Toxicity of copper on the growth of marine microalgae *Pavlova* sp. and its chlorophyll-*a*

T Purbonegoro¹, Suratno¹, R Puspitasari¹, and N A Husna²

¹ Research Center for Oceanography, Indonesian Institute of Sciences (LIPI) Jalan Pasir Putih I, Ancol Timur, Jakarta 11048, Indonesia
² Faculty of Chemistry, Gadjah Mada University, Indonesia
E-mail: purbonegoro@gmail.com

Abstract. Marine microalgae is the primary producer at the base of the marine food chain. Their sensitivity to metal contamination provides important information for predicting the environmental impact of pollution. Toxicity testing using marine microalgae *Pavlova* sp. was carried out to assess the toxicity of copper on the growth and chlorophyll-*a* content. Results of this study show that adverse effects were observed by the increase of copper concentration. Cell number began to decrease at the lowest concentration (13 μg/L) and reduced drastically at 98 μg/L. Minimum cell number was observed at the highest concentration (890 μg/L). The inhibition concentration (IC50) value of copper for *Pavlova* sp. was 51.46 μg/L and at concentrations >29 μgL⁻¹ the chlorophyll-*a* content decreased dramatically compared to the control. A variation in cell size and morphology was also observed at the higher concentration by the increase in the cell size and loss of setae compared to normal cells.

1. Introduction

Marine microalgae form the basis of the marine food chain. Any disturbance to this component due to the release and accumulation of toxicant have an impact on higher trophic level [1]. Their sensitivity to metal contamination has been used for years and provide important information for predicting the environmental impact of pollution. Some of the trace metals such as lead (Pb), copper (Cu), aluminium (Al), cadmium (Cd), boron (B), selenium (Se), chromium (Cr), manganese (Mn), cobalt (Co) and arsenic (As) are essential for organisms and can be toxic at more than the required level [2]. Copper is being a component of several proteins and enzymes involved in a variety of metabolic pathways in plants and algae [3]. The potential sources of copper in the environment include domestic and industrial discharges, agricultural run-off, and leachate from metal-based antifouling paints [4]. The toxicity of copper is mainly related to free ions and its sensitivity varies among microalgae [1].

As bioaccumulation of heavy metals occurs mainly through a food chain with phytoplankton at the base, it is important to determine the effects of heavy metals to microalgae species which have been used as food in mariculture, such as *Pavlova* sp. *Pavlova* sp. has a high potential as food in mariculture. It is characterized by a high content of both DHA and EPA in contrast to many other commonly used microalgae, which are only able to synthesize either DHA or EPA in relevant amounts [5][6]. Accumulation of heavy metal such as copper will have an impact on the higher trophic level and human eventually. Therefore, the aim of this study is to assess the toxicity of copper on the growth and chlorophyll-*a* content of *Pavlova* sp.
2. Materials and methods

2.1. Toxicity Test

The 96-hour toxicity test using phytoplankton was carried out following [7] and [8]. The stock solution of copper was prepared using its metallic salt of copper sulfate pentahydrate (CuSO$_4$.5H$_2$O) (Merck) dissolved in distilled water. Diluted water was a sterile filtered saltwater (using Sartorius cellulose nitrate filter, 0.45 µm pore size, 47 mm in diameter) and autoclaved for 15 minutes at 15 psi. All experiments were carried out in triplicate using different nominal concentrations including control (100, 180, 320, 560, and 1000 µg/L). The actual dissolved Cu concentration was determined using Hach DR2800 spectrophotometer, with the results as follows: 13, 29, 98, 201, and 890 µg/L. These actual concentrations were then used to determined IC$_{50}$ value. The age of Pavlova sp. culture as inoculum was four days old to ensure that the cultures at the appropriate age and growth phase. Walne’s medium without EDTA (Ethylene Diamine Tetraacetic Acid-chelating agents) for microalgae growth were added to all treatments including the control and inoculum to minimize nutrient limitation during the test. Each flask inoculated with 1 ml of Pavlova sp culture with a density of 1 x 10$^6$ cells/mL. This will result in an initial density of 1 x 10$^4$ cells/mL. All flasks were covered with aluminum foil to prevent contamination.

All flasks were placed in randomized position in the incubation chamber with continuous illumination (cool white fluorescent lighting, with the light intensity of 4000 lux) and swirled at least once daily to re-suspend all contents. Test condition were recorded (pH : 7.67±0.07, temperature : 22.9±0.25 °C, salinity : 30.5±0.26 ppt, and dissolved oxygen : 7.5±0.33 mg l$^{-1}$). Test were terminated after 96 hours by transferred 0.9 ml subsample from each Erlenmeyer flask into the labeled glass vial and preserved with 0.1 Lugol’s solution. Cell counts were performed using Haemocytometer (Improved Neubauer Assistant Germany) under high power microscope (Nikon Eclipse E600). Observation and photography of cell morphology were performed using Nikon DIAPHOT and Canon EOS 10D DSLR camera.

2.2. Chlorophyll-a analysis

The chlorophyll-a content was determined according to the method based on [9]. One hundred mL of algal culture in test solution was filtered using Whatman 40 filter paper. Seven mL of full strength acetone was added into Eppendorf tube with a lid that has been filled with filtered sample and kept it for 24-h. After 24-h of extraction, the solution of chlorophyll extract was taken and measured using Turner Trilogy® Laboratory Fluorometer AU-10.

2.3. Data analysis

Percent inhibition (I) of growth relative to the control was calculated using the following equation:

$$I\% = \frac{C - T}{C} \times 100$$

Where C = control response and T = Treatment response

The IC$_{50}$ value was calculated using linear interpolation analysis in ICPIN software application Version 2.0. [10]. Graphics analyses were performed using Microsoft Excel 2007.

3. Result

The exposed-growth of Pavlova sp. responses to the increasing concentration of Cu within 96-h are shown in Figure 1. Cell number began to decrease at the lowest concentration (13 µg/L) and reduced drastically at 98 µg/L. Growth inhibition was significantly increased from 28.05% (at 29 µg/L) to 95.47% (at 98 µg/L). Minimum cell number and maximum growth inhibition were observed at the highest copper concentration (890 µg/L). The calculated IC$_{50}$ value of copper for Pavlova sp. was 51.46 µg/L, indicated a concentration that caused a 50% reduction in cells number.
Figure 1. The exposed-growth of *Pavlova* sp. responses to the different concentration of Copper (Cu) within 96-hours.

![Figure 1](image1)

Figure 2. The exposed-chlorophyll-a content of *Pavlova* sp. responses to different concentration of Copper within 96-hours.

![Figure 2](image2)

Similar results were also observed for the chlorophyll-a content (Figure 2). The chlorophyll-a content began to decrease at the lowest copper concentration and reduced significantly from 16.12 μg/L at 29 μg/L to 0.70 μg/L at 98 μg/L. Microscopic photographs of cell morphology (Figure 3) shows no apparent change in cell morphology at the lower copper concentrations. A variation in cell size was observed at the concentration of 98 μg/L (length, 7.26 μm) relative to the control (length, 5.4 μm) and the number of cells without setae was increased at this concentration.
Figure 3. Microscopic photographs (400x magnification) of cell morphology of *Pavlova* sp. in control (a) and in the copper concentration of 98 μg/L (b) after 96-h.

4. Discussion

Previous studies showed the sensitivity of microalgae to copper varies among species. [11] reported the calculated IC$_{50}$ value of copper was 31.80 μg/L for *Isochrysis* sp. and 63.75 μg/L for *Chaetoceros* sp. Meanwhile, [2] and [4] showed that marine diatom *Odontella mobiliensis* and Chaetoceros calcitrans were less sensitive to copper compared to *Pavlova* sp., indicated by IC$_{50}$ value of 298.4 μg/L and 450 μg/L, respectively. However, [12] explained that the effect of copper to microalgae depends on the species used, the composition of culture medium and the experimental protocol. The differences in sensitivity may also be due to differences in uptake rates across the plasma membrane, internal binding mechanisms and/or detoxification mechanisms. Therefore, direct comparison of sensitivity among microalgae due to copper exposure is difficult. This can be minimized at least by using similar experimental protocol.

A major process in the inhibition of algal growth and photosynthetic processes by pollutants is the generation of reactive species (RS) [13]. It is well known that RS such as superoxide hydroxyl radicals (OH) and hydrogen peroxide (H$_2$O$_2$) is produced in cells when exposed to environmental stresses, e.g., exposure to high light intensities, UV radiation, and metals exposure [1]. [2] explained, that chlorophyll-a reduction at higher concentrations could be attributed to the ability of Cu$^{2+}$ ions to inhibit the synthesis of d-aminolevulinic acid and protochlorophyllide reductase, peroxidative breakdown of pigments and membrane lipids by reactive oxygen species and prevention of chlorophyll to integrate into chloroplast photosynthetic membranes. Increasing the levels of RS can lead to severe cellular injury or death [1].

Increase in cell size at the higher copper concentration (98 μg/L) in the present study may be due to inhibition of cell division and accumulation of photosynthesis products inside the cell [14]. This is confirmed by previous studies by [15] and [4] that also reported an increase in cell size during copper exposure in *Nitzschia closterium* and marine diatom, *Chaetoceros calcitrans*, respectively. High concentration of copper inhibited the cell division and fixed carbon produced in photosynthesis could
not be excreted or utilised in cell division. Photosynthetic products then accumulated, leading to enlarged cells.

5. Conclusions
In this study, increasing copper concentration could suppress the growth of *Pavlova* sp. after 96-h. Minimum cell number and maximum growth inhibition relative to the control was observed at a higher concentration. Similar results were also observed for the chlorophyll-α content, which showed a maximum reduced chlorophyll-α content at the highest concentration. The variation in cell size and morphology was also observed at the higher copper concentration by the increase in the cell size and loss of setae compared to normal cells. The sensitivity of Pavlova sp. is comparable to other species and suitable as a test organism for toxicity testing due to its sensitivity to metal contamination.

Acknowledgments
We thank Eston Matondang for the assistance during laboratory analysis and Dede Falahudin for the assistance and help during writing this paper. We thank the reviewers for useful reviews and comments.

References
[1] Li M, Hu C, Zhu Q, Chen L, Kong Z and Liu Z 2006 Copper and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in the microalga *Pavlova viridis* (Prymnesiophyceae) *Chemosphere* **62** 565–72
[2] Manimaran K, Karthikeyan P, Ashokkumar S, Ashok Prabu V and Sampathkumar P 2012 Effect of copper on growth and enzyme activities of marine diatom, *Odontella mobilisensis* *Bull. Environ. Contam. Toxicol.* **88** 30–7
[3] Morelli E and Scarano G 2004 Copper-induced changes of non-protein thiols and antioxidant enzymes in the marine microalga *Phaeodactylum tricornutum* *Plant Sci.* **167** 289–96
[4] Anu P R, Bijoy Nandan S, Jayachandran P R and Don Xavier N D 2016 Toxicity effects of copper on the marine diatom, *Chaetoceros calcitrans* *Reg. Stud. Mar. Sci.* **8** 498–504
[5] Volkmann J K, Dunstan G A, Jeffrey S W and Kearney P S 1991 Fatty acids from microalgae of the genus *Pavlova* *Phytochemistry* **30** 1855–9
[6] Rehberg-Haas S, Meyer S, Tielmann M, Lippemeier S, Vadstein O, Bakke I, Kjørsvik E, Evjemo J O and Schulz C 2015 Use of the microalga Pavlova viridis as enrichment product for the feeding of Atlantic cod larvae (*Gadus morhua*) *Aquaculture* **438** 141–50
[7] Asean-Canada CPMS 1995 Phytoplankton growth test *Protocol for sublethal toxicity tests using tropical marine organism.* (Asean-Canada Cooperative Programme on Marine Science Phase II) pp 14–20
[8] ASTM 2006 Standard Guide for Conducting Toxicity Tests with Microalgae *Annual Book of ASTM Standards* 2006. Section Eleven: Water and Environmental Technology. Volume 11.06 Biological effects and Environmental Fate; Biotechnology pp 278–91
[9] Cochlan W and Herndon J 2012 *Water Quality Methods* (San Fransisco: San Francisco State University)
[10] Norberg-King T 1993 *A Linear Interpolation Method for Sublethal Toxicity: The Inhibition Concentration (ICp) Approach Version 2.0.* (Duluth, MN)
[11] Suratno, Puspitasari R, Purbonegoro T and Mansur D 2015 Copper and Cadmium Toxicity to Marine Phytoplankton, *Chaetoceros gracilis* and *Isochrysis* sp. *Indones. J. Chem.* **15** (2) 172–8
[12] Cid A, Herrero C, Torres E and Abalde J 1995 Copper toxicity on the marine microalga *Phaeodactylum tricornutum* effects on photosynthesis and related parameters *Aquat Toxicol* **31** 165–74
[13] Melegari S P, Perreault F, Costa R H R, Popovic R and Matias W G 2013 Evaluation of toxicity and oxidative stress induced by copper oxide nanoparticles in the green alga *Chlamydomonas reinhardtii* *Aquat. Toxicol.* **142–143** 431–40

[14] Stoiber T L, Shafer M M, Perkins D A K, Hemming J D C and Armstrong D E 2007 Analysis of glutathione endpoints for measuring copper stress in *Chlamydomonas reinhardtii* *Environ. Toxicol. Chem.* **26** 1563–71

[15] Stauber J L and Florence T M 1987 Mechanism of toxicity of ionic copper and copper complexes to algae *Mar. Biol.* **94** 511–9