愛媛大学農学部紀要（Mem. Fac. Agr., Ehime Univ.）
56：41－45（2011）

Effect of Exposure of Zinc at Low Concentration to Bacterial Production in Seawater

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ABSTRACT

Zinc (Zn) is one of the essential elements as an enzyme cofactor, however high concentrations of Zn can be toxic. In the usual ocean environment, Zn is found at trace levels, whereas pollution of Zn has been sometimes found in coastal waters. Bacteria should express a differential response to various concentrations of Zn. Here we found that Zn gave a rapid impact on bacterial productivity, whereas proteolytic enzymes were not affected. The results showed that a low concentration of Zn (0.01 μM) decreased the bacterial abundance, growth rate and bacterial production, but not inhibited proteolytic enzymes, suggesting that acute effects of Zn are independent of protein utilization enzymes.

Key words: Zinc, bacterial productivity, protease, protein

Zinc (Zn) is an essential micronutrient and that has been shown to be essential for growth, development and differential of all types of biota including bacteria (29). The Zn ion plays a role as an essential factor for most classes of enzymes; however, Zn is potentially harmful at excessive levels. In the oceanic environment, Zn is a trace element in the pelagic ocean (0.00005 to 0.009 μM) (22) but is sometimes artificially released to coastal water as well as other metals (UNEP, 1996 UNEP. 1996. State of the Marine and Coastal Environment in the Mediterranean Region. MAP Technical Report Series No. 100. UNEP, Athens.UNEP 1996, State of the Marine and Coastal Environment in the Mediterranean Region. MAP Technical Report Series No. 100, UNEP, Athens).

Toxicity studies of Zn have mostly focused on higher trophic level species in the aquatic food web (24, 25), whereas the effects of metals on phytoplankton and bacteria, which are fundamental lower trophic level members, are relatively scarce with most having been carried out in the fresh water environment (23, 30). Toxicity studies applying laboratory-bioassays or mesocosms have found severe effects of Zn upon marine microorganisms (1, 23). Rochelle-Newall *et al*. (26) reported that even at low concentrations Zn showed a negative effect on the food web at the lower trophic level, and on carbon transfer in sensitive coastal environments. On the other hand, bacterial isolates from natural habitats showed Zn tolerance (4, 11). The mechanism of resistance may be based on 1) metal retention on the surface of cell, 2) intracellular transformation into less toxic forms, 3) the release of metals from cells accompanied with polymers, and 4) decreasing of permeability of the cell membrane (14).

Protein is an important source of carbon and nitrogen for bacteria and major component of high molecular weight DOM accounting for 20-35% of total DOM (5). As pointed out by Nagata (16), protein can be a model to study the major features of the complexity in polymer-bacteria interactions in the sea. Hydrolysis of high molecular weight protein is an initial step in microbial protein utilization. Proteases act as mechanism of protein utilization and some of the proteases need Zn for this activity (17). However, proteases of marine bacteria being inhibited by Zn at the 100 μM to 1 mM level are known (15, 21), although effect at lower concentrations is not known. Thus control of protein degradation by Zn is suspected to have an affect on the bacterial growth and/or production through proteolytic enzymes.

From this evidence, Zn possibly induces inhibition of essential processes associated with enzyme reaction (12, 27), which might cause drastic ecological consequences. So far, heavy metal effects at lower than toxic concentrations focusing on bacterial extracellular enzymes has not been well documented. Therefore, in this study we are interested in understanding the effect of Zn on extracellular activities and enzyme production in an aquatic microbial ecosystem since protein is a major component of biogenic organic matter. Hence, the aims of this study are to evaluate the effect of Zn exposure at low concentrations by acute exposure on the response of the bacterial assemblages and on extracellular protease

Received April 13, 2011
Accepted July 14, 2011
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activities. This study will help us to expand our understanding on the role of Zn on bacterial-relating matter cycling in the ocean.

Seawater samples were taken from Scripps Institution of Oceanography (SIO) pier (32°52'N, 117°15.4'W) in La Jolla, CA (USA) during wintertime in February 2009. Seawater was sampled and maintained using an acid washed, thoroughly rinsed 10-L carboy. The seawater was transported within 1 hr in the dark to the laboratory and samples were processed within 2 hr. Seawater samples were filtered using 8-μm pore size polycarbonate filter to remove particles. In order to represent the natural assemblage of bacteria from the marine environment, seawater was then filtered through 0.6-μm pore size polycarbonate filter to exclude essential grazers. The filtrates were distributed to three 1 L flasks, each of which was filled with to 0.6 L. Each flask was subject to either no addition (control) or an addition of Zn. Zinc chloride ($ZnCl_2$) was added to the samples to give a final concentration of 0.01 μM and 0.1 μM. The concentrations added were within the ranged previously reported in the San Diego water (0.02 - 0.2 μM). After the addition of Zn samples were then incubated in the dark at room temperature (20-25°C) and sub-samples were taken at 0, 2, and 8 hr for measurement of bacterial production, bacterial growth and protease assay.

The hydrolysis of analog methylcoumarylamide (MCA) peptide substrates were used to estimate potential proteolytic enzyme activity as described by Obayashi and Suzuki (20). Aminopeptidase and trypsin types were measured (Table 1). All of the substrate was completely dissolved in dimethy sulfoxide (superior grade; Wako Chemical) before being added to triplicate seawater samples to give the final concentration of 100 μM. The enzymatic reaction (0.3 ml volume) was run in a 96 well plate and incubated at 25°C for one hour. Fluorescence of hydrolytic product, 7-amino-4-methylcoumarin (AMC) was determined using SpectraMax M2/M2e Microplate Readers (Molecular Devices, Sunnyvale, CA). Excitation and emission wavelengths were 380 nm and 460 nm. Fluorescence units were converted to units of nmol L^{-1} h^{-1} with the aid of a standard curve generated from serial dilution with a known concentration of standard AMC.

Bacterial abundance (BA) in seawater was enumerated by nucleic acid staining (SYBR Gold) and epifluorescence microscopy according to Noble and Fuhrman (18). Subsamples were fixed with formalin (final conc. 2%. w/v) and stored at 4°C until analysis. A 2 ml sample was filtered through an Anodisc membrane filter (0.02-μm pore size), with a backing filter (0.8-μm Millipore type AA filter) and stained under darkness for 15 min. Bacteria were counted under blue light excitation under x100 magnification on an Olympus BX60 epifluorescene microscope. More than 200 cells were enumerated for each sample. Leucine incorporation rate was measured as an indicator of bacterial production (BP). Samples were processed according to Kirchman (12). [4, 5-^{3}H]-Leucine was added to triplicate samples to give a final concentration of 20 nM in 1.7 ml seawater. Triplicate and one trichloroacetic acid (TCA)-killed blank (89 μl 100% TCA) were incubated in 2 ml screw cap microcentrifuge tubes for one hr under dark conditions at 25°C. The incubation was terminated by the addition 89 μl of 100% TCA and then centrifuged at 16,000 x g for 10 min. The pellet was resuspended and washed by the addition of 1.5 ml of 5% TCA. Samples were centrifuged again and subsequently aspirated, and a 1 ml of liquid scintillation cocktail (Ecoscint, scintiverse BD, ultima gold) was added. The radioactivity was determined by scintillation counting. BP was calculated according to Kirchman (12). Cell-specific growth rates were calculated assuming exponential growth. Bacterial specific growth rates were calculated from BP/BA assuming a mean C content of 20 fg C $cell^{-1}$.

Regression and analysis of covariance (ANCOVA) were used to determine whether there were statistically significant differences in bacterial abundance, bacterial growth rates, bacterial production and protease activity between controls and treatments.

Concentration of Zn in the San Diego water has been reported to be 0.02 μM to 0.2 μM (8, 28), which is much lower than the EPA chronic WQC (1.2 μM; U.S Environmental Protection Agency 2002, National Recommended Water Quality Criteria EPA-822-R-02I-047) indicating the sampling area was relatively uncontaminated. Fig. 1 presents the response of heterotrophic bacterial abundance, bacterial production and growth rate against Zn. The initial number of total bacterial abundance was 4.62×10^5, which were slightly increased along the 8 hr incubation (Fig. 1A). The final bacterial counts in Zn-added group and control were not significantly different ($p>0.05$), indicating that the cell growth was not affected by low concentrations of Zn.

A marked effect was found in bacterial production measured by leucine incorporation rates (Fig. 1B). Bacterial production was lower in the Zn treatment group

Table 1: The list of substrates used in this study. Abbreviations: MCA=4-methyl-coumaryl-7-amide

Name	Type of substrate	Molecular formula	Molecular weight	Compound
Leu-MCA	Aminopeptidase	$C_{16}H_{20}N_2O_3$	288.34	L-Leucine MCA
Ala-MCA	Aminopeptidase	$C_{13}H_{14}N_2O_3$	246.26	L-Alanine MCA
Boc-Phe-Ser-Arg-MCA	Trypsin	$C_{33}H_{43}N_7O_8$	665.74	t-Butyloxycarbonyl-L-Phenylalanyl-L-Seryl-L-Arginine MCA
Boc-Val-Pro-Arg-MCA	Trypsin	$C_{31}H_{45}N_7O_7$	627.73	t-Butyloxycarbonyl-L-Valyl-L-Prolyl-L-Arginine MCA
Boc-Leu-Ser-Thr-Arg-MCA	Trypsin	$C_{34}H_{52}N_8O_{10}$	732.82	t-Butyloxycarbonyl-L-Leucyl-L-Seryl-L-Threonyl-L-Arginine MCA
Boc-Val-Leu-Lys-MCA	Trypsin	$C_{32}H_{49}N_5O_7$	615.76	t-Butyloxycarbonyl-L-Valyl-Leucyl-L-Lysine MCA

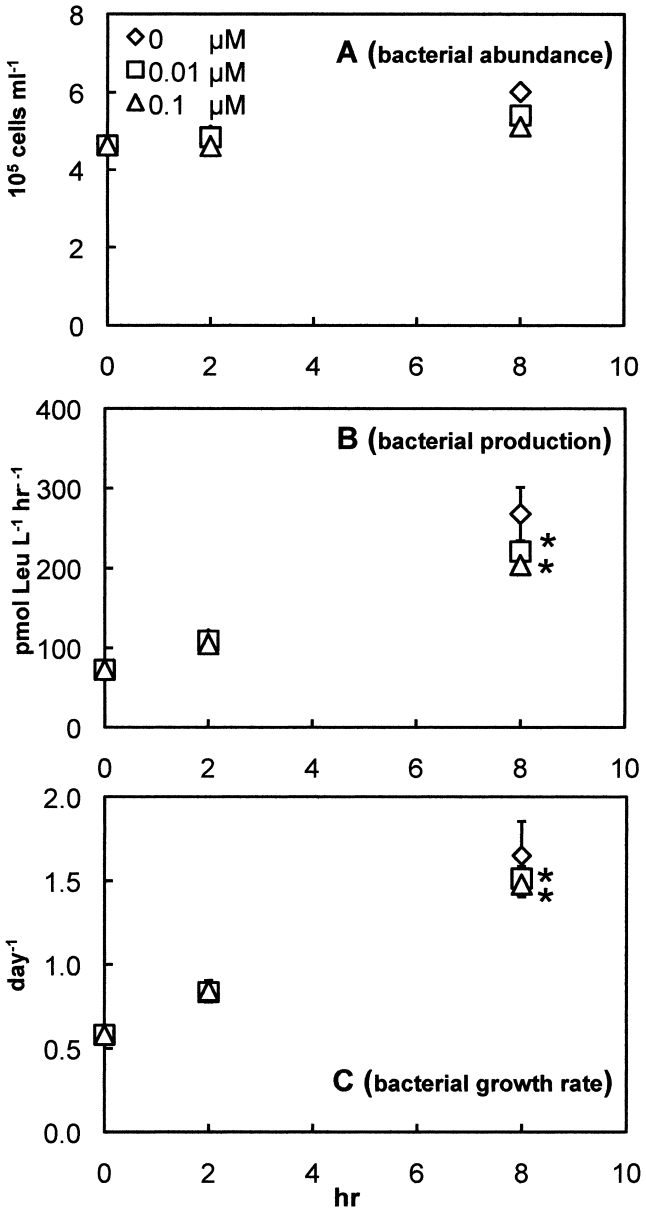

Fig 1. Temporal analysis of bacterial abundance (A), bacterial production (B) and bacterial growth rates (C) in 0.6-μm passing through seawater. Bars represent standard deviation of the mean from triplicates. * Showed lower than control (◇) with significant difference (p< 0.001).

than the control, showing reductions of 17% and 24% at 0.01 μM and 0.1 μM Zn, respectively (p<0.001). Zn is an inhibitor of respiratory electron transport in bacteria (10). Therefore, it is possible that the inhibition of amino acid (leucine) incorporation is caused by uptake system driven by ATP energy gained through the respiratory chain. However, the effect of Zn on substrate utilization needed for growth is virtually unknown. Since Zn is known to inhibit proteases in some marine bacteria (15, 21), it is of interested to determine the effect of Zn on bacterial production and protein utilization.

However, bacterial growth rates were observed to increase over the time as shown in Fig. 1C. The growth rate in Zn-treatment groups was significantly lower than the control (p<0.001) although the difference was not high, 8% at 0.01 μM and 10% at 0.1 μM. Theoretically specific growth rate is independent of the population size. Since the incubation time is short in this study, community structure changing is negligible. The aim of this experiment was to determine the immediate response of bacteria to low concentrations of Zn. The growth rates will be affected by bacterial abundance if the incubation is continued.

The effect of Zn on potential protease activities is shown in Fig. 2. The substrates for trypsin and aminopeptidase were hydrolyzed with different rates as described in Obayashi and Suzuki (20). In most trypsin substrates, activity was initially higher and subsequently decreased with time (p<0.01), whereas aminopeptidase activities slightly increased with time (p<0.001). This temporal change in enzyme activity was similar to our previous observations (19, 20). A significant difference was not observed in protease activity among Zn-treated and control groups during the 8 hr incubation. However, another experiment with increasing Zn concentration and incubation time (16 hr) showed dose dependent inhibition of aminopeptidase activity (6), suggesting that the Zn effect on proteases depended on the concentration and incubation time. Longer exposure effect on aminopeptidase may be explained by the fact that aminopeptidase is a family of Zn-dependent enzyme and thus it is predicted to be regulated by Zn (7). Zn at high concentrations may involve masking the catalytically active subunits of the substrate proteins, changing the conformation of enzyme structure and competing with caution activators connected with the formation of a substrate enzyme complex (9). If the source of enzyme would be various, for example flagellates and ciliates, it is hypothesized that various proteases would have different susceptibility to Zn. Various enzymes might play a role to decompose proteins in seawater as well as bacterial enzymes.

The results presented here show the addition of Zn clearly decreased the bacterial production rate (～24%) compared to the control within the 8 hr incubation (Fig. 1 B), suggesting that the bacterial productivity was more rapidly respond to Zn stress, which is indicator of total toxic effect including inhibition of respiratory and enzyme activities.

Alden Demoling and Bååth (2) applied leucine incorporation to study the toxicity of phenols toward bacterial communities extracted from sediment. Their results demonstrated that the leucine incorporation technique to the bacterial community is a rapid and sensitive method to evaluate toxicity with the comparison of thymidine incorporation.

Meanwhile our data also indicated that an increase in the concentration of Zn would have a serious affect on the carbon metabolism and respiratory activities in heterotrophic bacteria in aquatic ecosystems. Our findings are in agreement with Rochelle-Newall et al. (26), which reported that Zn addition at low concentrations could give negative results on the auto- and heterotrophic

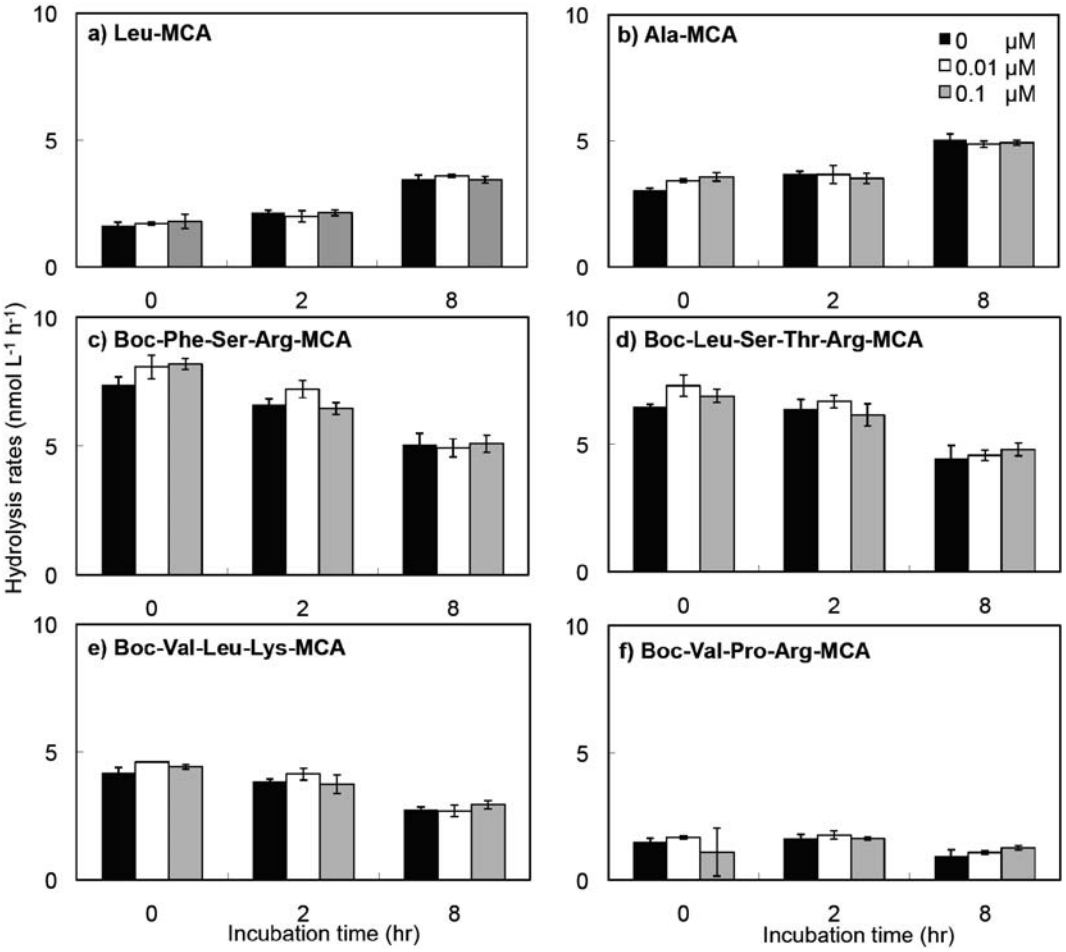

Fig 2. Hydrolysis rate of aminopeptidase (a-b) and trypsin (c-f) in 0.6-μm passing through seawater. Bars represent standard deviation of the mean from triplicates.

compartment and carbon transfer in aquatic ecosystem. Heterotrophic bacteria are the major degraders of both particulate and dissolved organic matter, and the rapid growth rates and production of bacteria represent an important link in the carbon cycle (3). The suppression of bacterial production at low concentrations of Zn or short periods might result in major shifts in the relative abundance of the bacterial community and consequently affecting the production of specific hydrolytic enzymes. The pollution in marine environment by heavy metals can affect the microbial carbon flux. These effects will link to the changing the quality of dissolved organic matter and biogeochemical cycle of organic matter especially in contaminated marine ecosystem.

Acknowledgments

We are grateful to the Marine Biology Research Division, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, California especially to Professor Farooq Azam and Dr. Francesca Malfatti for the use of their laboratory facilities for these experiments and useful advice and comments on our research. We also thank Dr. Todd W. Miller, CMES, for his critical review of this paper. This study was partly supported by Global COE Program at Ehime University. Chui Wei Bong was financially supported by the Government of Malaysia.

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摘　　要

ボン・チュイウェイ・大林由美子・鈴木　聡：海水中細菌生産に及ぼす低濃度亜鉛の影響

亜鉛は酵素反応の補因子として働くが，高濃度では阻害的に作用する金属で，天然海水中では微量しか存在しないが，時に高濃度汚染が起こることもある．細菌は様々な濃度の亜鉛に対して異なる応答を示す．本研究は，低濃度亜鉛は添加後数時間内に細菌生産性を阻害するが，タンパク質分解酵素には影響がないことを示した．このことは，亜鉛の効果は細菌のタンパク質利用系とは別の系に作用していることを示唆している．