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Growth and Extracellular Carbonic Anhydrase Activity of Zooxanthellae *Symbiodinium* sp. in Response of Zinc Enrichment

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Coral reef communities contain a wide variety of mutualistic associations none more important than the relationship between corals and their symbiotic dinoflagellates of the genus *Symbiodinium* sp., commonly referred to as zooxanthellae. The function of Zinc (Zn) as cofactor of several enzyme systems such as extracellular carbonic anhydrase (extracellular CA) which catalyzes the interconversion of HCO_3^- and CO_2. Concentrations of dissolved Zn in oligothropic waters are often very low therefore may limit the growth of zooxanthellae and their ability to fix CO_2 from seawater via the carbonic anhydrase. The aim of this research is to investigate the effect of various concentrations of Zn on the growth and extracellular CA activity in zooxanthellae. Cell density was monitored daily by enumeration with hemocytometer-type chamber (0.1 mm). Extracellular CA was measured in homogenized intact whole cell by a pH drift assay. Results revealed that Zn status strongly influences the growth rate and extracelullar CA activity in zooxanthellae. The specific growth rate and cell density increased two-fold whilst extracelullar CA activity increased 10.5 times higher than that in control with increasing concentrations of Zn from 0 to 80 nM, but decreased when Zn was over 80 nM. Under a concentration of 80 nM was not Zn limited culture, consequently the growth rate of zooxanthellae not dependent on CO_2 concentration yet offset by extracelullar CA activity.

Key words: zooxanthellae, coral, Zn, growth, extracelullar carbonic anhydrase activity

INTRODUCTION

Coral reef communities contain a wide variety of mutualistic associations none more important than the relationship between corals and their symbiotic dinoflagellates of the genus *Symbiodinium* sp., commonly referred to as zooxanthellae (Veron 1995). The harboring of these intracellular and phototrophic dinoflagellates (zooxanthellae) is credited for the long-term success and dominance of coral in shallow, tropical, nutrient-poor environments since the Triassic (Muscatine & Porter 1997; Stanley 2003). A prolonged loss of these photosynthetic symbionts can lead to coral mortality (Brown 1997; Hughes *et al*. 2003).

At pH 8.2 to 8.3 of seawater, most of the inorganic carbon in seawater is in the form of HCO_3^-. Yet CO_2, not HCO_3^-, is the primary substrate for ribulose bisphosphate carboxylase/oxygenase (RUBISCO), the enzyme assumed to be responsible for photosynthetic carbon assimilation in zooxanthellae (Miller & Colman 1980; Cook *et al*. 1986). Additionaly, unlike freeliving microalgae, those that exist in symbiosis do not have direct access to the relatively constant seawater dissolved inorganic carbon (DIC) pool (2.2 mM). Therefore, the small pool of CO_2 (aqueous) in seawater available for direct uptake by the zooxanthellae is insufficient to sustain high photosynthetic rates (Muller-Parker & D'Elia 1997). One of mechanism for overcoming the limitation of CO_2 is carbonic anhydrase (CA) (Yellowlees *et al*. 1993). Carbonic anhydrase is a large and diverse collection of Zn metalloenzymes that catalyze the equilibration of dissolved inorganic carbon species according to the following equation (Moroney *et al*. 2001):

$$HCO_3^- + H^+ \leftrightarrow CO_2 + H_2O \qquad (1)$$

As a Zinc (Zn) metalloenzyme, CA is constituted of Zn in its active centre. Zn is an essential nutrient and functional component of many cellular enzymes (Anderson *et al*. 1978; Da Silva & Williams 1991). Recently, extremely low concentrations of Zn free ion, determined particularly in oceanic surface waters, have prompted a revaluation of its role with respect to phytoplankton productivity (Morel *et al*. 1994). Uptake of zinc by zooxanthellae is related to its free ion concentration rather than organically complexed zinc (Anderson *et al*. 1978; Sunda & Huntsman 1992). More than 98% of zinc is organically complexed in surface seawater of central North Pacific, which results in a concentration of Zn free ion as low as 1 pM (Bruland 1989; Bruland *et al*. 1991). Since the optimum Zn free ion concentration for microalgae is often higher than this

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(Ellwood & Van den Berg 2000), such low zinc availability may limit growth of zooxanthellae and their ability to fix CO_2 from seawater via the enzyme carbonic anhydrase (Sunda & Huntsman 1992; Morel *et al.* 1994).

The aim of this research was to investigate the effect of various concentrations of Zn on the growth and extracellular carbonic anhydrase (CA) activity in zooxanthellae.

MATERIALS AND METHODS

Culture of the Zooxanthellae *Symbiodinium* sp. Axenic cultures of zooxanthellae *Symbiodinium* sp. isolated from macroalga surface *Padina* sp. (Payung Island, Jakarta) were used in the experiments. Bioassay nutrient medium consisted of filtered seawater enriched to make f/2 medium. Test solutions were made by adding $ZnSO_4$ to the cultures medium in various amounts. Test solution of 40, 60, 80, 100 nM $ZnSO_4$ and control were used for this experiment. Aliquot of cell suspensions (15 ml) were then placed in sterile culture tubes and lightly capped. Three replicate culture tubes were filled for each test concentration and control. All experimental cultures were placed under countinous fluorescent lighting (2500 lux) and kept at a constant temperature of 28 °C through the experiment. Zooxanthellae cell density in each culture tube was estimated on alternate days.

Cell Density and the Growth Rates. Cell density was monitored daily by enumeration with a Hemocytometer-type chamber (0.1 ml). The growth rates were computed from a plot of cell densities versus cultivation day that resulted a linear regression. Thus, the growth rates equals the angle of that linear regression.

Extracellular Carbonic Anhydrase (CA) Activity Assays. Culture experiment was centrifuged at 4000 rpm and suspended in Tris HCl buffer (pH 8.3). The pellet consisting of intact cells. Measurements of the enzymatic activity were made using the potentiometric method of Wilbur and Anderson (1948) with modifications: 1.5 ml of double deionized water, saturated with CO_2 at 2 °C, were added to a volume of 3 ml of zooxanthellae material resuspended in the buffer (Ta), or buffer without microalgae (Tb). This reaction was performed in closed flask kept at a temperature between 0-4 °C. The time necessary for the CO_2 saturated deionized water to lower one unit of pH of both solution was measured and the enzymatic activity was calculated using the equations:

$$\frac{T_b}{T_a} - 1 = UA/\Sigma cell \qquad (2)$$

Equation 1 expresses the enzymatic activity as Units of activity (UA) per cell.

Cell density and activity of extracellular CA were analysed using one-way analysis of variance (ANOVA) to determine significant difference between controls and various treatments. Significant treatment effects were further analysed using least significance difference to identify Zn concentration that were significant affected to the growth and extracellular CA activity. Statistical tests were performed with statistical analysis system (SPSS 17.0) software.

Determination of Concentration of Dissolved Inorganic Carbon Composition. Dissolved inorganic carbon (DIC) was determined by pH method of the cultivation medium in which acidified to about pH 3.5 and the alkalinity can be calculated from the difference between the amount of acid added and the excess acid present (Anderson & Robinson 1946). This computation was performed with the measured pH and the alkalinity values using Strickland and Parsons's procedure (1968) in which a portion (5 ml) of the sea water sample is mixed with 1 ml of exactly 0.01 N hydrochloric acid. The pH of the resulting solution is measured. The standard acid in excess of that required to titrate the sample to the carbon dioxide inflection point is computed from a knowledge of this pH and an empirical factor. This excess acid is then substracted from 2.5 milliequivallent/l l and the total alkalinity of the sample is thus evaluated.

RESULTS

Effect of Zn Enrichment to Cell Density and Growth Rates. Increasing of Zn concentration from 0 to 80 nM, in laboratory cultures increased the specific growth rate of zooxanthellae from approximately 2.5 to 4.6 day^{-1}, and it was decreased when Zn addition at a concentration over 80 nM (Figure 1). The highest cell density and spesific growth rate were on Zn concentration under 80 nM in culture medium whereas it was two-fold higher than that of control (Table 1). However, the spesific growth rate and cell densities of zooxanthellae were reduced at Zn

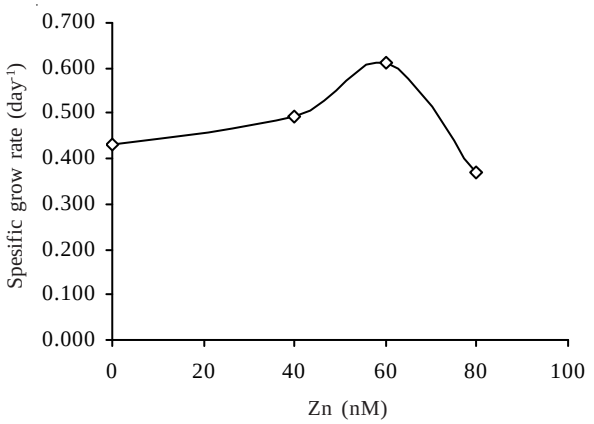

Figure 1. Growth rate of Zooxanthellae at different concentrations of Zn.

Table 1. Cell density and the growth rate of Zooxanthellae grown under different concentrations of Zn

Zn (nM)	Specific growth rate (d^{-1})	Cell density (x10^5 cells ml^{-1})
control	2.46	0.34
40	3.38	0.43
60	3.79	0.49
80	4.60	0.61
100	2.91	0.37

Values representing cell density and the growth rate of Zooxanthellae grown under different concentrations of Zn. Values statistically different from control were determined by Least significant difference's test: $P < 0.05$.

addition of 100 nM. One-way analysis of variance (ANOVA) of density data revealed significant differences in the densities of cells between controls and all treatments over the entire duration of the experiment ($F = 8.071$; $df = 4$, $P < 0.05$). Least significant difference test indicated that mean cell densities recorded in controls were significantly lower than the densities of cells under 40, 60, and 80 nM Zn treatments, respectively ($P < 0.05$).

The Effect of Zn Enrichment to Extracellular Carbonic Anhydrase (CA) Activity. The effect of Zn addition in cultures medium on enzymatic activities found (Figure 2) that extracellular CA activity was increased with increasing of Zn concentration from 0 to 80 nM, and decreased when Zn concentration was over 80 nM. The lowest extracellular CA activity was at control and the highest was at Zn concentration of 80 nM, it was 10.5 times higher than that of control. At concentration of 100 nM, the activity of extracellular CA was dropped (Table 2). One-way ANOVA on the pooled enzymatic activities data of controls and experimental cultures revealed significant treatment effects ($F = 20.515$; $df = 4$, $P < 0.05$). Further analysis using least significant difference test indicated that enzymatic activities 40, 60, and 80 nM Zn treatment cultures had significantly higher enzymatic activities than that of control ($P < 0.05$).

Cell Density and Extracellular CA Activity Interaction. Exponentially relationship between cell densities and enzymatic activities showed that index of determination coefficient (R^2) for all treatments was 0.80-0.92 (Figure 3). This was indicated that there was a strong relationship among cell density and enzymatic activity of Extracellular CA.

Consentration of DIC Composition. The pH of the medium had similar responds for all treatments which

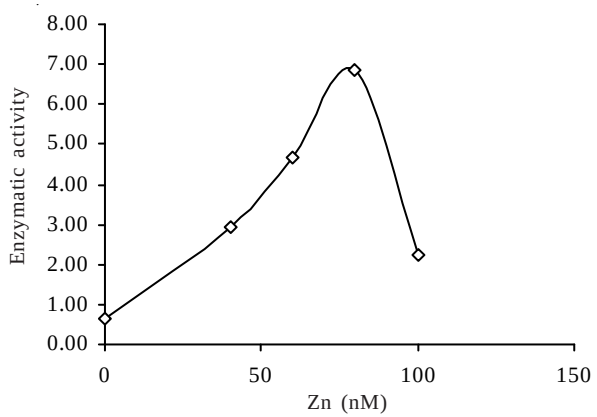

Figure 2. Relative amounts of extracellular CA activity of Zooxanthellae at different concentrations of Zn.

Table 2. Extracellular carbonic anhydrase activity of Zooxanthellae grown under different concentrations of Zn

Zn (nM)	Enzymatic activity (UA/Σ cell)
control	0.65
40	2.93
60	4.67
80	6.85
100	2.26

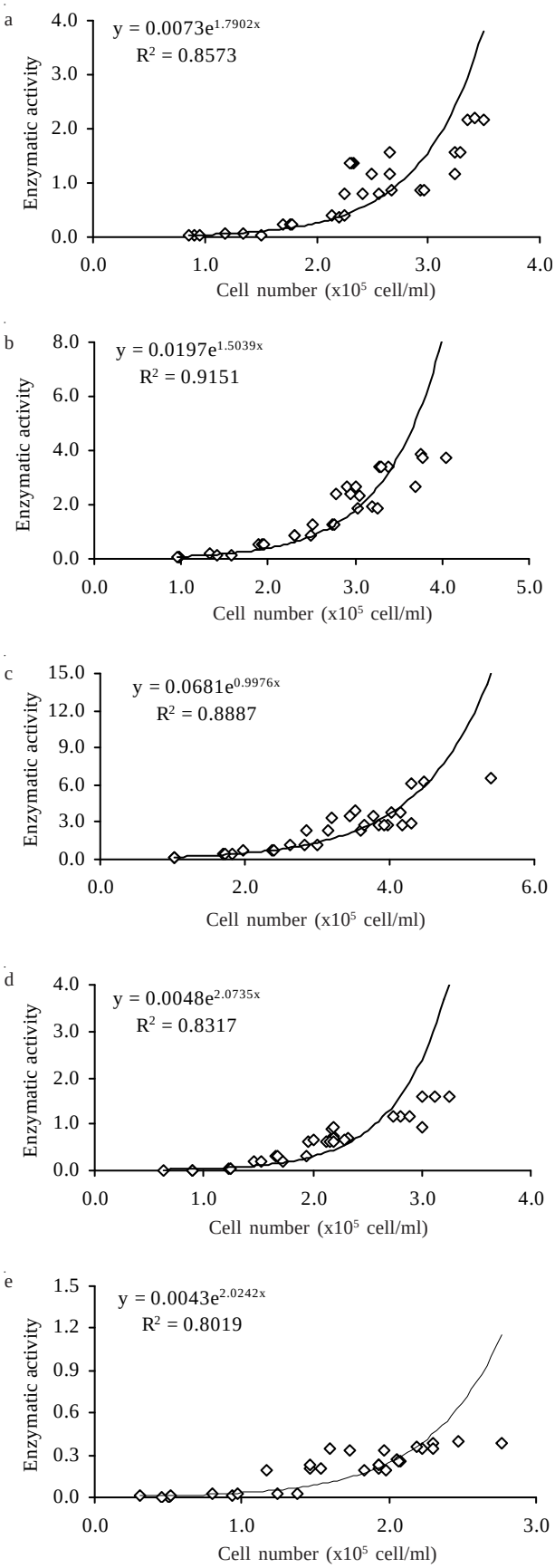

Figure 3. Relationship between cell density (10^5 x cell/ml) and CA_{ext} activity (unit of activity/Σ cell) at different concentrations of Zn; a. 40 nM, b. 60 nM, c. 80 nM, d. 100 nM, and e. control. Linear regressions were performed for each data point plotted. The regression equations and R^2 values are shown.

was increased from 7.72 in the first cultivation day to 8.1 and 7.91 at the tenth day for treatment of 80 nM Zn and control repectively (Figure 4). Both of HCO_3^- and CO_2 concentration showed different responds (Figure 5 & 6), it was decreased since the second day of cultivation. The highest decreasing of HC was in treatment of 80 nM Zn, it was from 2.18 to 2.01 mM, whilst control treatment was a slow decreasing, it decreased from 2.18 to 2.06 mM. Dissolved CO_2 was the first species of inorganic carbon available, its concentration was significantly decreased compared to HCO_3^- in the second day of cultivation. The concentration of CO_2 had a significant decreasing at the treatment of 80 nM Zn which was decreased from 0.034 to 0.011 mM, whilst control showed insignificant decreasing, it was decreased from 0.034 to 0.018 mM.

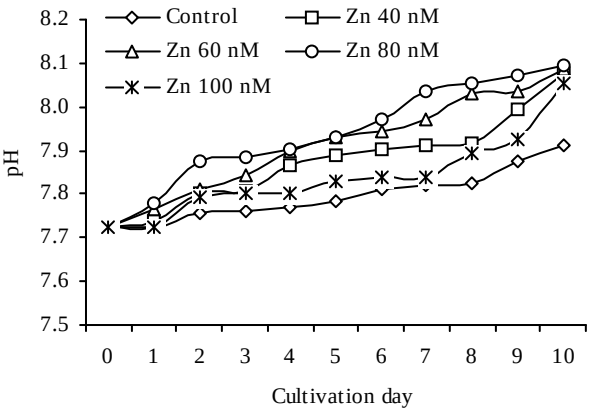

Figure 4. pH of growth media at different concentrations of Zn.

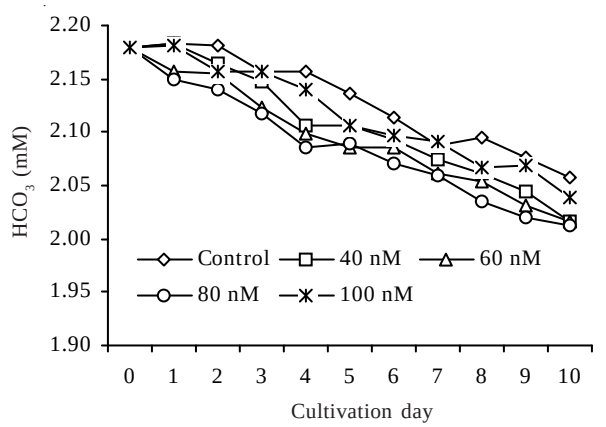

Figure 5. Concentration of HCO_3 at different concentrations of Zn.

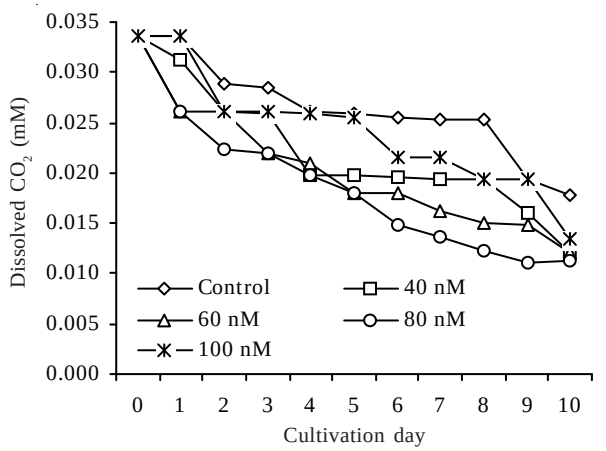

Figure 6. Concentration of dissolved CO_2 at different concentrations of Zn.

DISCUSSION

Plots of spesific growth rate versus the concentration of Zn in culture medium (Figure 1) showed that the largest increases of spesific growth rate occured at relatively high Zn concentration (80 nM). Suggesting at this concentration may be a concentration of Zn was needed to various metabolisms of zooxanthellae thus achieve the highest spesific growth rate. A similar pattern was observed for 40 and 60 nM treatment cultures, but exhibited much lower cell densities and spesific growth rate compared to control (Table 1), indicating that at these Zn concentrations may not be sufficient to meet Zn demand of zooxanthellae. As an inorganic trace metal, Zn is an essential nutrient stimulated the growth of microalgae, since it is cofactor of several enzyme systems such as carboxypeptidase, alkaline phosphatase (which is needed to acquire organic forms of phosphorus), and carbonic anhydrase (Anderson & Morel 1978; Morel *et al*. 1994), essential in the functioning of living organisms as micronutrients serving as structural proteins and pigment, used in the redox processes, regulation of the osmotic pressure, maintaining the ionic balance and enzyme component of the cells (Kosolapov *et al*. 2004). A second important function of zinc is in proteins involved in DNA metabolism, include DNA and RNA polymerases, reverse transcriptase, and zinc finger transcription factors (Da Silva & Williams 1991). Because of the critical role of zinc in many different metabolic function, therefore zinc addition in cultures medium should affect the growth and in this experiment presented that at a Zn concentration of 80 nM met the growth requirement of zooxanthellae. This finding is in agreement with that of Anderson *et al*. (1978) who found under the added Zn treatment of 80 nM in culture medium resulted Zn free ion activity which was not limited the growth of a coastal diatom *Thalassiosira weissflogii*.

Photosynthetic process involving nitrogen assimilation and in this experiment, we used Nnitrogen type. N assimilation in the photosynthetic process increased pH of medium. The typical stoichiometry of nitrogen assimilation is given by the following (Geider & Osborne 1992):

$$106CO_2 + 138H_2O + 16N \leftrightarrow (CH_2O)_{106}(NH_3)_{16} + 6OH^- + 138O_2 \quad (3)$$

The pH of all treatments was gradually increased as a result of the utilization of N assimilation and dissolved CO_2 by the growing zooxanthellae in photosynthetic process (Figure 4). The highest pH culture was in 80 nM Zn, whilst the lowest was in controls. Consequently, pH value of culture medium represented metabolism process in the cell, assuming that Zn addition of 80 nM was the optimum Zn concentration require in order to obtained

high spesific growth rate and cell densities, therefore increased pH of culture media from 7.72 in the first day to 8.10 in the end of cultivation. Moreover, pH shifting resulted in inorganic carbon species altered in culture medium that is present mostly as HCO_3^-. Our results and those of Moroney *et al.* (2001) indicated when the pH level is below 6.4, concentration of CO_2 predominates; at pH between 6.4 and about 10.3, concentration of HCO_3^- predominates; whereas above pH of 10.3, predominated by concentration of CO_3^-. HCO_3^- and CO_2 dissolved concentrations in all treatments were decreased from the second day and steepest dropping in 80 nM Zn. This decrease, in combination with a significant increase in cell number and spesific growth rate compare with other treatments, suggesting the Zn addition of 80 nM stimulated the growth of zooxanthellae. Increasing of pH culture medium represents photosynthesis process hence altering the inorganic carbon speciation in the culture medium, perhaps due to the low of dissolved CO_2 concentration on the outside of cell surface induce the activity of extracellular CA as can be seen in Figure 6 where most of the extracellular CA activities occured in the second day are associated with a decreased dissolved CO_2 concentration (Figure 6). The highest enzymatic activity was in 80 nM Zn reflecting this concentration could meet the Zn demand of zooxanthellae's cell to synthesize extracellular CA and its activity affected by the low of dissolved CO_2 concentration in the outside of cell membrane. The effect of Zn availability in CA activity induction also demonstrated by Lane and Morel (2000).

Addition of Zn higher than 80 nM slightly decreased the cell number, spesific growth rate (Figure 1) and extracellular CA activity (Figure 2). Based on one-way ANOVA test demonstrated that at concentration higher than 80 nM (in this experiment was 100 nM), was not differ in growth and extracellular CA activity than without Zn (control), indicating at a concentration higher than 80 nM, Zn inhibited growth and might be toxic to the zooxanthellae. This result agrees with earlier finding by Sunda (1991) that Zn is an essential nutrient for microalgae in trace metal concentration (0.05-50 nM), but it can also inhibit growth at higher concentrations since it might forms dangerous free radical. At a high Zn concentration, it could masking the catalytically active subunits of the enzyme or substrate proteins, changing the conformation of the enzyme structure and competing with cation activators connected with the formation of a substrate enzyme complex Ji and Silver (1995). This is mainly due to the fact that heavy metals alter the conformational structures of nucleic acids and proteins, and consequently form complexes with protein molecules, which render them inactive therefore slowing down the growth and destruction cell membrane integrity (Bong *et al.* 2010). However, the Zn addition of 100 nM in this study has not been in toxic level to zooxanthellae since the cells were able to grow with cell densities 2.91×10^5 cell/ml and spesific growth rate 0.37 day^{-1}.

The lowest growth occured in the controls indicate that Zn concentration at the sea surface inadequate to meet the Zn requirement in order to synthesize many cellular enzymes and proteins to support zooxanthellae growth. In this experiment, seawater from Pari Island, Jakarta as medium for control and all treatment cultures had a mean background concentration of 30 nM. Uptake of Zn by phytoplankton is related to its free ion concentration rather than organically complexed zinc (Anderson *et al.* 1978; Sunda & Huntsman 1992). As Bruland reported (1989), that Zn characteristic's is tend to form complexes with inorganic ligands such as Cl^-, OH^-, CO_3^-, SO_4^{2-} hence it could weak Zn free ion activity which is needed by microalgae. In this experiment, we applied *ethylenediaminetetraaceticacid* (EDTA), a complexes trace metals for precise regulation of trace metal availability in studies of trace metal uptake, limitation, and toxicity (Sunda 1989; Price *et al*. 1989). At equilibrium, virtually all of the zinc is present as biologically complexed zinc with Zn-EDTA chelates, and only ~1 in 6.500 zinc atoms is present as free ion Zn species (Zn^{2+}) (Bruland 1989). Therefore if total Zn concentration in the medium is 30 nM, the equilibrium concentration of biologically available inorganic zinc species (Zn^{2+}) is only 0.005 nM, such low zinc availability may limit growth of oceanic phytoplankton. Anderson *et al.* (1978) reported that the onset of limitation is around 0.01 nM Zn^{2+} in a coastal diatom *Thalassiosira weissflogii*. Therefore, if we only rely on Zn concentration in the seawater, its extremely low concentrations may limit the optimal growth of zooxanthellae especially in the high pH value and low dissolved CO_2 concentration in sea surface and their ability to fix CO_2 from seawater via the enzyme CA (Sunda & Huntsman 1992; Morel *et al.* 1994). Coale (1991) noted that Zn addition in the growth medium was needed since its characteristic as a trace element which produced the least response to the growth rate of natural phytoplankton populations from subarctic Pacific surface waters.

The relationship between cell densities and extracellular CA activity (Figure 3) represents that amount of cell zooxanthellae influenced the enzymatic activity. Similar behavior was observed by Weis and Reynolds (1999) that amounts of activity and enzyme CA were greatly enhanced in symbiotic animals (with zooxanthellae) compared with aposymbiotic (without zooxanthellae) to a temperate anemone *Anthopleura elegantissima*. Our results indicate that increase of densities, resulting decreased of dissolved CO_2 concentration in the medium (Figure 6), thus stimulated the activity of extracellular CA to supply CO_2 toward cell for photosyntesis prosess. If dissolved CO_2 concentration is relatively high for photosyntetic process, thus extracellular CA activity is low as shown in culture medium without Zn addition (control). A low CO_2 concentrations at the cell surface is one of the major factor to induce CA activity (Badger & Price 1994). According to concentration of dissolved CO_2 in all treatments (Figure 6), significant decreased of dissolved CO_2 concentration was in 80 nM. The lowest concentration of CO_2 in this cultures medium were not affected the growth of zooxanthellae as canbe seen in the cell densities and spesific growth rate that 2 times higher

than control. It indicated that at this concentration, Zn concentration did not limit the growth and hence did not affected the low concentration of CO_2 but was restored by the high of extracellular CA activity. CA activity was not influenced because there were sufficient supply of Zn as the major component to synthesize CA. Several factors regulate CA activity in microalgae *Chlorella kessleri* are the availability of inorganic carbon, nitrogen species, photosynthesis, light and metabolites of respiratory path such as glycolate and pH of the media (Marcus *et al*. 1983; Dionisio *et al*. 1989; Dionisio-Sese *et al*. 1990; Matsuda *et al*. 1996; Nimer *et al*. 1999; Bozzo *et al*. 2000).

Our results here agree with other results obtained from work on the effect of zinc on zooxanthellae and other marine algae. Goh and Chou (1997) studied the effect of $ZnCl_2$ addition on the rate of growth of zooxanthellae and discovered that at Zn concentrations of 1.53×10^{-6}–1.53×10^{-5} M significantly lowered rates of growth. The specific growth rate, cell final yields and extracellular CA activity of the red tide alga *Skeletonema costatum* were found to increased with increasing Zn^{2+} concentrations from 0 to 12 pM (Hu *et al*. 2003). This study has also shown that Zinc addition preferentially stimulated the growth but it can also inhibit growth at elevated concentrations.

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