Inhibitory effect of zinc on the remineralisation of dissolved organic matter in the coastal environment

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ABSTRACT: To understand the role of zinc (Zn) in the biogeochemical cycle in coastal environments, we examined the bacterial remineralisation of dissolved organic matter (DOM) with 2 composite experiments using microcosms supplemented with Zn. In Expt 1, using samples collected from 2 stations in the Seto Inland Sea, Japan, we found that a decrease in DOM due to bacterial remineralisation during a 14 d experimental period had negative responses to Zn at both sites, but we found an inhibitory effect on bacterial abundance only at a station in the western part of the Seto Inland Sea. In Expt 2, comparison of the response of the remineralisation process to Zn among 3 kinds of organic substrate showed that Zn has little effect on 2 authentic standards (laminarin and bovine serum albumin) and that remineralisation of DOM originating from natural seawater was significantly suppressed by the addition of Zn. Based on the regression curves, we estimated the potential impact of Zn on the remineralisation of DOM. At a water quality standard of Zn concentration (86 μg Zn l⁻¹), DOM concentrations at the end of the experimental period (Day 14) increased 2.4 to 6.9%, and turnover time prolonged with a timescale of weeks to months. These potential shifts induced by Zn suggest that the allochthonous input of Zn into the coastal environment leads to suppression of energy flow in the microbial loop and enhances transport of DOM from coastal to offshore areas.

KEY WORDS: Zinc · Zn · DOM · Remineralisation · Bacterial abundance

INTRODUCTION

The coastal zone is a highly productive area, and its ecosystem is made up of complex biological, physical and chemical processes. Dissolved organic matter (DOM) is one of the important components in the biogeochemical cycle of coastal ecosystems because it is often found as a major constituent of organic matter in various environments (Williams 1995, Bodineau et al. 1999). DOM has a wide continuum of bioavailability, and its roles and fates in marine environments strongly depend on bacterial remineralisation processes (Bennet 2002, Carlson 2002). In general, the labile DOM, which is a fraction rapidly cycled, accounts for less than a small percentage of the bulk DOM (Rich et al. 1997, Skoog et al. 1999, Carlson 2002), but it acts as the major energy source for microbial food webs (Azam et al. 1983, Rich et al. 1997, Pavés & González 2008). On the other hand, the relatively refractory fraction of DOM remains in the water column for a long period, and contributes to processes of carbon sequestration to the ocean interior (Hansell et al. 1997, Carlson et al. 2000).

Although various factors, such as temperature, chemical composition, and bacterial activity, have been considered to be involved in the control of DOM remineralisation in coastal environments (Kirchman & Rich 1997, Miller & Moran 1997, Amon et al. 2001), allochthonous chemicals originating from both anthropogenic and natural sources have seldom been considered to date. In coastal areas there is an input of massive amounts of chemicals such as heavy metals and organic pollutants (Hoshika & Shiozawa 1988, Mandalakis et al. 2005, Kar et al. 2008), and significantly
higher concentrations of pollutants have been detected in coastal areas (Reddy et al. 2005, Cuong et al. 2008, Siddique et al. 2009) when compared to oceanic regions (Ellwood & Van den Berg 2000, Fukuda et al. 2000). Because some of the chemicals can alter biological activities (e.g. Pinto et al. 2003, Pereira et al. 2009), numerous researchers have focused on the toxic effects of such chemicals on organisms, including humans (e.g. Dell’Anno et al. 2003, Bielmyer et al. 2006, Thompson & Bannigan 2008, Mishra 2009). Based on their toxicity, it is recommended that the environmental concentrations of some chemicals be restricted to low levels (Bielmyer et al. 2006).

Because zinc (Zn) is one of the heavy metals regarded as being less toxic to human health unless the concentration is abnormally high (Walsh et al. 1994), its concentration is loosely regulated (Water Quality Standards: 10 to 86 μg Zn l−1) (Chongpraisith et al. 1994), allowing considerable emissions of Zn from anthropogenic sources. In addition, much of the runoff of Zn occurs not only from human activity but also derives from natural environments (Hoshika & Shiozawa 1986). These findings indicate concentrations of Zn that are several orders of magnitude higher in many coastal environments when compared to other toxic heavy metals such as cadmium, copper and lead (Shitashima & Tsubota 1990, Reddy et al. 2005, Cuong et al. 2008).

As mentioned above, Zn (compared with other heavy metals) is regarded as being a relatively less toxic element for humans and higher organisms, but it exerts a strong inhibitory effect on bacterial activity (Paulsson et al. 2000). Most studies on the effect of Zn on bacteria have been carried out in freshwater environments (Nweke et al. 2006, 2007, Vega-López et al. 2007); the results have shown a strong suppression of bacterial growth at low concentrations (>0.1 μg Zn l−1), which is about an order of magnitude lower than the effective level on phytoplankton (Paulsson et al. 2000). Although there have been just a few studies on the effects of Zn on marine bacterial communities (Kušpilić et al. 1989, Caroppo et al. 2006, Rochelle-Newall et al. 2008), these studies, too, have suggested that the activity of marine bacteria is also inhibited by the addition of Zn at 1 to 100 μg Zn l−1. However, the impact on marine bacteria might be a little weak when compared with that on phytoplankton (Caroppo et al. 2006, Rochelle-Newall et al. 2008), and it was suggested that Zn induces a shift to a bacteria-dominated heterotrophic system. Considering that bacterial activity is closely related to DOM remineralisation (Kirchman et al. 1991, Rich et al. 1997), Zn input into coastal zones will result in an alteration of DOM dynamics.

In addition, Zn is involved in enzymatic activity in natural environments. While more than 300 kinds of enzyme are enhanced by Zn (Hayashi 2004), Zn inhibits the activities of some other enzymes (Choudhury & Srivastava 2001). Because the remineralisation of marine DOM is partly regulated by the activities of various enzymes (Karner & Herndl 1992, Obayashi & Suzuki 2008), alterations in the remineralisation of DOM are also induced by the effect of Zn on enzymatic activities.

Although the effects of Zn on bacterial activity and enzymes suggest that Zn plays key roles in the remineralisation of DOM in coastal regions, there have been few studies to date on the relationship between DOM dynamics and Zn. Most of the studies on the impact of Zn on biogeochemistry have focused on the role of Zn as an essential trace element for phytoplankton (Morel et al. 1994) or enzymatic activity (Fukuda et al. 2000) in the open ocean. In the present study, we carried out 2 composite experiments on the decomposition of DOM to better understand the impact of Zn on the remineralisation of DOM in coastal environments. In Expt 1, we evaluated the effect of Zn on the remineralisation of DOM using samples from 2 coastal areas in the Seto Inland Sea, Japan, in order to determine the role of Zn in DOM dynamics. In Expt 2, we compared the effects of Zn on the remineralisation of 3 kinds of organic substrate: carbohydrate, protein and natural DOM. Because the chemical composition of marine DOM varies, both temporally and spatially, depending on its source and diagenetic state (Skoog & Benner 1997, Amon et al. 2001, Hama et al. 2004), it is important to determine the variability of the Zn impact among substrates. From these experiments, we achieved novel insights into the potential impact of Zn on DOM remineralisation based on the quantitative relationship between Zn and the parameters related to DOM remineralisation. The roles of Zn in the biogeochemical cycle are also discussed.

**MATERIALS AND METHODS**

**Expt 1: Effect of Zn on samples from 2 coastal areas in the Seto Inland Sea.** We collected seawater samples, which were passed through a 150 μm mesh, from sites on 2 coasts of the Seto Inland Sea—Matsuyama in Ehime Prefecture (Stn Matsuyama; salinity: 32.5; temperature: 24.9°C; September 2008) and Suminoe in Osaka Prefecture (Stn Osaka; salinity: 23; temperature: 9.7°C; February 2009). Stn Matsuyama is on the western coast of the Seto Inland Sea (Iyo-Nada), where relatively lower concentrations of Zn have been detected previously (Shitashima & Tsubota 1990). On the other hand, Stn Osaka is in a semi-enclosed area in
Concentrations of OC were measured by a total carbon analyzer (TOC-V, Shimadzu) after acidification with HCl and bubbling with air. Sample water was injected into the combustion column 4 to 6 times, and the values of the coefficient of variance were <2%. A calibration curve was drawn using an external standard (potassium hydrogen phthalate). The blank (value of Milli-Q water) and fluctuation range (standard deviation of Milli-Q water) were evaluated as (value of Milli-Q water) and fluctuation range (standard deviation, respectively.

**Analysis.** Concentrations of OC were measured by a total carbon analyzer (TOC-V, Shimadzu) after acidification with HCl and bubbling with air. Sample water was injected into the combustion column 4 to 6 times, and the values of the coefficient of variance were <2%. A calibration curve was drawn using an external standard (potassium hydrogen phthalate). The blank (value of Milli-Q water) and fluctuation range (standard deviation of Milli-Q water) were evaluated as 0.075 and ±0.031 mg C l⁻¹, respectively.

**Expt 2: Effect of Zn on various organic substrates.** Seawater samples were collected in 500 ml polycarbonate bottles after passing through a 150 μm mesh at Stn Matsuyma (salinity: 33; temperature: 23.2°C; OC concentration: 1.29 mg C l⁻¹; September 2009), and ZnCl₂ solution was added to the samples at 7 successive concentration levels (0 to 475 μg l⁻¹; see Fig. 1) (maximum final conc. 420 μg Zn l⁻¹). In addition, we added 3 kinds of organic substrate, described below.

To one series of samples we added laminarin with a calculated concentration of 8.0 mg l⁻¹. Another series was supplemented with bovine serum albumin (BSA) with a calculated final concentration of 10 mg l⁻¹. In addition to these 2 series using authentic standards, we conducted a third series of experiments with the addition of concentrated DOM (collected from Stn Osaka in February 2009, and stored at −20°C until the start of the decomposition experiment) in sizes ranging from 0.2 μm to 3 kDa (preparation described in the section ‘Analysis’ below). Samples for the measurement of Zn concentration were stored in low-density polyethylene bottles with the addition of ultra-pure HNO₃ (PlasmaPURE, GL Science) for acidification to pH 1. All the sampling equipment was washed with Milli-Q water after immersing for more than 12 h in 1 N HNO₃ solution.

**Expt 1: Effect of Zn on remineralisation in coastal waters**

**Wada & Suzuki: Effect of zinc on remineralisation in coastal waters**

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Bacteria were counted using epifluorescence microscopy after staining with 4',6-diamidino-2-phenylindole (DAPI) (Kitayama et al. 2007).

Carbohydrate and protein measurements were carried out using the phenol–sulfuric acid method (Handa 1966) and Bradford method (Bradford 1976), with glucose and BSA as standards, respectively; concentrations were expressed as glucose equivalent (mg glc equiv. l–1) and BSA equivalent (mg BSA equiv. l–1), respectively.

For measurements of total Zn concentration, the seawater samples were acidified to pH 1, and preserved for >1 wk at room temperature. For the desalination and concentration of Zn, we used a solid-phase extraction column (InertSep ME-1, GL Science). After loading the column with 2 N HNO3, the remaining acid was rinsed with 20 to 30 ml Milli-Q water, and the pH was adjusted around neutral using 0.1 M CH3COONH4. About 50 to 200 ml of sample seawater were neutralized by the addition of NH3 (aqueous ammonia) and loaded into the column. Because some metals, including Zn, and salt ions absorb resin, we eliminated the salt ions by washing with 0.5 N CH3COONH4 and Milli-Q water. After these procedures, the absorbed metals were recovered by loading 2 N HNO3, and diluting to 0.68 N (5% HNO3). The recovery yield of Zn throughout the procedure was reported as nearly 100% in a previous study (Furusho et al. 2008). The Zn concentration of the desalted samples was determined by Inductively-Coupled Plasma Mass Spectrometry (HP-4500, Hewlett-Packard) with yttrium as an internal standard.

RESULTS

Expt 1: Effect of Zn on the remineralisation of DOM at Stns Matsuyama and Osaka

The concentration factors of high-molecular-weight fractions of DOM from Stns Matsuyama and Osaka, obtained by ultrafiltration, were 429- and 111-fold, respectively. The concentrations of OC, carbohydrate, protein and Zn in the concentrates from Stns Matsuyama and Osaka were 60.0 and 31.5 mg C l–1, 42.8 and 35.8 mg glc equiv. l–1, 3.08 and 4.44 mg BSA equiv. l–1, and 67.4 and 21.4 μg Zn l–1, respectively (Table 1). Assuming that the respective carbon contents of carbohydrates and protein were 40% (based on the molecular formula of glucose: C6H12O6) and 53% (Ohlenbusch & Frimmel 2001), the carbohydrate accounted for the major fraction (28.5 and 45.4%), while protein was the minor fraction of OC (2.72 and 7.47%) in the samples from Stns Matsuyama and Osaka, respectively. Such high contributions of carbohydrate were, in general, comparable to the organic composition of high-molecular-weight DOM in other marine environments (Biersmith & Benner 1998, Benner 2002). Zn concentrations in the concentrates were 67 and 21 μg Zn l–1, which include both contamination during ultrafiltration and Zn in the high-molecular-weight fraction in natural seawater, for the samples from Stns Matsuyama and Osaka, respectively. Because 10 and 25 ml of the high-molecular weight fractions were added to 500 and 750 ml of GF/F filtrates, the amount of Zn originating from the concentrates was calculated as 1.32 and 0.690 μg Zn l–1, respectively. These values were not especially high compared to the amount of ZnCl2 added in the experiments (1.36 to 475 μg Zn l–1).

At the start of the experiments, concentrations of OC in the samples from Stns Matsuyama and Osaka were 2.12 ± 0.0644 and 2.74 ± 0.0188 mg C l–1 (in situ concs. 1.13 and 1.93 mg C l–1), respectively. The time course showed that the concentrations of OC in samples from both Stns Matsuyama and Osaka declined during 14 d, and the concentrations remaining on Day 14 (CR14d) compared with the value on Day 0 were 85.8 to 98.0 and 77.0 to 85.4% of the initial OC concentrations, respectively (Fig. 1, Table 2). Fitness of the time course decrease in OC to the exponential model is known, thanks to previous studies (Middelburg 1989, Hopkinson et al. 2002), and we applied the model using Eq. (1) as described below. In the present study, weak (p < 0.1) or significant (p < 0.05) relationships were found in most cases (Table 2).

\[ \text{OM}_t = \text{OM}_0 \times e^{-kt} \]  

(1)

where \( \text{OM}_0 \) and \( \text{OM}_t \) are the substrate concentrations on the initial (Day 0) and on a certain day (Day \( t \)), respectively, and \( k \) is the remineralisation rate constant.
stant. The $k$ values and turnover times (reciprocal of $k$ value) for samples from Stns Matsuyama and Osaka were 0.00210 to 0.0107 and 0.0114 to 0.0193 d$^{-1}$, and 94 to 290 and 52 to 88 d, respectively (Table 2). In the present study, the relationships of CR$_{14d}$ and $k$ values to Zn were verified using Eq. (2) to assess the quantitative relationship between DOM remineralisation and Zn:

$$C(z) = \alpha \times \log z + \beta$$  

(2)

where $C(z)$ is the value of CR$_{14d}$ or $k$ at a certain Zn concentration ($z$), and $\alpha$ and $\beta$ are constants (Table 3). For both samples, CR$_{14d}$ values increased with a Zn addition, while providing only a weak fit with Eq. (2) ($p < 0.1$) (Figs. 2a & 3a). Decreases in $k$ value with Zn additions were found for both samples, but a weak fit with the above logarithmic equation was obtained for the sample from Stn Matsuyama ($p < 0.1$) (Fig. 2b). Although the values on Day 14 (CR$_{14d}$) increased with Zn as described above, the values on other days (e.g. Day 7) had no significant relationship with Zn, probably due to relatively smaller decreases in DOC concentration from Days 0 to 7.

For the latter samples, the bacterial cell number on Day 0 was 4.0 to 4.7 $\times$ 10$^6$ cells ml$^{-1}$. These values increased once on Day 1, and decreased thereafter to between 29 and 58% of the initial values at 14 d (Fig. 1a, Table 2). A comparison of the values on Day 14 with Zn concentrations showed a clear decline in bacterial abundance (BA$_{14d}$) with the addition of Zn (Fig. 2c). The bacterial cell number on Day 0 of the sample from Stn Osaka was 2.0 to 2.4 $\times$ 10$^6$ cells ml$^{-1}$, and decreased with time (Fig. 1b). Unlike the sample from Stn Matsuyama, the BA$_{14d}$ value showed no relationship with the addition of Zn (Fig. 3c). We did not fit a curve to the time course of bacterial abundance because the bacterial cell number temporally increased in some samples probably due to a response to the addition of organic substrates.

**Expt 2: Effect of Zn on the metabolism of various organic substrates**

The carbohydrate concentration of the sample supplemented with laminarin was estimated by the
phenol–sulfuric acid method to be 10.1 ± 0.700 mg glc equiv. l–1. This value rapidly decreased before Day 9, and the values of CR 14d were 2.58 to 12.4% of the initial carbohydrate concentration (Fig. 4a). Although the number of plots (on Days 0, 9 and 14) was too small to verify the fit to the exponential model and calculated k value, the turnover time proved to be <9 d because most of the fraction, as well as BSA (described below), had been remineralised within 9 d.

Protein concentrations in the sample with addition of BSA also decreased rapidly in the initial period of the experiment (Fig. 4b); the time course of protein concentration was a significantly good fit to the exponential equation (p < 0.05), with the CR 14d and k values being 0.740 to 1.59% and 0.306 to 0.374 d–1 (turnover time: 2.67 to 3.27 d), respectively. For both samples, using authentic standards, the CR 14d and k values exhibited no significant relationship with Zn.

In contrast, the response to Zn addition of the sample supplemented with natural DOM differed from that in the samples with laminarin and BSA because the time course of OC concentrations showed an exponential decrease (Fig. 4c), with CR 14d and k values of 77.0 to 89.6% and 0.00909 to 0.0212 d–1 (turnover time: 47 to 110 d), respectively (Table 2). CR 14d showed a significant logarithmic relationship (p < 0.05) with the addition of Zn, while the k values only weakly fit the logarithmic curve (p < 0.01) (Fig. 5a,b).

The bacterial count on Day 0 was 1.4 ± 0.29 × 10⁶ cells ml–1. These values in the samples supplemented with

Table 2. Concentration of organic carbon remaining on Day 14 (CR 14d), bacterial abundance on Day 14 (BA 14d), and parameters (k, r², and p) calculated from an exponential curve fitted to the time course of organic carbon. k: remineralisation rate constant, r²: coefficient of determination, and p: rejection probability. ns: not significant

<table>
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<tr>
<th>Actual concentration of Zn (μg l–1)</th>
<th>CR 14d (%)</th>
<th>BA 14d (%)</th>
<th>k (d–1)</th>
<th>r²</th>
<th>p</th>
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Table 3. Constants of logarithmic equation (C(z) = α × log z + β). ns: not significant

| CR 14d values at Stn Matsuyama (Expt 1) | 1.51 | 86.3 | Weak (<0.1) |
| k value at Stn Matsuyama (Expt 1) | −0.000895 | 0.00845 | Weak (<0.1) |
| CR 14d values at Stn Osaka (Expt 1) | 1.34 | 74.3 | Weak (<0.1) |
| k value at Stn Osaka (Expt 1) | −0.00109 | 0.02090 | ns |
| CR 14d values of sample with natural DOM (Expt 2) | 1.73 | 76.2 | <0.05 |
| k value of sample with natural DOM (Expt 2) | −0.00125 | 0.0207 | Weak (<0.1) |
laminarin and BSA increased once (~290% against the initial value) (Fig. 4a, b), probably due to a response to the abundant inputs of organic substrates. After depletion of the substrates, the bacterial cell numbers decreased to between 55 and 99% and between 52 and 110% of their initial values, respectively. On the other hand, the time course of the sample supplemented with natural DOM showed a monotonic decline in bacterial cell numbers (Fig. 4c), suggesting lower bioavailability of natural DOM compared with laminarin and BSA, a finding consistent with previous studies (Nagata & Kirchman 1996, Arnosti 2000, Nunn et al. 2003). In this experiment, we found no significant correlation between bacterial cell numbers and Zn additions (Fig. 5c).

**DISCUSSION**

**Effect of Zn on the remineralisation of natural DOM**

Uptake and remineralisation of DOM by heterotrophic bacteria is one of the most important driving forces in the energy flow of marine ecosystems (Anderson & Ducklow 2001, Pavés & González 2008), and factors controlling DOM remineralisation, such as bacterial activity (Kirchman & Rich 1997, Rich et al. 1997), have been intensively studied. Although the role of Zn in DOM remineralisation has been neglected to date, some previous studies have suggested that Zn exerts a strong inhibitory effect on bacterial activity at concentrations ranging from

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**Fig. 2. Stn Matsuyama. Relationships between (a) concentrations of organic carbon remaining on Day 14 (CR14d), (b) k value, (c) bacterial abundance on Day 14 (BA14d) and concentrations of zinc (Zn) in the sample from in Expt 1. Error bars show ±SD between duplicate samples**

![Graph 1](image1)

**Fig. 3. Stn Osaka. Relationships between (a) concentrations of organic carbon remaining on Day 14 (CR14d), (b) k value, (c) bacterial abundance on Day 14 (BA14d) and concentrations of zinc (Zn) in the sample from in Expt 1. Error bars show ±SD between duplicate samples**

![Graph 2](image2)
<5 μg Zn l⁻¹ (Kušpilić et al. 1989, Paulsson et al. 2000). In addition, Zn is related to the activities of various kinds of enzyme, such as metalloprotease (Hase & Finkelstein 1993, Ogino et al. 1999, Fukuda et al. 2000), and dehydrogenase (Choudhury & Srivastava 2001, Vega-López et al. 2007), which would be involved in the DOM rem-

Fig. 4. Time course of changes in the concentration of carbohydrate, protein and organic carbon (OC), and bacterial abundance, in the presence of each of a range of concentrations of zinc (Zn additions from 0 to 419 μg l⁻¹) in Expt 2. •: percentages of (a) carbohydrates, (b) protein, and (c) OC compared with values on Day 0. □: percentages of bacterial abundance compared with values on Day 0. Note that both symbols overlap to a black square on Day 0. Curves: exponential regression curves in the samples with a significant relationship between Zn and each parameter.

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Fig. 5. Relationships between (a) the concentrations of organic carbon remaining on Day 14 (CR₁₄d), (b) k value, and (c) bacterial abundance on Day 14 (BA₁₄d) and the concentrations of zinc (Zn) in the sample with natural dissolved organic matter (DOM) in Expt 2.
ineralisation process. Considering that Zn concentrations >5 μg Zn l⁻¹ have been reported in some coastal environments (Reddy et al. 2005, Cuong et al. 2008), Zn potentially alters coastal carbon cycling.

In Expt 1, we examined the effect of Zn on the remineralisation of DOM collected from Stn Matsuyama (near Iyo-Nada) and Stn Osaka (near Osaka Bay). The concentrations of Zn in the mixtures of GF/F filtrates and high-molecular-weight DOM concentrates originating from Stn Matsuyama and Osaka were 1.49 and 9.74 μg Zn l⁻¹, respectively. Because small volumes (10 and 25 ml) of high-molecular-weight concentrates (Zn concentration: 67 and 21 μg Zn l⁻¹) were mixed with 500 and 750 ml of GF/F filtrates of the samples from Stns Matsuyama and Osaka, respectively, the Zn originating from the high-molecular-weight fraction was calculated to be 1.32 and 0.690 μg Zn l⁻¹, respectively. Although we did not measure the actual Zn concentration in natural seawater at those sampling locations, we calculated them to be 0.167 and 9.05 μg Zn l⁻¹ by subtracting the values of Zn originating from high-molecular-weight concentrates (1.32 and 0.690 μg Zn l⁻¹) from those of the mixture of the concentrates and GF/F filtrates (1.49 and 9.74 μg Zn l⁻¹) in Stns Matsuyama and Osaka, respectively. Previous studies have already reported Zn concentrations in various regions of the Seto Inland Sea, and showed concentrations of Zn in surface waters in Iyo-Nada (0.16 to 0.46 μg l⁻¹) and Osaka Bay (0.49 to 24 μg l⁻¹) (Shitashima & Tsubota 1990; Research Institute of Environment, Agriculture and Fisheries, Osaka Prefectural Government 2004) consistent with the results in the present study.

Both samples collected from Stns Matsuyama and Osaka showed a decrease in CR14d values and an increase in k values with Zn concentrations. The correlations of these values with Zn were not significant, but the values of CR14d had a weak relationship (p < 0.1). A similar weak relationship of k values was found for the samples from Stn Matsuyama (Figs. 2 & 3). Overall, addition of Zn appeared to alter the parameters relating to DOM remineralisation, suggesting the inhibitory effect on DOM remineralisation. The range in the amount of Zn addition was quite large in the present study (~475 μg Zn l⁻¹), and it would be useful to evaluate the effect of Zn in highly polluted areas (e.g. Reddy et al. 2005, Cuong et al. 2008). However, the level of Zn concentration in unpolluted areas would be relatively lower (around 1 μg Zn l⁻¹), implying that it will be an important issue to investigate the impact of Zn at the lower concentration level (e.g. ~10 μg Zn l⁻¹) in order to apply the Zn–DOM relationship in unpolluted coastal environments.

The in situ temperature was quite different between the 2 locations (Stn Matsuyama: 24.9°C; Stn Osaka: 9.7°C), and the incubation temperature (25°C) was set at a level similar to that at Stn Matsuyama. Although temperature is one of the important factors controlling the rate of remineralisation, we considered that the concentration level for the inhibitory effect of Zn is independent of temperature because the effective level of Zn on bacterial uptake of organic matter did not induce significant change in a previous study (Díaz-Roviño & Bååth 1996).

We considered that the effects of Zn on remineralisation could be attributed to bacterial abundance because, in many cases, DOM remineralisation is closely related to bacterial growth and abundance (Amon et al. 2001, Kitayama et al. 2007). Zn addition led to a clear decrease in bacterial cell numbers on Day 14 for the sample from Stn Matsuyama (Fig. 2c), confirming the inhibitory effect of Zn on bacteria. Such a trend was not commonly found because there was no correlation between bacterial abundance and Zn at Stn Osaka (Fig. 3c). One possible factor accounting for such a difference could be a technical feature of cell counting, because the DAPI staining method detects both living and dead bacterial cells. Because it is not necessarily the case that cell numbers reflect bacterial activity, those numbers for Stn Osaka might actually bear no relationship to the Zn addition. In conclusion, the regional difference in the Zn effect on bacterial cell numbers between locations was unclear, but the negative effect on the sample from Stn Matsuyama suggests the potential impact of Zn on the activity of marine bacteria.

Variability of the effect of Zn among different organic substrates

The effect of Zn on the metabolism of different organic substrates showed that an alteration in the remineralisation process was found only when using natural DOM in Expt 2. This result would suggest that Zn may selectively affect some metabolic pathways involved in the remineralisation process of natural DOM. However, it is possible that a high concentration of laminarin or BSA, compared with natural DOM, could affect the results because some organic material could bind with metals. No affinity of laminarin to metals has been shown (Strmečki et al. 2010), while some studies have shown that BSA binds to Zn. Considering that 1 molecule of BSA has 1 binding site (Masuoka et al. 1993), Zn bound with BSA would be theoretically equal to the number of BSA molecules. Because the BSA concentration in the present study was 10 mg l⁻¹ (0.15 μM), the amount of Zn bound to BSA would be calculated as 9.6 μg Zn l⁻¹ (0.15 μM Zn). Although this binding capacity of BSA could be ignored in the sample with the highest concentration of Zn (~419 μg Zn l⁻¹), no inhibitory effect was found even in this sample.
Consequently, the affinity of the BSA posed no serious problem in the analysis of the effect of Zn in the present study.

Numerous studies have reported the inhibitory effect of Zn on various metabolic pathways of bacteria (Choudhury & Srivastava 2001, Nguyen et al. 2006, Dementin et al. 2007), and the most conceivable pathways to be affected by Zn would be respiratory electron transport systems (Choudhury & Srivastava 2001) because Zn exerts strong inhibition on dehydrogenase activity (Choudhury & Srivastava 2001, Nweke et al. 2006, 2007). Because the respiratory system is involved in the remineralisation of most organic substrates, we expected that the remineralisation of all substrates (laminarin, BSA and natural DOM) in this experiment would be suppressed by Zn. However, the findings in Expt 2 produced the opposite result, in which the effect of Zn was found only in samples supplemented with natural DOM. We consider that this result indicates an inhibitory effect on specific metabolic pathways related to natural DOM remineralisation.

Before the present study, only a few studies had examined the impact of Zn on the remineralisation of natural DOM. However, Rochelle-Newall et al. (2008) recently tried to evaluate the effect of Zn on the uptake of organic matter based on the incorporation of 3H-labeled leucine. In a long-term (7 d) experimental period, they concluded that there was no significant effect of Zn on the incorporation of leucine, which seems to conflict with our results. We consider that such a discrepancy with previous findings (Rochelle-Newall et al. 2008) is due to the specific effect of Zn on natural DOM remineralisation, as shown in Expt 2 in the present study. The uptake of leucine might be independent of a Zn-regulated pathway, given that a free amino acid, such as leucine, is just a minor component of marine DOM. Another possible factor is the presence of phytoplankton in the study of Rochelle-Newall et al. (2008), in which a stronger inhibitory effect on phytoplankton was found. Because phytoplankton produce a large amount of labile organic material in seawater (Hama et al. 2004), the substrates available for bacteria in ambient seawater in the present study might be quite different from those available in Rochelle-Newall et al. (2008). In conclusion, it was difficult to determine in detail the mechanism of the inhibitory effect of Zn in the present study, but at least we can be confident that a Zn input alters the natural DOM dynamics.

**Potential implication of a Zn impact on coastal ecosystems**

The decomposition experiments in the present study showed that Zn induces a decrease in DOM remineralisation, but the relationship between the parameters (CR14d and k) and Zn was weak in most cases (p < 0.1). Based on the logarithmic regression curve between parameters related to DOM remineralisation (CR14d and k) and Zn, it is possible to calculate the potential impact of Zn on DOM dynamics. Because several water-quality criteria for Zn concentration in seawater were defined as 10 to 86 μg Zn l⁻¹ (e.g. Canada, Association of Southeast Asian Nations [ASEAN], USA and Japan; Chongprasith et al. 1999, Nagpal 1999, FDEP 2005, National Institute of Technology and Evaluation in Japan 2008), we calculated the potential inhibitory effect at the highest concentration of 86 μg Zn l⁻¹. The CR14d values of the samples without Zn addition were 88.6 and 77.8% (Tables 2 & 3), while the calculated CR14d values at 86 μg Zn l⁻¹ were 93.0 and 80.2% for the samples from Stns Matsuyama and Osaka in Expt 1, respectively. In addition, we applied the same analysis to the results of samples supplemented with natural DOM in Expt 2, and also showed the increase in CR14d values from 77.0% (for samples without Zn addition) to 83.9% (calculated at 86 μg Zn l⁻¹).

In order to understand the impact of Zn on the energy flow in the coastal environment, we should consider the shift of the remineralised fraction (RF14d: 100% × RP14d), because DOM utilised by bacteria supports the microbial loop as an energy source. In Expt 1, the respective values of RF14d in the samples without Zn addition were 11.4 and 22.2% at Stns Matsuyama and Osaka, while the calculated values at 86 μg Zn l⁻¹ were 7.0 and 19.8%. For the samples supplemented with natural DOM in Expt 2, the RF14d value would shift from 23.0% (without Zn addition) to 16.1% (at 86 μg Zn l⁻¹). From these calculations, we estimate the inhibitory effect of Zn on the RF14d value as 9.60 to 38.8% using the equation below:

\[
\text{Inhibitory effect (\%) } = \left( 1 - \frac{\text{RF14d at } 86 \mu g \text{ Zn l}^{-1}}{\text{RF14d without Zn addition}} \right) \times 100%
\]

These estimations show that the Zn concentration at a water-quality criterion (86 μg Zn l⁻¹) leads to a weak decrease in the energy supply to microbial loops in coastal environments.

In addition to DF14d values, we calculated the potential shifts of k values at 86 μg Zn l⁻¹. Results from Expt 1, and those from the sample supplemented with natural DOM in Expt 2, showed significant correlations of k values with Zn addition (p < 0.1), except for Stn Osaka in Expt 1 (Figs. 2b, 3b & 5b). The k values of the Stn Matsuyama samples in Expt 1 without Zn addition, and those supplemented with natural DOM in Expt 2, were 0.0188 and 0.00550 d⁻¹, respectively (turnover times: 53 and 180 d) (Table 2), and the calculated values at 86 μg Zn l⁻¹ were 0.0151 and 0.00446 d⁻¹ (turnover times: 66 and 220 d), respectively (Table 3),...
showing that turnover times are prolonged by periods ranging from weeks to months. In some coastal areas where the residence time of the water mass is ≤1 mo (Yanagi 1996, Wada et al. 2008), such a prolongation of turnover times will enhance the transport of DOM to oceanic areas.

Analyses based on the relationships obtained in our experiment suggest the potential impacts of Zn on the microbial loop and transport processes of DOM in coastal environments. Although the present study has implications as the first trial to evaluate the impact of chemical pollutants on the biogeochemical cycle in a coastal area, several issues remain to be addressed to further our understanding. One important issue would be the consideration of metal speciation, because a part of the organic matter in seawater has a capacity to bind metal ions (Yang & Van den Berg 2009). Most of the Zn (>95%) had bound to organic matter in the open ocean (Ellwood & Van der Berg 2000), while 20 to 30% of the total Zn had a free form in coastal environments (Galceran et al. 2007), showing a more complex state of metal speciation in coastal environments. Considering these findings, it is important to investigate the effect of speciation on the dynamics of organic matter in the coastal environment.

Another issue is to calculate the impact based on the effect of Zn alone, even though some coastal environments are subjected to inflows of various kinds of chemical, such as heavy metals and organic contaminants originating from both anthropogenic and natural sources (Hoshika & Shiozawa 1986, Reddy et al. 2005, Cuong et al. 2008). Considering that other kinds of chemical would also have significant effects on bacterial metabolites (e.g. Fabiano et al. 1994, Caroppo et al. 2006), we should carefully evaluate the effect of such chemicals from allochthonous sources so as to better understand the biogeochemical cycle in coastal ecosystems.

CONCLUSION

We reported a novel finding on the effects of Zn on the remineralisation of natural DOM based on 2 decomposition experiments. Responses to Zn could vary among organic substrates, but inhibition of the remineralisation processes of natural DOM would commonly occur in coastal environments. Based on the regression curves, we calculated the potential impacts of Zn on a coastal ecosystem. Increases in the values of RP14d, k and DF14d suggested the importance of further studies on the impact of allochthonous chemicals on the biogeochemical cycle and ecosystems in polluted coastal environments.

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