Chemical Geology 366 (2014) 42–51

Contents lists available at ScienceDirect

Chemical Geology

journal homepage: www.elsevier.com/locate/chemgeo

Zinc isotope fractionation during surface adsorption and intracellular incorporation by bacteria

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ARTICLE INFO

Article history:
Received 14 June 2013
Received in revised form 26 October 2013
Accepted 15 December 2013
Available online 25 December 2013

Editor: Carla M. Koretsky

Keywords:
Zn
Zinc
Isotopes
Adsorption
Uptake
Bacteria

ABSTRACT

Zinc (Zn) isotopes are fractionated during biogeochemical processing by microorganisms. Uncertainties remain, however, regarding the roles of cell surface adsorption and speciation of aqueous Zn on the extents of isotopic fractionation. In this study, we conducted bacterial surface adsorption and intracellular incorporation experiments using Zn and representative Gram-positive (*Bacillus subtilis*) and Gram-negative (*Pseudomonas mendocina*, *Escherichia coli*) bacterial species, as well as a natural bacterial consortium derived from soil. Under conditions of high Zn:bacteria ratio, surface complexes preferentially incorporated the heavier isotopes of Zn, resulting in an average $\Delta^{66}Zn_{adsorbed\text{-}solution}$ of +0.46‰ ($\alpha_{adsorbed\text{-}solution} \approx 1.00046$). Adsorption experiments conducted under conditions of low Zn:bacteria ratio appear to have been complicated by the presence of dissolved organic exudates that competed with surface functional group sites for Zn. We were able to empirically model this process to show that very small amounts of Zn-organic complexes with fractionation factors in the range of $\alpha = 1.002$ to 1.003 could account for the observed $\delta^{66}Zn$ of the experimental solutions. For the intracellular incorporation experiments, the presence of 0.2 and 2 mg/L of Zn (as Zn-citrate) resulted in a $\Delta^{66}Zn_{incorporated\text{-}solution}$ ranging from −0.2‰ to +0.5‰, depending upon the bacterial species and the growth phase. The addition of 0.2 and 2 mg/L Zn^{2+} to the growth medium appeared to create a metal stress response (or at least a change in metal processing) in *P. mendocina* that resulted in a positive $\Delta^{66}Zn_{incorporated\text{-}solution}$ of up to +2.04‰. Our study suggests that Zn isotopes have the potential to be used to elucidate metal-binding pathways associated with microorganisms in natural systems, but that the interpretation of these effects is likely complicated by factors such as competing surface interactions and differences in bacterial species and metal speciation.

1. Introduction

Zinc (Zn) is a critical element for biological functioning (Olhaberry et al., 1983; Shankar and Prasad, 1998; Hambidge, 2000; Andreini et al., 2006a,b; Maret, 2009), and the stable isotopes of Zn are substantially fractionated during biological processing (e.g., John et al., 2007). For these reasons, a number of investigations have examined Zn isotopic fractionation during plant uptake (Weiss et al., 2004; Viers et al., 2007; Moynier et al., 2009; Arnold et al., 2010; Caldelas et al., 2011; Jouvin et al., 2012; Tang et al., 2012) and interaction with yeast (Zhu et al., 2002), diatoms (Gélabert et al., 2006; John et al., 2007) and bacteria (Wanty et al., 2013). Moreover, Peel et al. (2009) and Andersen et al. (2011) have shown that Zn isotopes of particulate matter and diatom frustules, respectively, can serve as proxies for the biochemical cycling of Zn.

Despite the increasing interest in understanding how Zn isotopes fractionate during biological cycling, there are still substantial uncertainties. In plant systems, for example, variable concentrations of Zn, as well as differences in biochemical pathways among different plant species, can lead to substantially different Zn isotopic signatures among plants (Caldelas et al., 2011; Jouvin et al., 2012; Tang et al., 2012). In microorganism systems, the relative importance of surface complexation versus intracellular incorporation is uncertain. For example, Gélabert et al. (2006) showed that during sorption and uptake, freshwater and seawater diatoms were preferentially associated with heavier Zn isotopes ($\Delta^{66}Zn_{cell\text{-}solution}$ of +0.35‰ and +0.27‰, respectively), while in a separate study John et al. (2007) found that seawater diatoms incorporated the lighter Zn isotopes ($\Delta^{66}Zn_{cell\text{-}solution}$ ranged from −0.20‰ to −0.80‰, depending on the Zn homeostasis pathway), but adsorbed the heavier Zn isotopes. In addition, there is still uncertainty regarding the magnitude of Zn isotopic fractionation among different bacterial species and under differing conditions of Zn stress.

In order to try and address some of these uncertainties, we investigated the isotopic fractionation of Zn during surface adsorption and intracellular incorporation by representative Gram-positive (*Bacillus subtilis*) and Gram-negative (*Pseudomonas mendocina* and *Escherichia coli*) bacterial species and a natural bacterial consortium isolated from

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http://dx.doi.org/10.1016/j.chemgeo.2013.12.007

soil. The laboratory bacteria strains were chosen because of their different surface chemical properties. Moreover, Zn homeostasis mechanisms for each of these bacterial species have been described previously (e.g., Beard et al., 2000; Outten and O'Halloran, 2001; Cánovas et al., 2003; Moore and Helmann, 2005). We conducted separate batch adsorption experiments and bacterial growth experiments under a variety of chemical conditions. Samples of the associated fluids and/or bacterial cells (collected as a function of pH, time, or growth phase) were prepared and analyzed for their concentrations and isotopic compositions of Zn. Here we present the results of these experiments and discuss their implications for understanding Zn isotope variations in natural systems.

2. Methods

All volumetric flasks, sample bottles, test tubes, and pipette tips used for experiments were acid-washed in sub-boiling 10% HCl and rinsed 3 times with ultra-pure (18.2 MΩ) water. Chemical reagents and growth media were prepared using ultra-pure water. Ultra-pure acids and bases were used for experiments and sample digestion and preparation.

2.1. Bacteria collection, growth, and harvesting

B. subtilis (Gram-positive), *P. mendocina*, and *E. coli* (both Gram-negative) were chosen as representative laboratory strains of bacteria and were acquired through the Fein lab at the University of Notre Dame. The cellular surface of Gram-positive bacteria consists principally of a thick and rigid peptidoglycan layer (25 to 30 nm) bound together with secondary polymers such as teichoic acids (Konhauser, 2006). The surface of Gram-negative bacteria consists of a flexible outer membrane of lipopolysaccharides covering a thin (3 nm) peptidoglycan layer (Beveridge and Koval, 1981; Konhauser, 2006). *B. subtilis* was grown by inoculating (via sterile loop) an autoclaved medium of 30 g of Trypticase Soy Broth (TSB) plus 10 g of yeast extract dissolved in 2 L of ultra-pure water (Kenney et al., 2012). *P. mendocina* and *E. coli* were grown in the same manner but in a basal medium modified slightly from that described by Hersman et al. (2001). The nutrient medium included the addition of the following solutes per 1 L of ultra-pure water: 0.5 g K_2HPO_4, 1.0 g NH_4Cl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g $CaCl_2 \cdot 2H_2O$, 8.33 g succinate disodium salt, 30 mM Fe-EDTA, 4.77 g HEPES buffer, 1 g of glucose (for *E. coli* only), and 0.125 mL of trace elements solution (5 mg $MnSO_4 \cdot H_2O$, 6.5 mg $CoSO_4 \cdot 7H_2O$, 3.3 mg $ZnSO_4$ and 2.4 mg MoO_3 per 100 mL of water; Hersman et al., 2001). Zn is an essential trace nutrient and the bacteria cannot grow effectively without it. It was eliminated from the growth experiments but replaced with another form of Zn (Zn-citrate or Zn^{+2}). The elimination of the $ZnSO_4$ was important so we could have an isotopically homogeneous starting point. For all experiments, bacteria were grown aerobically at 25 °C in an incubator-shaker. All inoculations and sample handling were conducted under sterile conditions in a Class 100 clean bench.

A consortium of natural bacterial species was obtained from near-surface soils in the El Paso, Texas, region. Five hundred grams of soil were sampled using a sterile plastic bag and nitrile gloves. Thirty grams of the soil sample were used to inoculate 2000 mL of sterilized basal medium. After several days of growth, the suspended soil was allowed to settle and 1 mL of the top part of the solution was used to inoculate a second sterilized flask of basal medium. This transfer "diluted out" the soil used for the initial inoculation (e.g., Borrok et al., 2004). Even though the natural consortium was used as an analog for natural bacterial species, we cannot exclude the possibility that other microorganisms like fungi may have been present. Furthermore, it is likely that the microbial populations shifted slightly from batch to batch based on small changes in soil conditions and inoculation.

Bacteria used for adsorption experiments were harvested from the media after 48 h through centrifugation. Previous work has shown that the laboratory strains are near the stationary growth phase after 48 h (e.g., Guiné et al., 2006; Wei et al., 2011). It was not possible to determine the growth phase for the natural consortium because multiple bacterial species were present. After the bacteria pellet was removed from the growth medium, it was re-suspended in a 15 mL test tube in a wash solution of 0.02 M $MgSO_4$ adjusted to a pH of 1.5. The bacterial suspension was centrifuged (7 min at 3800 *g*) and the supernatant removed and replaced with fresh wash solution. This washing process was repeated 5 times to remove any surface-bound Zn acquired from the growth medium (e.g., Navarrete et al., 2011; Kenney et al., 2012). During the final wash, the bacterial pellet was centrifuged at 5000 *g* for 1 h and the supernatant removed. The moist mass of the bacteria pellet was recorded and the pellet was used for experiments. The effectiveness of the washing procedure was evaluated by measuring the concentrations of Zn in the supernatant solutions using an ICP-OES.

2.2. Variable pH surface adsorption experiments

Batch adsorption experiments were designed to isolate Zn complexation reactions associated with bacterial surfaces. Bacterial surfaces contain a variety of organic-acid functional group sites (phosphoryl, carboxyl, hydroxyl, and some sulfhydryl, etc., depending on the species and Gram-type) that deprotonate as a function of pH (e.g., Beveridge, 1989; Kelly et al., 2002; McClure et al., 2003; Guiné et al., 2006; Mishra et al., 2010). The deprotonated sites form surface complexes with Zn and other metals, impacting the transport and fate of metals in natural systems (e.g., Yee and Fein, 2001; Ngwenya et al., 2003; Ginn and Fein, 2008). These surface interactions are also a first step in intracellular uptake of metal (e.g., Borrok et al., 2005a). Batch adsorption experiments were conducted as a function of pH in experimental solutions with constant Zn/bacteria ratios using live cells of *B. subtilis* or the natural consortium and using live and dead cells of *P. mendocina*. Experimental component concentrations are summarized in Table 1.

Although the live cells were not thought to be actively metabolizing in the nutrient-free experimental solutions, the dead cell experiment was done to further test this possibility. In this case, *P. mendocina* cells were suspended in the experimental stock solution and treated for 12 h with UV radiation prior to the addition of Zn. We tested the effectiveness of the UV treatment by trying to culture the affected cells and no growth was observed. All batch adsorption experiments were performed in a stock solution with a 0.01 M $NaClO_4$ electrolyte. A measured volume of stock solution was amended with Zn (from an in-house Zn wire standard) and a pre-weighed bacterial pellet was suspended in the mixture. A control experiment where no bacterial pellet was added to the stock solution was also conducted to confirm that Zn did not interact with the experimental apparatus. The control experiment was subjected to the same procedures, including filtration and sample processing, that were part of the other experiments.

The final Zn and bacteria concentrations for each experiment are provided in Table 1. Thirty milliliters of the homogenized experimental stock solution were added to each of eight 50 mL reaction vessels. The pH of each vessel was individually adjusted between pH 2.5 and ~7 using 0.2 mL aliquots of dilute HNO_3 or dilute NaOH (this small volume addition had a negligible impact on Zn concentrations). The pH of the experimental solutions was measured using a Thermo Scientific Orion Star Log™ pH Meter that was calibrated prior to each experiment. The reaction vessels were placed on a shaker table for 2 h after which time the final (equilibrium) pH of each vessel was recorded. Previous work has shown that a period of 2 h is enough time for bacteria–metal surface complexes to reach apparent equilibrium (e.g., Fowle and Fein, 2000). The reaction vessels were then centrifuged and the supernatant filtered through a pre-cleaned (with 2% nitric acid) 0.45 μm nylon filter. The supernatant was preserved for later analyses by adding 0.2 mL of concentrated HNO_3. The solutions were later analyzed for their Zn concentrations using an ICP-OES and were further prepared for isotopic analysis (see below).