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COPPER AND CADMIUM TOXICITY TO MARINE PHYTOPLANKTON, *Chaetoceros gracilis* AND *Isochrysis* sp.

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ABSTRACT

In Copper (Cu) based antifouling (AF) paints Cu was largely used as booster biocide after organotin was banned. Cu is micronutrient which is important in photosynthesis process because Cu is an essential metal as component of enzyme and electron transport chain. But in certain dosage, Cu could be toxic to marine organism. Chaetoceros gracilis and Isochrysis sp. are dominant microalgae in aquatic ecosystem. In this study the effect of Cu and Cadmium (Cd) on two marine microalgae, C. gracilis and Isochrysis sp. were compared. Toxicity test was based on American Standard for Testing Material (ASTM). IC_{50}-96 h of Cd as reference toxicant was 2,370 $\mu g.L^{-1}$ for C. gracilis and 490 $\mu g.L^{-1}$ for Isochrysis sp. IC_{50}-96 h of Cu to growth of C. gracilis was 63.75 $\mu g.L^{-1}$ and Isochrysis sp. was 31.80 $\mu g.L^{-1}$. Both Cd and Cu were inhibited growth of microalgae. Based on IC_{50}-96 h value, it could be concluded that Cu was more toxic than Cd. Toxicity of Cu was 37 times stronger than Cd for C. gracilis and 15 times for Isochrysis sp. It was estimated that at concentration 10 $\mu g.L^{-1}$ Cu does not show observable effect (NOEC) to C. gracilis and 5 $\mu g.L^{-1}$ to Isochrysis sp. The lowest observable effect of Cu (LOEC) to C. gracilis was at concentration 17 $\mu g.L^{-1}$ and 10 $\mu g.L^{-1}$ for Isochrysis sp.

Keywords: *Cd; Cu; toxicity; Chaetoceros gracilis; antifouling*

ABSTRAK

Penggunaan cat antifouling dengan bahan aktif tembaga (Cu) semakin meningkat semenjak dilarangnya penggunaan senyawa organotin (golongan TBT) sebagai biosida. Cu merupakan mikronutrien yang memegang peranan penting dalam proses fotosintesis karena merupakan komponen penting dalam rantai enzim dan transpor elektron. Akan tetapi, dalam dosis tertentu Cu dapat bersifat toksik terhadap biota laut. Dalam penelitian ini, telah dibandingkan pengaruh Cu dan Cd terhadap pertumbuhan dua mikroalga laut Chaetoceros gracilis dan Isochrysis sp. Prosedur pengujian toksisitas didasarkan pada standar metoda dari American Standard for Testing Material (ASTM). Nilai IC_{50}-96 jam dari Cd sebagai toksikan acuan sebesar 2370 $\mu g.L^{-1}$ terhadap C. gracilis dan 490 $\mu g.L^{-1}$ terhadap Isochrysis sp. Nilai IC_{50}-96 jam Cu terhadap pertumbuhan C. gracilis sebesar 63,75 $\mu g.L^{-1}$ dan 31,80 $\mu g.L^{-1}$ terhadap Isochrysis sp. Dari nilai IC_{50}-96 jam terlihat bahwa Cu lebih bersifat toksik dibandingkan Cd untuk mikroalga laut. Toksisitas Cu 37 kali lebih besar dibandingkan dengan Cd untuk C. gracilis dan 15 kali untuk Isochrysis sp. Cu tidak memberikan efek yang signifikan (NOEC) terhadap pertumbuhan C. gracilis pada konsentrasi 10 $\mu g.L^{-1}$ Cu dan 5 $\mu g.L^{-1}$ Cu pada Isochrysis sp. Konsentrasi Cu terendah yang memberikan efek signifikan terhadap pertumbuhan C. gracilis pada konsentrasi 17 $\mu g.L^{-1}$ Cu dan 10 $\mu g.L^{-1}$ Cu pada Isochrysis sp.

Kata Kunci: *Cd; Cu; toksisitas; Chaetoceros gracilis; antifouling*

INTRODUCTION

Anthropogenic pollutants in marine and estuarine environments have significantly increased over the last few decades. Among these pollutants, heavy metals, which tend to accumulate in bottom sediment and release slowly into water bodies, have long been recognized as major marine pollutants [1]. The algae are generally responsible for a large percentage of primary production. Reduction in this primary production can affect the amount of food available to organisms of other trophic levels especially the aquatic herbivores in the same marine ecosystem [2]. This is due to the phytoplanktons being the main source of food uptake to various trophic levels in the aquatic ecosystem [3]. Reduction in algal production could be caused by several factors including deterioration in the water quality of the surrounding environment. For example,

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elevated metal levels in the seawater may adversely affect the growth of marine algae [4-5].

Due to their ecological and commercial importance, considerable research on the effect of heavy metal pollutants on various microalgae has been conducted over the last few decades [6-8]. Several metals are essential for living organisms at very low concentrations, but at high concentrations most are toxic and have a direct and adverse influence on various physiological and biochemical processes. Cu belongs to the category of ‘‘essential metals’’ and participates in growth, metabolism and enzyme activities. The effect of Cu is of interest, as this element has become a wide spread contaminant due to its use as an algaecide and a fungicide in agriculture [9].

Cd has been ranked as one of the major metal hazards. It is present in aquatic and terrestrial environments at levels that are sufficient to produce biological effects to various organisms. Cd exerts harmful effects on aquatic organisms in many ways that affect the properties of many biological molecules (enzymes, etc.), often by blocking and reducing the thiol sites on proteins. Moreover, Cd can be accumulated via the food chain, posing a serious threat to human health [10].

After the ban on toxic organotin (TBT, TPT, etc.) compounds, usage of Cu based Anti Fouling (AF) paints have largely increased [11], especially in the form of Cu oxide. Heavy metals released from AF coatings also tend to cause change in the growth, biochemical metabolism and reproductive potential of the marine organisms. Among the metals, Cu is having an essential role in the algal metabolism [12-13]. However, in higher concentrations Cu tends to damage the cell wall membrane function causing reduction in potassium ion concentration inside the cells [14]. Cu is also universally used as biocide as it is lethal to microorganism at higher concentrations [15].

The toxic chemicals used in the AF paints cause severe environmental pollution. It is one of the most toxic metals to micro algae, and can be toxic at concentrations as low as 1 mgL^{-1}. [8,16]. Excessive Cu is accumulated on the cell wall and then absorbed into the cell, and affects the enzymes (SH-groups) causing reduction in reproduction [17]. The members of Chlorophyceae are sensitive to Cu toxicity, at higher concentrations [18]. The leachate from AF coating is considered as one of the major sources of increased Cu levels in the marine environment [11,19-20]. At community level, chronic Cu pollution alters the dominance and influences the biodiversity of algae [21].

Since Cu based AF coatings have been largely used as booster biocide, effects of Cu and Cd on growth of *C. gracilis* and *Isochrysis* sp. were investigated.

EXPERIMENTAL SECTION

Materials

Single culture of *C. gracilis* and *Isochrysis* sp. were obtained from Mariculture Laboratorium, Research Center for Oceanography Indonesian Institute of Sciences. Micro algae were cultured with Walne’s media and acclimation during 7 days under 12 h/12 h illumination. *Isocrhysis* sp. and *C. gracilis* were used in this test because it could grow fast, high sensitivity and high abundance in tropic ocean [22]. All seawater that used in test were filtered with 0.45 µm (cellulose nitrate) filter papers and sterilize by 15 min autoclaved 1.5 Pa pressure. Glasswares were washed with nitrate acid 10% and acetone and also rinsed with aquadest [23].

Instrumentation

The equipments utilized in this experiment were pH/DO meter Eijkelklamp, ATAGO refractometer for water parameter measurement. Autoclave and Heareus oven for sterilize all glassware. Computer windows XP based for statistical analysis using ICPIN and TOXSTAT program.

Procedure

Reference toxicant was a substance that already had known effect to organism by previous research. Reference toxicant should be used as positive control in bioassay test to check relative sensitivity of organism that used in bioassay test. Cd usually used as reference toxicant because its toxicity effect to organism [24]. Cd solution stock (1000 $mg.L^{-1}$ Cd) prepared using Cd chloride salt ($CdCl_2$) diluted in aquadest. Range of Cd concentration that used in this test are 0, 0.56, 1.00, 1.8, 3.2 and 5.6 $mg.L^{-1}$ Cd for *C. gracilis* and *Isochrysis* sp.

Cu solution stock (100 $mg.L^{-1}$) prepared using Cu sulfate salt ($CuSO_4$) diluted in aquadest. Series of concentration that used in this test were 0, 10, 18, 32, 56 and 100 $\mu g.L^{-1}$ for *C. gracilis* and *Isochrysis* sp. Actual concentration of Cu was measured using HACH DR2800 spectrophotometer based on porphyrin method. 500 mL solution of Cd and Cu concentration prepared with autoclaved filtered natural seawater. 100 mL solution filled into 250 mL Erlenmeyer flask (3 replicate) and 200 mL for water parameter measurement (pH, dissolve oxygen, salinity and temperature). 0.1 mL Walne’s non EDTA media added to each erlenmeyer flask as source of nutrient for phytoplankton growth. One milliliter (1 mL) of *C. gracilis* and *Isochrysis* sp. were inoculated to each concentration

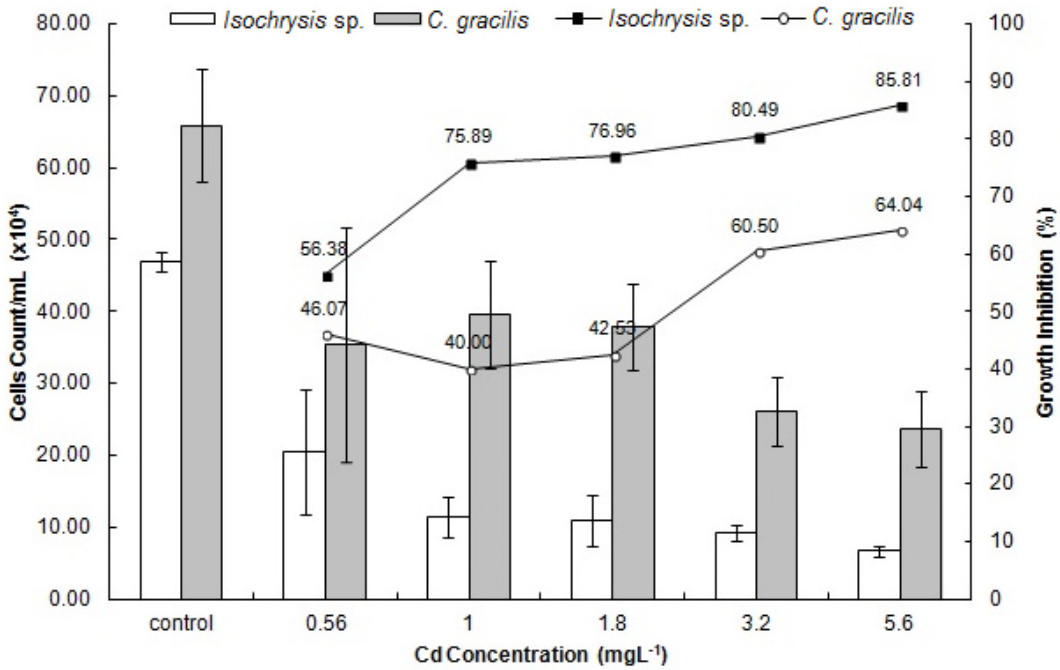

Fig 1. Growth Inhibition and Cells Count of *C. gracilis* and *Isochrysis* sp. after 96 h exposure to Cd

of Cd or Cu. This procedure based on standard method from ASTM (1992) with salinity and temperature modified according to tropical condition. Dissolve oxygen were measured by DO meter YSI 55, salinity with refractometer, pH and temperature measured by Eijkelklamp pH meter.

Data Analysis

According to procedure, after 96 h 0.9 mL of each concentration was sampled and mix with 0.1 mL Lugols as preservative compound. Phytoplankton was counted using Haemocytometer under microscope. Test were assumed valid if cells count in control after 96 h ≧ 2 x 10^5 cells/mL [23]. Percentage Inhibition (I) or Stimulation (S) were measured from average of cells count each treatment (T) compare with average cells count in control (C), according to this equation:

$$I\% = \frac{C-T}{C} x100\% \quad (1)$$

$$S\% = \frac{T-C}{C} x100\% \quad (2)$$

Effect of Cd and Cu to marine phytoplankton growth, LOEC (Lowest Observed Effect Concentration) and NOEC (No Observed Effect Concentration) value were analyzed with ANOVA and Dunnett test that already installed in TOXSTAT 3.2 program. LOEC are lowest concentration of toxicant that gives significant effect relative to control. NOEC are highest concentration of toxicant that have no significant effect relative to control. IC_{50}-96 h is a concentration of toxicant that have significant effect to inhibit 50% after 96 h relative to control. IC_{50}-96 h was measured using ICPIN 2.0 program [25].

RESULT AND DISCUSSION

Cd and Cu Toxicity Test

Phytoplankton growth toxicity test was assumed valid if cells count at negative control after 96 h ≥ 2 x 10^5 cells/mL [23]. According to this method, both of the test were valid because average cells count at control Cd for *C. gracilis* was 6.583 x 10^5 cells/mL and 4.70 x 10^5 cells/mL for *Isochrysis* sp., meanwhile control Cu was 7.367 x 10^5 cells/mL for *C. gracilis* and 3.783 x 10^5 cells/mL for *Isochrysis* sp. Typical growth of *C. gracilis* and *Isochrysis* sp. after 96 h exposure to Cd and Cu described at Fig. 1 and 2.

C. gracilis growth decreased between 42.53–66.67% and 56.38–85.82% for *Isochrysis* sp. from concentration range 560–5,600 µg/L Cd. Concentration range from 5–82 µg/L Cu could decrease 9.05–55.88% growth of *C. gracilis* and 13.66–78.85% growth of *Isochrysis* sp. According to Fig. 1 and 2, Cd could give inhibition growth 46.07% for *C. gracilis* and 56.38% for *Isochrysis* sp. at 560 µg/L Cd, meanwhile the highest Cu concentration was 82 µg/L gave 55.88% inhibition growth of *C. gracilis* and 78.85% for *Isochrysis* sp. Based on this information, Cu 7 times power full to inhibit growth of phytoplankton than Cd. Water parameters measurement was used to ensure the

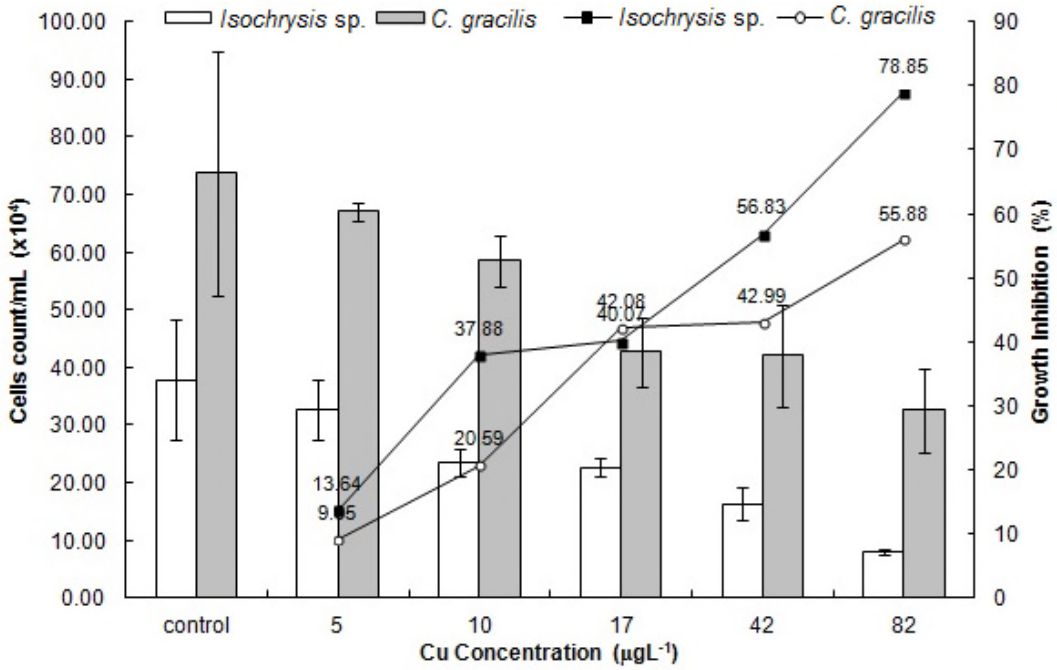

Fig 2. Growth Inhibition and Cells Count of *C. gracilis* and *Isochrysis* sp. after 96 h exposure to Cu

Table 1. Condition test and minimum requirement that should be filled for chronic test using phytoplankton (ACCPMS, 1995)

No	Parameter	Test Condition
1	Type of Test	Static
2	Temperature	27 ± 1 °C
3	Quality of lighting	Laboratorium light condition
4	Photoperiod	Continues
5	Size of Test Tube	Erlenmeyer 250 mL
6	Volume of solution	100 mL
7	Age of stock phytoplankton culture	4-7 days
8	Initial density	10,000 cells/mL
9	Replicate	3
10	Shaking period	Twice a day
11	Nutrient	Walne's non EDTA media
12	Dissolution factor	0.5
13	Duration of test	96 h
14	Effect that observed	Growth (cells count)
15	Validity of test	Density at control ≥ 2 x 10^5 cells/mL

Table 2. Water parameter with Cu toxicant test for *C. gracilis* and *Isochrysis* sp

Conc. (µg L^{-1})	DO (mg/L)	pH	Temp	Salinity (ppt)
Control	3.15	7.96	33.2	30
5	3.55	7.87	33.4	30
10	3.75	7.79	33.5	30
17	3.65	7.79	33.4	30
42	3.50	7.82	33.5	30
82	3.50	7.80	33.4	30

Table 3. Water parameter with Cd toxicant test for *C. gracilis* and *Isochrysis* sp.

Conc. (mg L^{-1})	DO (mg/L)	pH	Temp	Salinity (ppt)
Control	5.55	7.89	33.2	30
0.56	4.65	7.87	33.1	30
1.00	4.55	7.96	33.1	30
1.80	4.60	7.94	33.1	30
3.20	4.75	7.94	33.0	30
5.60	4.70	7.94	33.2	30

validity of toxicity test procedure. Test condition and minimum requirement that should be filled for phytoplankton toxicity test based on ACCPMS describe in Table 1. Water parameter conditions in this study were described in Table 2 and 3. Based on water

parameters, we cloud say that this study already had minimum requirement for phytoplankton toxicity test.

From the result of ICPIN software analysis, we could found that IC_{50}-96 h of Cd to *C. gracilis* was 2,370 μg/L and 490 μg/L for *Isochrysis* sp. IC_{50}-96 h of Cu to *C. gracilis* was 63.75 μg/L and 31.80 μg/L for *Isochrysis* sp. Liu et al. [26] reported motility of *I. galbana* was significantly retarded at 100 μmol/L of Cd. At 10 μmol/L, Cd significantly inhibited the relative values of motility percentage (MOT), Straight line velocity (VSL) and linearity (LIN) of *Tetraselmis chui*. All other motility traits of *T. chui* were significantly reduced at 31.62 μmol/L of Cd. Motility was completely arrested at 1000 and 316.23 μmol/L for *I. galbana* and *T. chui*, respectively. EC_{50} values for Cd equivanet to 13.67 mg/L and 4.25 mg/L for *I. galbana* and *T. chui*, respectively. In this study, IC_{50} values of Cd more sensitives comparable to IC_{50} values that Liu et al. [26] reported. Yap et al. [16] reported IC_{50}-5 d of *I. galban*a was 740 μg/L and *I. galbana* have capability to accumulate Cd due to induction of heavy metal sequestration peptides (phytochelatin) and detoxifying metals in vegetal cells. Nassiri et al. [27] found correlation of the concentrations of Cd and Cu with the metal-binding polypeptides phytochelatin in *Tetraselmis sueica*. Hu et al. [28] demonstrated that phytochelatin is the major intracellular cd chelator in a microalga *C. reinhartii*.

The EC_{50} value for Cu in the marine Haptophyte *Isochrysis* sp. have been reported by several authors: Cu 110–1000 μg/L [29]; Cu 30–410 μg/L [30]; Cu 910 μg/L [16]; Cu 4200 μg/L [8]. Comparing the values of Table 4 with those cited, this study shows lower IC_{50} values for Cu.

Table 4. Comparison result of Cd and Cu toxicity test to *C. gracilis* and *Isochrysis* sp.

Species	Toxicant	End Point	Value (μg L^{-1})
Isochrysis sp.	Cd	IC_{50}-96 h	490
		NOEC	0
		LOEC	560
	Cu	IC_{50}-96 h	31.80
		NOEC	5
		LOEC	10
C. gracilis	Cd	IC50-96 h	2,370
		NOEC	1,000
		LOEC	1,800
	Cu	IC50-96 h	63.75
		NOEC	10
		LOEC	17

Table 5. $IC_{50}/EC_{50}/LC_{50}$ Cu values for different microalgaa, obtained by different authors

Microalgae species	$EC_{50}/IC_{50}/LC_{50}$	LOEC	NOEC	References	Additional information
Isochrysis galbana	31,4 μmol/L (EC_{50})			Liu et al. [26]	MOT (percentage of motile)
Tetraselmis chui	1,3 μmol/L (EC_{50})			Liu et al. [26]	MOT
Dunaliela tertiolecta	530 μgCu/L (IC_{50})	42 μgCu/L	8 μgCu/L	Levy et al. [31]	72 h growth
Tetraselmis sp	47 μgCu/L (IC_{50})	22 μgCu/L	7 μgCu/L	Levy et al. [31]	72 h growth
Gephyrocapsa oceanic (non cocoliths)	>25 μgCu/L (IC_{50})	2.6 μgCu/L	1.3 μgCu/L	Levy et al. [31]	72 h growth
Emiliania huxleyi (non cocoliths)	20 μgCu/L (IC_{50})	-	9 μgCu/L	Levy et al. [31]	72 h growth
Nitzia closterium	18 μgCu/L (IC_{50})	5.8 μgCu/L	4.4 μgCu/L	Levy et al. [31]	72 h growth
Emiliania huxleyi (cocoliths)	17 μgCu/L (IC_{50})	1 μgCu/L	<1 μgCu/L	Levy et al. [31]	72 h growth
Phaeodactylum tricornutum	8 μgCu/L (IC_{50})	1.5 μgCu/L	<1.5 μgCu/L	Levy et al. [31]	72 h growth
Heterocapsa niei	4.8 μgCu/L (IC_{50})	-	-	Levy et al. [31]	72 h growth
Phaeodactylum sulcata	4.2 μgCu/L (IC_{50})	-	<5 μgCu/L	Levy et al. [31]	72 h growth
Isochrysis sp	4.0 μgCu/L (IC_{50})	1.1 μgCu/L	<1.1 μgCu/L	Levy et al. [31]	72 h growth
Micromonas pusilla	1.2 μgCu/L (IC_{50})	0.6 μgCu/L	0.3 μgCu/L	Levy et al. [31]	72 h growth
Minutocellus polymorphus	0.6 μgCu/L (IC_{50})	0.2 μgCu/L	<0.2 μgCu/L	Levy et al. [31]	72 h growth
Isochrysis galbana	910 μgCu/L (EC_{50})			Yap et al [16]	5 d growth
Chlorococcum litorale	10,200 μgCu/L (IC_{50})	-	-	Satoh et al. [8]	72 h growth
Chlorococcum sp	11,700 μgCu/L (IC_{50})	-	-	Satoh et al. [8]	72 h growth
Prasinococcus sp	5,400 μgCu/L (IC_{50})	-	-	Satoh et al. [8]	72 h growth
Tetraselmis tetathele	7,400 μgCu/L (IC_{50})	-	-	Satoh et al. [8]	72 h growth
Isochrysis galbana	4,200 μgCu/L (IC_{50})	-	-	Satoh et al. [8]	72 h growth
Heterocapsa sp	11,600 μgCu/L (IC_{50})	-	-	Satoh et al. [8]	72 h growth
Cylindrotheca	7,700 μgCu/L (IC_{50})	-	-	Satoh et al. [8]	72 h growth
Synechoccus sp	5,300 μgCu/L (IC_{50})	-	-	Satoh et al. [8]	72 h growth
LPP-group	5,400 μgCu/L (IC_{50})	-	-	Satoh et al. [8]	72 h growth
Tetraselmis chuii	330 μgCu/L (IC_{50})	-	-	Debelius et al. [38]	72 h growth
Nannochloropsis gaditana	137 μgCu/L (IC_{50})	-	-	Debelius et al. [38]	72 h growth
Isochrysis galbana (T-iso)	58 μgCu/L (IC_{50})	-	-	Debelius et al. [38]	72 h growth
Chaetoceros sp	88 μgCu/L (IC_{50})	-	-	Debelius et al. [38]	72 h growth
Rhodomonas salina	48 μgCu/L (IC_{50})	-	-	Debelius et al. [38]	72 h growth
Isochrysis sp	31.80 μgCu/L (IC_{50})	10 μgCu/L	5 μgCu/L	In this study*)	96 h growth
Chaetoceros gracilis	63.75 μgCu/L (IC_{50})	17 μgCu/L	10 μgCu/L	In this study*)	96 h growth

Based on TOXSTAT calculation result showed *C. gracilis* NOEC and LOEC for Cd were 1,000 µg/L and 1,800 µg/L, respectively. NOEC and LOEC Cd for *Isochrysis* sp. were 0 µg/L and 560 µg/L, respectively. NOEC and LOEC Cu for *C. gracilis* were 10 µg/L and 17 µg/L, respectively. NOEC and LOEC Cu for *Isochrysis* sp. were 5 µg/L and 10 µg/L, respectively. Table 4 showed information about NOEC and LOEC some microalgae and the result of this study compare to the current Australasian marine water quality guideline and also Indonesian marine water quality guideline for Cu was more than 1.4 µg Cu/L and 8 µg Cu/L, respectively (ANZEC/ARMCANZ, 2000; KMNLH, 2004). Levy et al. [31] reported four alga species had LOEC values less than Australasian marine water quality guideline for Cu and this suggests that the guideline may be under-protective for many sensitive marine microalgae.

Algal sensitivity to Cu is more likely to be related to Cu internalization than to adsorption to non-specific surface binding sites. Binding of Cu to the biotic ligand, as yet unknown in algae but assumed to be on the plasma membrane [32]. Growth inhibition in microalgae has also been related to intracellular Cu concentration [7,33].

The induction of proteins due to stress (toxicant-induced or via nutrient deficiency) has been noted in some algal cells [34] and the induction of the antioxidant superoxide dismutase has been noted for the marine prasinophyte *Tetraselmis gracilis* when exposed to Cd. Finally, efflux mechanisms may be used to pump metal back into solution, potentially as a different, less toxic metal species. Population dynamics and growth rates can also play a role, as an increase in cell density will provide a greater surface area, effectively diluting the concentration of toxicant per cell [33,35].

Interspecies differences in sensitivity of microalgae to Cu may also be related to their habitat (estuarine versus coastal versus oceanic environments) and their prior exposure to Cu. Sunda [36] showed that small changes in metal bioavailability in the open ocean affected the type of algal species that occurred. In contrast, Quigg et al. [37] showed that Cu accumulation rates in algae were not related to geographic position, e.g. coastal versus oceanic environments.

CONCLUSION

C. gracilis less sensitive than *Isochrysis* sp. toward Cd and Cu toxicity, respectively. IC_{50}-96 h of Cd as reference toxicant was 2,370 µg.L^{-1} for *C. gracilis* and 490 µg.L^{-1} for *Isochrysis* sp., respectively. IC_{50}-96 h of Cu to growth of *C. gracilis* was 63.75 µg.L^{-1} and *Isochrysis* sp. was 31.80 µg.L^{-1}, respectively. Toxicity of Cu was 37 times stronger than Cd for *C. gracilis* and 15 times for *Isochrysis* sp. It was estimated that at concentration 10 µg.L^{-1} Cu does not show observable effect (NOEC) to *C. gracilis* and 5 µg.L^{-1} to *Isochrysis* sp. Mean while lowest observable effect of Cu (LOEC) to *C. gracilis* was at concentration 17 µg.L^{-1} and 10 µg.L^{-1} for *Isochrysis* sp.

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