle furnace. The ash samples were dissolved in 1:1 HNO_3 acid solution and diluted to 10 mL in a volumetric flask. This was analyzed with a Thermo Jarell Atomic Absorption Spectrometer (LOD = 0.02 mg L^{-1} or 0.02 ppm) and the algal cadmium uptake was reported as total Cd^{2+} per cell. Chlorophyll and cadmium analysis were done at the Research and Analytical Services Laboratory, Natural Sciences Research Institute, University of the Philippines, Diliman, Quezon City.

RESULTS AND DISCUSSION

Toxic metal ions are known to affect adversely the growth of dinoflagellates (Fernandez-Leboranz et al. 2007) and other phytoplankton (Miao & Wang 2006). Using the biotoxic metal cadmium as a model, we determined the immediate (72 h) and long-term (60 d) toxic effects of exposing cultures of *Pbc* to 1 ppm and 50 ppm Cd^{2+} solutions by counting the density of the cells (Figure 1). After 24 h, the 50 ppm Cd^{2+}-treated *Pbc* cultures cell count began to drop (100 cells mL^{-1}), whereas, no significant change (820~880 cells mL^{-1}) was observed in the 1 ppm Cd^{2+}-treated cultures even beyond 48 h (Figure 1a). At longer exposure period (~60 d), the cell density in 1 ppm Cd^{2+}-treated cultures eventually declined to about less than half of the original, maintaining about 200~400 cells mL^{-1} throughout the counting period (Figure 1b). Meanwhile, the 50-ppm Cd^{2+}-treated cultures were not able to recover from the initial shock of its exposure to Cd^{2+} with approximately ~1-2 cells mL^{-1}.

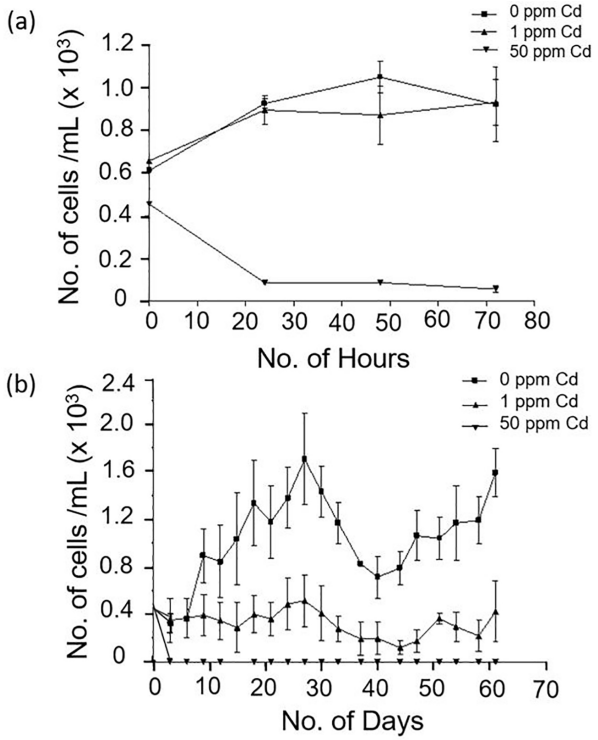

Figure 1. (a) Immediate effect (72 hours) and (b) long-term effect (61 days) of cadmium on the cell density (growth) of *P. bahamense* var. *compressum.*

In this same experiment, we also observed the effect of Cd^{2+} on the growth of *Pbc* upon nutrient depletion of the media by starving the cultures (i.e., no fresh media was added) for 12 d. The growth of the control *Pbc* cultures declined dramatically due to competition by the growing cells, but eventually regained upon fresh addition of the media on the 40th day. Interestingly, the 1-ppm Cd^{2+}-treated cultures did not experience reduction in growth even in such stressful condition (Figure 1b). It maintained a steady cell density (150-250 cells mL^{-1}) within this period until fresh culture medium was added, after which they eventually regained their original state (~400 cells mL^{-1}). The 50-ppm Cd^{2+}-treated cultures, however, maintained cell density of 1-2 cells mL^{-1} throughout the experiment.

Phytoplankton is a primary producer in the aquatic ecosystems; and like in higher plants, food is manufactured by absorbing sunlight through the chlorophylls present in its system. Chlorophylls are not only good indicators of phytoplankton biomass (Ryther & Yentsch 1957), but also of the phytoplankton's uptake of metals by substitution of the essential metal ion (i.e., Mg^{2+}) in the chlorophyll molecule (Kupfer et al. 2002). To examine the reproductive response of *Pbc*, both chlorophylls *a* (measure of phytoplankton biomass) and c_2 or chlorofucine (specific for diatoms and dinoflagellates biomass), were measured after 0, 3 and 30 d of exposure to Cd. Right after the addition of Cd solution at day 0, there was an increase in the *Pbc* biomass of the 50-ppm Cd^{2+}-treated cell cultures (1.2 x 10^{-4} µmol Chlorophyll *a* $cell^{-1}$; 3.5 x 10^{-4} µmol Chlorophyll *c2* $cell^{-1}$). There was no significant change in the 1 ppm Cd^{2+}-treated and control cell cultures (<1 x 10^{-4} µmol Chlorophyll $cell^{-1}$) (Figure 2). Three days after treatment, the biomass of the 50 ppm Cd^{2+}-treated cells were significantly amplified to about seven- to fifty-fold (chlorophyll $a = 6.0 \times 10^{-3}$ µmol/cell; chlorophyll $c_2 = 2.3 \times 10^{-3}$ µmol/cell), with little improvement in the biomass of the 1 ppm Cd^{2+}-treated and control cell cultures (Figure 2). Thirty days after exposure to cadmium, the amount of chlorophylls in the 1 ppm Cd^{2+}-treated cell cultures decreased significantly (chlorophyll *a*, 5 x 10^{-6}; chlorophyll c_2, 1 x 10^{-5}), while the 50 ppm Cd^{2+}-treated cells were photo-bleached, that is, the cells became colorless or transparent to light exposure. Torres et al. (1997) showed that 50 ppm Cd^{2+} is lethal to the marine algae *P. tricornutum* but not 1 ppm Cd^{2+}.

Metal ions are important in the homeostasis of phytoplankton (Twining & Baines 2013). As this study suggests, *Pbc* can survive at low level (1 ppm) of Cd metal ions in culture medium, which indicates an adaptive mechanism in response to the stress condition applied. Hence, we examined the intracellular uptake in

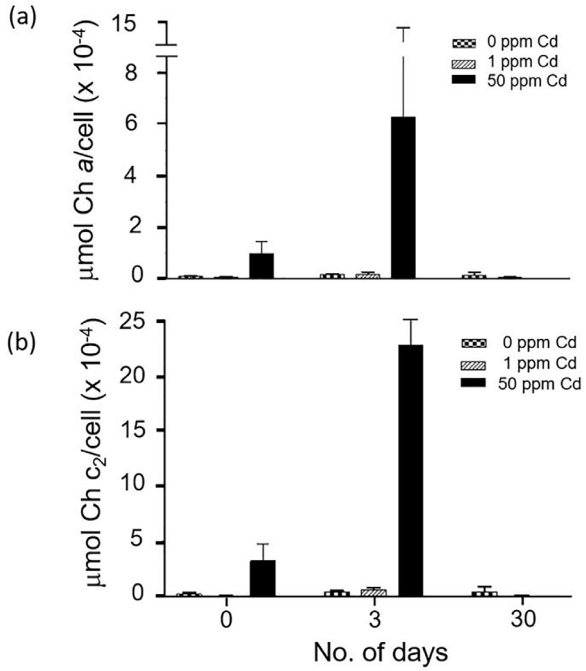

Figure 2. Effect of Cd to total biomass of *P. bahamense* var. *compressum* cell cultures after 30-day exposure. (a) chlorophyll *a* and (b) chlorophyll c_2 concentrations per cell.

Pbc controlled cultures under two-week cadmium ion exposure. As shown in Figure 3, there was an average intracellular uptake of 5.5 x 10^{-6} mg L^{-1} Cd^{2+}/cell in the 1 ppm Cd^{2+}-treated cells, which was a ~10-fold increase in the intracellular cadmium. This result suggested that *Pbc* has adapted to low Cd level allowing it to survive during their two-week exposure. However, intracellular cadmium uptake of *Pbc* cell cultures treated with 50-ppm Cd^{2+} was low (~0.75 x 10^{-6} mg L^{-1} Cd^{2+}/cell). This implies that most of the cadmium ions in the latter case might be present only in the extracellular matrix and the too low number of cells because of their premature death (Figure 1b) might have limited that uptake. Those who have survived, however, may have managed to thrive on the depleted and contaminated medium by "nutrient substitution".

In the cell density experiment, cadmium caused the death of much *Pbc* cells within three days (72 h) at 50 ppm concentration, but those who survived the initial shock showed up to 50-fold increase in chlorophylls, and thus, in their total biomass. Seemingly, the photosynthetic mechanism or apparatus of *Pbc* was initially unaffected by the heavy metal ion. Interestingly, prolonged exposure of the cells to high cadmium level was too lethal for photosynthesis to proceed as this could saturate all the available chlorophylls in the photosynthetic machinery. Some dinoflagellates and phytoplankton can survive at very trace amount of metals, but high amount can inhibit their growth (Wang & Wang 2009). Cadmium has been implicated in the survival and growth of some phytoplankton (Xu & Morel 2013). Some marine phytoplankton showed adaptive response to stress induced by Zn and Cd (Zeng et al. 2009; Wang & Wang 2011). At shorter period and low metal exposure, the organisms could acclimatize or recover from the initial shock but longer exposure was toxic (Zeng et al. 2009; Wang & Wang 2011). Toxic metals induce the production of some metal-binding proteins phytochelatins and metallothioneins as a stress

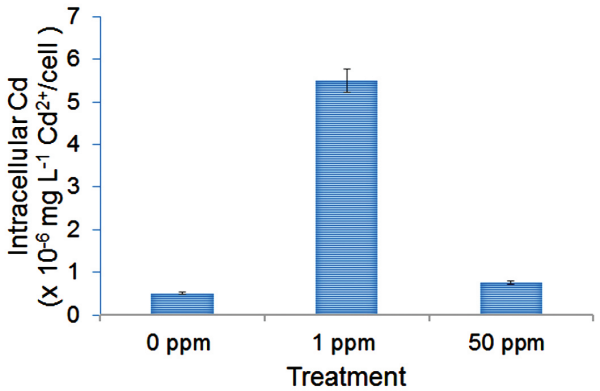

Figure 3. Uptake of Cd by *P. bahamense* var. *compressum* cell cultures after two weeks of treatment.

response in phytoplankton (Perez-Rama et al. 2001; Wang &Wang 2011).

Although cadmium has been implicated in the cellular homeostasis of some transition metals (Co, Zn, Mn, Fe) (Moulis 2010) and may substitute for nutrient requirement of some marine organisms (Fitzwater et al. 2000), there has been no report yet on the importance of cadmium on the physiology, growth and metal homeostasis of any dinoflagellates, especially *P. bahamense* var. *compressum*. In this study, we suppose that the tolerance of *Pbc* to 1-ppm cadmium might be due to its inherent adaptation to environmental stress (Wang & Wang 2011). At some tolerable level of the metal ion, this might be considered as advantageous to the bloom dynamics of *Pbc*. Tolerance of phytoplankton to toxic metal ions such as Cd^{2+} was associated to the production of extra- and intra-cellular metal-ligating molecules in the cells (Pistocchi et al. 2000). Its adaptive response at low concentration and short-term exposure to metal stress may be due to some survival mechanism (Smayda & Reynolds 2003). This is provided by metal binding proteins or peptides, such as carbonic anhydrase (Park et al. 2007) or phytochelatins (Pawlik-Skowrońska 2001), as well as thiol-induction, adaptation or acclimatization (Wang & Wang 2011), which is probably a detoxification mechanism of the organism and control of Cd homeostasis in cells (Xu & Morel 2013).

On the other hand, nutrient depletion and longer exposure might also have a great impact at higher metal concentration and longer period of exposure (Wang & Wang 2011). Moreover, the remarkable reduction of both cell density and chlorophylls, together with the cadmium uptake, indicate that cadmium might have some role in the bloom dynamics of this dinoflagellate. The ability of *Pyrodinium bahamense* var. *compressum* to adapt at low concentration of cadmium suggests some important physiological, geochemical and ecological implications, including its ability to survive in heavy metal-laden oceanic waters, bloom dynamics related to the growth of other marine organisms such as *Pbc*-grazing bivalves, metal cycling, and pollution control in marine waters. The increase of metal pollution in aquatic and marine waters has been associated with the occurrence and distribution of dinoflagellates (Sætre et al. 1997). For instance, *P. bahamense* was found to thrive in heavily polluted Pasig River in the Philippines, where significant amounts of Ni, Pb and Zn were found (Azanza et al. 2004). Hence, *Pbc* can be a potential bioindicator of heavy metal pollution in the aquatic or marine environment and further investigation of the role of other heavy metals to *Pbc* growth and dynamics is desirable.

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REFERENCES

AZANZA RV, SIRINGAN FP, SAN DIEGO-MCGLONE ML, YÑIQUEZ AT, MACALAD NH, ZAMORA PB, AGUSTIN MB, MATSUOKA K. 2004. Horizontal dinoflagellate cyst distribution, sediment characteristics and benthic flux in Manila Bay, Philippines. Phycol Res 52(4):376–386.

AZANZA RV, TAYLOR FJHM. 2001. Are *Pyrodinium* blooms in the Southeast Asian Region recurring and spreading? A view at the end of the millennium. Ambio 30(6):356–364.

CORRALES RA, HALL S. 1993. Isolation and culture of *Pyrodinium bahamense var. compressum* from the Philippines. In: Toxic phytoplankton blooms in the Sea. Smayda TJ and Shimizu Y, eds. Berlin, Heidelberg: Springer-Verlag. p. 725–730.

ECHEVESTE P, AUSTI S, TOVAR-SANCHEZ A. 2012. Toxic thresholds of cadmium and lead to oceanic phytoplankton: cell size and ocean basin-dependent effects. Envi Toxicol Chem 31(8):1887–1894.

FERNANDEZ-LEBORANS G, GABILONDO R, RUIZ-ALVAREZ S. 2007. Inter-annual depth-dependent toxicity and bioaccumulation of cadmium in marine benthic protist communities. Oceanologia 49(3):305–336.

FITZWATER S, JOHNSON KS, GORDON RM, COALE KH, SMITH WO. 2000. Trace metal concentrations in the Ross Sea and their relationship with nutrients and phytoplankton growth. Deep-Sea Res II 47(15–16):3159–3179.

FUKUYO Y, KODAMA M, OMURA T, FURUYA K, FURIO EF, CAYME M, TEEN LP, HA DV, KOTAKI Y, MATSUOKA K, et al. 2011. Ecology and oceanography of harmful marine microalgae (Project-2). In: Coastal Marine Science in Southeast Asia —Synthesis Report of the Core University Program of the Japan Society for the Promotion of Science: Coastal Marine Science (2001–2010). Nishida S, Fortes MD and Miyazaki N, eds. Tokyo, Japan: TERRAPUB. p. 23-48.

KNAUER K, BEHRA R, SIGG L. 1997. Effects of free Cu^{2+} and Zn^{2+} on growth and metal accumulation in freshwater algae. Environ Toxicol Chem 16(2):220–229.

KUPFER H, ŠETLIK I, SPILLER M, KŰPPER FC, PRAŠIL. 2002. Heavy metal-induced inhibition of photosynthesis: targets of i*n vivo* heavy metal chlorophyll formation. J Phycol 38:429–441.

LAVOIE I, CAMPEAU S, ZUGIC-DRAKULIC N, WINTER JG, FORTIN C. 2014. Using diatoms to monitor stream biological integrity in Eastern Canada: An overview of 10 years of index development and ongoing challenges. Sci Total Environ 475:187–200.

LIU D, SHI Y, DI B, SUN Q, WANG Y, DONG Z, SHAO H. 2012. The impact of different pollution sources on modern dinoflagellate cysts in Sishili Bay, Yellow Sea, China. Mar Micropaleon 84–85:1–13.

LLWELLYN LE. 2006. Saxitoxin, a toxic marine natural product that targets a multitude of receptors. Nat Prod Rep 23(2):200–222.

MATSUOKA J, FUKUYO Y. GONZALES CL. 1985. A new discovery of cyst of *Pyrodinium bahamense* var. *compressum* from the Samar Sea, Philippines. In: Red tides, biology, environmental science and toxicology. Okaichi T, Anderson DM and Nemoto T, eds. New York: Elsevier. p. 301–304.

MERTENS KN WJ, CARBONELL-MOORE C, BOGUS K, ELLEGAARD M, LIMOGES A, DE VERNAL A, GURDEBEKE P, OMURA T, AL-MUFTAH A, MATSUOKA K. 2015. Taxonomic re-examination of the toxic armored dinoflagellate *Pyrodinium bahamense* Plate 1906: Can morphology or LSU sequencing separate *P. bahamense* var. *compressum* from var. *bahamense*?. Harmful Algae 41:1–24.

MIAO AJ, WANG WX. 2006. Cadmium toxicity to two marine phytoplanktons under different nutrient conditions. Aquat Toxicol 78(2):114–126.

MOULIS JM. 2010. Cellular mechanisms of cadmium toxicity related to the homeostasis of essential metals. Biometals 23(5):877–896.

PARK H, SONG BA, MOREL FM. 2007. Diversity of the cadmium-containing carbonic anhydrase in marine diatoms and natural waters. Environ Microbiol 9(2):403–413.

PAWLIK-SKOWROŃSKA B. 2001. Phytochelatin production in freshwater algae *Stigeoclonium* in response to heavy metals contained in mining water; Effects of some environmental factors. Aquat Toxicol 52(3–4):241–249

PEREZ-RAMA M, HERRERO C, ALONZO JA, VAAMONDE ET. 2001. Class III metallothioneins in response to cadmium toxicity in the marine microalga *Tetraselmis suecica* (Kylin) Butch. Envi Toxicol Chem 20:2061–2066.

PISTOCCHI R, MORMILE AM, GUERRINI F, ISANI G, BONI LR. 2000. Increased production of extra- and intracellular metal-ligands in phytoplankton exposed to copper and cadmium. J Appl Phycol 12(3):469–477.

RYTHER JH, YENTSCH CS. 1957. The estimation of phytoplankton production in the ocean from chlorophyll and light data. Limnol Oceanogr 2(3):281-286.

SADIQ M. 1992. Toxic metals chemistry in marine environments. New York: Mercel Dekker. 392p.

SÆTRE MLL, DALE B, ABDULLAH MI, SÆTRE GP.1997. Dinoflagellate cysts as possible indicators of industrial pollution in a Norwegian fjord. Mar Environ Res 44(2):167–189.

SMAYDA TJ, REYNOLD CS. 2003. Strategies of marine dinoflagellate survival and some rules of assembly. J Sea Res 49(2): 95–106.

STERMAN NT. 1998. Spectrometric and fluorometric chlorophyll analysis. In: Experimental phycology: A laboratory manual. Lobban CS, Chapman DJ, and Kilmer BP, eds. Cambridge University Press. p. 35-41.

TORRES E, CID A, FIDALGO P, HERRERO C, ABALDE J. 1997. Long chain class III metallothionein as a mechanism of cadmium tolerance in the marine diatom *Phaedactylum tricornutum* Bohlin. Aquat Toxicol 39(3–4): 231–246.

Q TWINING BS, BAINES SB. 2013. The trace metal composition of marine phytoplankton. Ann Rev Mar Sci 5:191–215.

USUP G, KULIS DM, ANDERSON DM. 1994. Growth and toxin production of the toxic dinoflagellate *Pyrodinium bahamense var. compressum* in laboratory cultures. Nat Toxins 2(5):254–262.

WANG MJ, WANG WX. 2009. Cadmium in three marine phytoplankton: Accumulation, subcellular fate and thiol induction. Aquat Toxicol 95:99–107.

WANG MJ, WANG WX. 2011. Cadmium sensitivity, uptake, subcellular distribution and thiol induction in a marine diatom: Recovery from cadmium exposure. Aquat Toxicol 101:387–395.

XU Y, MOREL FMM. 2013. Cadmium in marine phytoplankton. In: Cadmium: From toxicity to essentiality. Sigel A, Sigel H, Sigel RKO, eds. Metal Ions in Life Sciences Series. Volume 11. Netherlands: Springer. p. 509–528.

ZENG J, YANG L, WANG WX. 2009. Acclimation to and recovery from cadmium and zinc exposure by a freshwater cyanobacterium, *Microcystis aeruginosa*. Aquatic Toxicol 93:1–10.