

The Effect of Zinc Exposure on the Bacteria Abundance and Proteolytic Activity in Seawater

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Abstract—Zinc (Zn) is an essential trace element for growth and enzyme activities of heterotrophic bacteria. However, excess load of Zn shows toxicity and inhibition to microbial processes. The effect of Zn on marine microbial function has not been studied focusing on bacterial and enzymatic activities. In this study, we examined the short-term effect of Zn on bacterial abundance and respective proteolytic activity. Seawater sample was filtered through 0.6- μm filter to remove particle and grazers, varying concentrations of Zn (0.01 and 10 μM) were added to the filtrates. Aliquots were withdrawn at 0, 4, and 16-hr. Bacteria were enumerated using SYBR Gold staining and epifluorescence microscopy. Extracellular protease activities (aminopeptidase-, trypsin- and chymotrypsin-type activities) were measured by methylcoumarylamide (MCA)-substrates. Results showed that, the addition of Zn did not cause any significant changes on the bacteria abundance. However, the addition of Zn clearly decreased the aminopeptidase activity compared to control. Trypsin activity was high at 0 hr and no change was observed along the 16 hr incubation period in conditions either with or without Zn. Chymotrypsin activity was also not affected by Zn. This suggests that Zn has direct impact on aminopeptidase. Overall, Zn contamination seems to have effect on the microbial activities and transformation of proteins in aquatic system.

Keywords: zinc, bacteria, protease, seawater

INTRODUCTION

Organic matter in seawater is composed of a complex mixture of organic compounds with various chemical compositions, physical structures, and reactivity (Nagata, 2008). Within the aquatic system, heterotrophic bacteria are ubiquitous and the most abundant, which is recognized to be a critical component of the marine biogeochemical cycles and food web dynamics. Dissolved organic matters (DOM) are passively transported by bacteria, nevertheless the molecular size of the DOM is restricted to small and chemically simple substrates with molecular

weight of roughly 600 Da or less (Weiss *et al.*, 1991). For the efficient utilization of the high molecular weight DOM by bacteria, bacterial extracellular- or ecto-enzymes are important to reduce the molecular size of these compounds. Such enzymes play an important role when nutrients are limited condition (Hoppe, 1983). Therefore, bacterial and its enzymatic activities are of prime importance, and factors such as those that affect bacterial metabolic activity, distribution and abundance are of major concern.

Many metals and metalloids (e.g., Zn, Cu, Mn) are 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). Although some heavy metals such as Zn are essential trace elements for bacterial growth, at high concentrations of Zn, most bacteria are inhibited. Community diversity is severely reduced by high levels of Zn and only a very limited number of resistant bacteria can survive Goulder *et al.* (1980) and Kelly *et al.* (2003). 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, (e.g., inactivation of enzymes, slow growth and destruction cell membrane integrity). Heavy metals are both naturally and artificially present in ecosystems and have a high ecological significance due to their toxicity and accumulative behavior.

Proteins are sources of organic C, N and energy for heterotrophic bacteria (Rosso and Azam, 1987). In some cases, proteins possess functional groups that have strong affinity for metals (Fe^{2+} , Cu^{2+} and Zn^{2+}). Furthermore metals are needed for enzymes at catalytic or functional sites, and as co-factors in catalytic process. So far the effect of heavy metals at lower than toxic concentrations and, focusing on bacterial extracellular enzymes is not well documented. In this study, we examined the short-term effect of Zn on proteolytic activity, bacterial abundance and bacterial community.

MATERIALS AND METHODS

Sampling

Seawater was collected from the Scripps Institution of Oceanography (SIO) pier in La Jolla, CA (USA) in February 2009. The Zn content of the SIO pier water was less than $0.2 \mu\text{M}$ (Shaffer *et al.*, 2004), which is much lower when compared to other polluted coastal water (Reddy *et al.*, 2005). Seawater was kept in a 10-litre carboy for less than 1 hr during transportation to the laboratory experiment. To represent the natural assemblage of bacteria from the seawater, natural seawater sample was filtered through $0.6\text{-}\mu\text{m}$ pore size polycarbonate filter to remove particle and grazers. To the filtrates ZnCl_2 was added at various concentrations (0.01 and $10 \mu\text{M}$); filtrate without Zn acted as the control. The samples were then incubated in the dark at room temperature ($20\text{--}25^\circ\text{C}$) and sub-samples were taken at 0, 4, and 16-hr.

Table 1. List of substrates used in this study.

Name	Type of substrate	Molecular formula	Molecular weight	Compound
Leu-MCA	Aminopeptidase	$C_{16}H_{20}N_2O_3$	288.34	L-Leucine 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
Suc-Ala-Ala-Pro-Phe-MCA	Chymotrypsin	$C_{34}H_{39}N_5O_9$	661.70	Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine MCA

Table 2. Regression analysis on the effect of Zn on the enzymatic activity.

	Zn con.											
	0 μ M				0.01 μ M				10 μ M			
	<i>n</i>	Slope	<i>F</i>	<i>p</i>	<i>n</i>	Slope	<i>F</i>	<i>p</i>	<i>n</i>	Slope	<i>F</i>	<i>p</i>
Aminopeptidase	9	-1.17	214.48	<0.001	9	0.74	299.12	<0.001	9	-0.02	2.25	>0.05
Trypsin	9	-0.20	52.78	<0.001	9	-0.20	52.51	<0.001	9	-0.03	0.89	>0.05
Chymotrypsin	9	-0.02	1.07	>0.05	9	-0.02	30.12	0.001	9	-0.01	4.37	>0.05

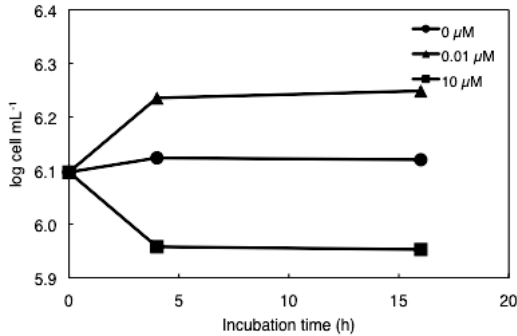


Fig. 1. Bacterial cell counts in responses to different Zn concentration.

Protease activities

Proteolytic activity was measured using analog methylcoumarylamide (MCA) peptide substrates (Obayashi and Suzuki, 2005). This method can separately measure enzyme activities of aminopeptidase, trypsin and chymotrypsin (Table 1). Final substrate concentration was 100 μM and the assay was performed triplicate. The enzyme reaction (0.3 ml) was placed in a 96 well plate and incubated at 25°C for one hr. Fluorescence was measured with excitation at 380 nm and emission at 460 nm using SpectraMax M2/M2e Microplate Readers (Molecular Devices, Sunnyvale, CA).

Bacterial counts

Subsamples were fixed with formalin (final conc. 2% w/v) and stored at 4°C until analysis. Bacterial counts were enumerated by SYBR Gold staining (Noble and Fuhrman, 1998) using epifluorescence microscopy (Olympus BX51, Japan). Two milliliters of the subsample were filtered through an Anodisc membrane (0.02- μm pore size), with a backing filter (0.8- μm Millipore type AA filter) and stained in the dark for 15 minutes. More than 200 cells were counted in each sample under the epifluorescence microscope.

Statistical analysis

Statistically significant difference in protease activity between controls and treatments were determined by regression and analysis of covariance (ANCOVA).

RESULTS AND DISCUSSION

Bacterial counts in the presence of different concentrations of Zn is shown in Fig. 1. Cell number of control group remained constant along the 16h incubation period. The cell number of 0.01 μM -Zn group slightly increased after 4h exposure, whereas that of 10 μM -Zn group decreased. However, significant

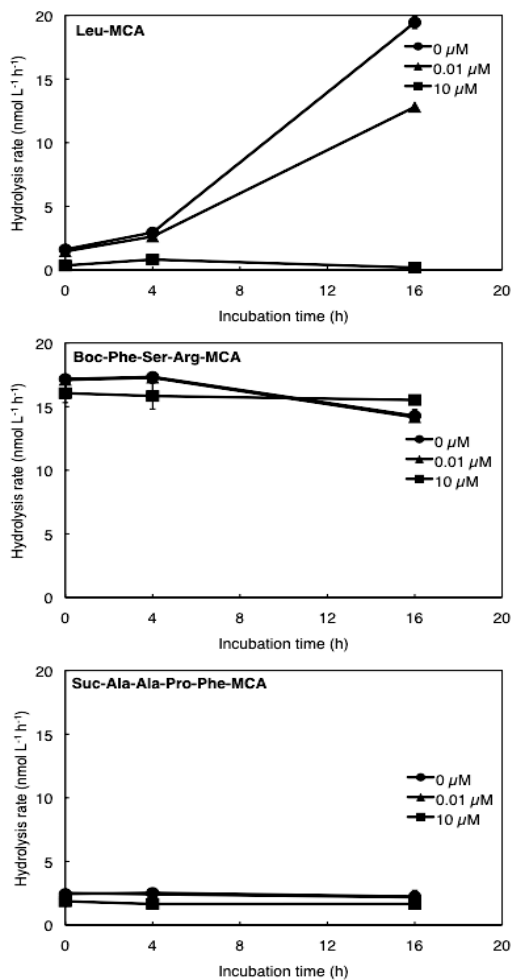


Fig. 2. Effect of different concentration of Zn on the hydrolysis pattern of aminopeptidase (A), trypsin (B) and chymotrypsin (C). Error bar are S.D. of triplicate measurement.

differences were not observed among the three groups ($p > 0.05$).

The effect of Zn concentrations on enzyme activities is shown in Fig. 2. Trypsin activity was the highest (Fig. 2B). There was an insignificant ($p > 0.05$) decreased in 10 μM -Zn exposed group but difference was not found in control and 0.01 μM groups. Chymotrypsin activity remained constant throughout the period (Fig. 2C). In aminopeptidase activity, quite a different profile was observed. A significant increase on enzyme activity was observed with time ($p < 0.001$) in the control and 0.01 μM -Zn groups (Fig. 2A) with significantly difference ($p < 0.001$) (Table 2). However, under the same condition, 10 μM -Zn completely inhibited

the aminopeptidase activity. This result indicates that at high concentrations of Zn, inhibition of aminopeptidase activity is observed whereas no changes for trypsin and chymotrypsin. The inhibition of proteolytic enzyme observed in this study is consistent with the previous report on toxic effect of Zn at high concentration that could involve 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). Some aminopeptidases are known to be metalloenzymes that cleave NH₂-terminal basic residues from proteins and oligopeptidase substrates. Aminopeptidase in seawater is a Zn-dependent enzyme and thus it is predicted that it is regulated by Zn (Choudhury and Srivastava, 2001). Azam *et al.* (1977) have reported that an adaptation or selection of bacteria to heavy metal occurs in the marine environment. Other studies on toxicity of metals to bacteria frequently report that the bacteria have a wide range of resistance mechanisms to—and intracellular uptake of—trace metals from seawater (Ross, 1988; Ferris *et al.*, 1989). For example, cadmium tolerant communities are likely to show co-tolerant to Zn (Paulsson *et al.*, 2000) and organotin tolerance simultaneously occurs associated with cadmium tolerance (Suzuki *et al.*, 1992). Such multi-tolerance to metals is a common phenomenon for both algae and bacteria in aquatic system (Takamura *et al.*, 1989; Pennanen *et al.*, 1996). Whether each species can benefit of this multi-tolerance or bacterial species are selected in community that is not yet very clear. The present study, suggests that high concentration of Zn has an effect on the enzyme level, especially on aminopeptidase activity. Therefore Zn contamination would have affect the biogeochemical cycle in aquatic system.

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