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Interactions between heterotrophic marine bacteria and trace metals

A Dissertation Presented

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Catherine Vogel

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The Graduate School

Catherine Vogel

We, the dissertation committee for the above candidate for the Doctor of Philosophy degree,
hereby recommend acceptance of this dissertation

Nicholas S. Fisher, Dissertation Advisor
Professor, School of Marine and Atmospheric Sciences

J. Kirk Cochran, Chairperson of Defense
Professor, School of Marine and Atmospheric Sciences

Gordon T. Taylor
Professor, School of Marine and Atmospheric Sciences

Christopher J. Gobler
Associate Professor, School of Marine and Atmospheric Sciences

Charles G. Trick
Professor, Department of Biology
University of Western Ontario

This dissertation is accepted by the Graduate School

Lawrence Martin
Dean of the Graduate School

Abstract of the Dissertation

Interactions between heterotrophic marine bacteria and trace metals

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The bioconcentration of metals in marine bacterioplankton has largely gone unexplored, even though bacterial cells represent the largest pool of living carbon in the ocean. Radioisotopes of several metals were used to investigate the magnitude of metal accumulation with different cultured bacteria, to determine the potential of metal transfer from bacteria to protistan grazers through trophic interactions, and to address the role of viral lysis on the release and bioavailability of bacterially-bound metals.

In order to quantify the accumulation of metals by marine bacteria, the radioisotopes ^{54}Mn, ^{55}Fe, ^{65}Zn, ^{109}Cd, ^{137}Cs, and ^{241}Am were used in laboratory experiments with 5 bacterial species. The bacteria were exposed to environmentally realistic metal concentrations in natural, unamended seawater. Typically, metal uptake proceeded rapidly for the first 10 to 24 h, which was often followed by a steady-state in metal accumulation. Upon reaching steady-state, the rank order of bioconcentration of metals in cells was Fe > Am $\simeq$ Mn $\simeq$ Cd > Zn > Cs. Volume concentration factors of metals were 0.6-2.8 x 10^6 for Fe, 0.6-3.5 x 10^4 for Mn and Am, 0.2-4.8 x 10^4 for Cd, 0.5-

1.6×10^3 for Zn, and 1.9×10^2 for Cs. Interspecific differences in metal uptake tended to be small and were proportional to surface:volume ratios of the cells. The Q_{10} of Zn uptake in the two bacterial species examined was about 1, suggesting passive uptake of this metal. In contrast, the Q_{10} of Mn uptake was in the range of 1.8 – 3.6, indicating active uptake and transport into the cells. Given typical bacterial biomass in surface waters, I calculate that <1% of most metals, but ~20% of Fe, should be associated with bacterial cells; these cells may serve as enriched sources of some metals for those organisms that consume them.

The radioisotopes ^{55}Fe, ^{65}Zn, ^{109}Cd, and ^{241}Am were used to assess the release of bacterially-bound metals and their bioavailability from virally lysed and unlysed cells of another bacterial species and to diatoms in experiments conducted in natural seawater. Viral lysis of radiolabeled *Vibrio natriegens* cells released more Cd and Zn into the dissolved phase than did unlysed cells, but it did not affect the release of Fe and Am from the bacterial cells. The bacterium *Pseudoalteromonas atlantica* and the diatom *Thalassiosira pseudonana* were exposed to the metals released from both lysed and unlysed bacteria, and metal accumulation was measured over 3 d. For all metals in *T. pseudonana* and for Zn and Cd in *P. atlantica*, differences in accumulation of the released metals were negligible. However, *P. atlantica* concentrated 11 times more Fe and 3 times more Am when the metals were released from unlysed bacteria compared to the lysed cells. The data indicate that viral lysis can enhance the release of at least some metals from bacterial cells to ambient seawater, but no consistent influence of viral lysis was observed on the relative bioavailability of released metals to bacterioplankton or phytoplankton.

Marine bacteria may serve as an enriched source of essential and non-essential metals for organisms that eat them. I evaluated this trophic transfer by measuring the accumulation of Fe, Zn, and Am by a planktonic ciliate isolate (*Uronema* sp.) following the ingestion of radiolabeled bacteria (*Vibrio natriegens*) in laboratory experiments. *V. natriegens* cells were allowed to accumulate ^{55}Fe, ^{65}Zn, and ^{241}Am from seawater for 5 d and were subsequently fed to the ciliates. Uptake and depuration of the metals was monitored in the ciliates over time. Measured assimilation efficiencies were 79% for Fe,

29% for Zn, and 24% for Am; corresponding efflux rates were 0.1 d^{-1} for Fe, 0.9 d^{-1} for Zn, and 0.7 d^{-1} for Am. These results suggest that bacteria present an alternate source to phytoplankton for some metals, especially Fe, to be introduced into metazoan food webs.

The accumulation of ^{55}Fe by three species of cultured heterotrophic bacteria (*Roseobacter litoralis*, *Vibrio natriegens*, and *Halomonas aquamarina*) was assessed in laboratory experiments using natural seawater from an oligotrophic ocean region by adding environmentally realistic levels of Fe in form of the radioisotope. An oxalate rinse was used to determine partitioning of extracellular and intracellular fractions of Fe. Fe accumulation proceeded rapidly over the first 10 h of the experiments, after which it approached a steady-state for all three bacterial species. Volume concentration factors varied from 1.3 x 10^6 to 7.4 x 10^7, with the highest values obtained for the bacterial species characterized by the smallest cell size and highest relative surface area. The maximum fraction of Fe removed using oxalate was 11%, indicating efficient Fe transport into the cells. Results are compared to results of Fe accumulation by the same bacterial species grown in more eutrophic natural seawater and to results on Fe uptake by naturally occurring picoplankton cells in the Equatorial Pacific Ocean.

Heterotrophic marine bacteria accumulate metals to varying degrees and can become greatly enriched in certain particle-reactive and essential elements, for example Fe. This enrichment can further result in an efficient transfer of metals to higher trophic levels upon ingestion of the bacterial cells, thereby affecting food web dynamics. Alternatively, bacterially accumulated metals may also be released back into the water column in dissolved form, for example from virally lysed cells or even from unlysed bacteria. There is some evidence that metals that are released from bacterial cells may be more bioavailable than their inorganic forms. Therefore, marine bacteria appear to play a significant role in ocean food web dynamics, metal speciation and bioavailability, as well as in the biogeochemical cycling of the metals.

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Chapter 1:

Introduction

Heterotrophic prokaryotes are the mediators for several important processes in marine ecosystems. Heterotrophic prokaryotes continually produce new cells by assimilating dissolved organic matter. They possess extracellular enzymes that enable them to hydrolyze complex organic polymers or particulate matter of various size classes. They may also facilitate the formation of phytoplankton aggregates (Grossart et al. 2006), which in turn affects the sinking flux of particulate matter to the deep ocean. One of the most crucial characteristics of these prokaryotes is that they are responsible for the remineralization of dissolved organic matter and for the recycling of nutrients that can then be assimilated by primary producers in the euphotic zone (Azam et al. 1983; Tortell et al. 1999). The effect of microbial processes on particulate matter fluxes is of interest because of the potential to transport and store surface-derived carbon at depth when particles sink. With a large degree of bacterial remineralization of biogenic particles, the flux of carbon is expected to be reduced and more of it recycled in surface water instead of being lost on sinking matter. Because of their small size and negligible weight, the sinking velocity of single bacterial cells is close to zero. Therefore, prokaryotes are directly involved in particle fluxes to the deep ocean only when they are associated with larger particles, such as marine snow, or incorporated into zooplankton fecal pellets (Azam et al. 1992). Approximately half of the total particulate organic carbon in the world's oceans is contained in marine prokaryotes (Tortell et al. 1996; Azam 1998; Hirose and Tanoue 2001). It is therefore not surprising that heterotrophic bacteria are considered to play an essential role in the carbon cycle, as well as in the cycling of essential metals and trace elements.

Most marine planktonic bacteria are Gram negative and over 80% of the bacterial assemblage are free-living cells without a strong attachment to particles (Cho and Azam 1988). In the surface waters these microorganisms oxidize organic matter and facilitate the recycling of dissolved nutrients, thereby prolonging their residence time in the surface. Bacteria are only able to assimilate low molecular weight compounds, such as free amino acids and simple sugars, which are small enough to be transported directly across their cell membranes. Molecules larger than 500-1000 Da, such as proteins and polysaccharides, cannot be transported across membranes by bacterial permeases and

need to be hydrolyzed prior to uptake with the help of extracellular enzymes (Ducklow 2000; Sherr and Sherr 2000). Little information is available on what type of dissolved organic matter bacteria preferentially hydrolyze, but there is some indication that high molecular weight organic matter may be less refractory, in general, and therefore more readily degraded than the bulk of low molecular weight organic matter (Amon and Benner 1996).

Planktonic prokaryotes are abundant in surface waters, with an average of about 10^6 cells per ml. This number can fluctuate by up to two orders of magnitude depending on depth and the type of habitat that is investigated. Nutrient-rich coastal waters and estuaries, for example, can support bacterial densities up to 10^8 cells per ml (Azam et al. 1983). It is difficult to measure bacterial carbon biomass directly, however estimates are routinely derived from observed abundances of cells and mean conversion factors of 10 to 20 fg of carbon content per cell in open ocean and coastal settings, respectively (Cho and Azam 1990; Christian and Karl 1994; Fukuda et al. 1998). A general observation is that integrated bacterial biomass equals or exceeds that of phytoplankton, except during phytoplankton blooms (Cho and Azam 1990; Li et al. 1992; Gasol et al. 1997).

Much of the research addressing the interactions between prokaryotes and metals has considered the role of terrestrial and aquatic bacteria in the remediation and transport of toxic metals and radionuclides in the environment, especially in groundwater and soils. For this purpose, bacteria are used in order to stabilize or remove toxic substances (Francis 1994). Soil and groundwater bacteria in radioactive waste repositories, for example, have been investigated for their ability to sorb actinides (Francis et al. 1998; Gillow et al. 2000). In the marine realm, the genera *Alteromonas*, *Vibrio*, *Pseudomonas*, and *Flavobacterium* have been shown to possess binding sites for thorium (Hirose and Tanoue 2001) and sorption of Pb, Cu, Ni and Zn to several strains of aquatic species has been demonstrated (Cervantes and Gutierrez-Corona 1994; Salehizadeh and Shojaosadati 2003; Savvaidis et al. 2003; Guine et al. 2006). The uptake of Cd by marine and aquatic bacteria is well demonstrated (Flatau et al. 1988; Borrok and Fein 2004), as well as the uptake of and development of resistance to Hg in coastal bacterial communities (Ramaiah and De 2003). The presence of Mn on spores of *Bacillus subtilis* that were suspended in

natural seawater has also been demonstrated (Rosson and Nealson 1982). Results based on extended X-ray absorption fine structure spectroscopy (EXAFS) illustrate that the binding process is due mainly to extracellular polymeric substances that include nucleic acids, proteins, polysaccharides, and lipids (Guiné et al. 2006).

Another research focus has been on investigating types of bacteria that are able to oxidize various metals in the marine environment, particularly manganese and cobalt. Manganese oxidizing bacteria have been shown to be important in enhancing the mineralization rate of manganese to a great degree compared to abiotic oxidation processes (Tebo et al. 2005). While Mn (II) oxidizing bacteria are not generally found in surface waters, they are very abundant at hydrothermal vents and in the boundary layers of oxic-anoxic interfaces, for example in sediments (Tebo et al. 2005). The oxidation of manganese is of importance because, once formed, the particulate oxides are capable of sorbing trace metals, as well as serving as electron acceptors during anaerobic respiration (Francis et al. 2001). Furthermore, it has been demonstrated that at least some of the manganese oxidizers are also able to oxidize cobalt, indicating a common pathway for the oxidation of both metals (Lee and Tebo 1994; Moffett and Ho 1996), although separate cobalt oxidizing species of bacteria also occur (Lee and Fisher 1993). Similarly, considerable interest exists in the microbially mediated reduction and oxidation of uranium (Francis et al. 2000; Beller 2005; Gao and Francis 2008).

While these studies focus on the interactions between bacterial cells and metals, little attention has been given to the role that marine bacteria may play in competing with phytoplankton for essential trace metals, as well as an alternate point of entry of metals into food webs. Traditionally, phytoplankton are thought of as the base of the food web and effectively concentrate metals from the aqueous phase and introduce them to animal communities (Fisher 1986). However, bacteria may also play an essential role in accumulating metals and facilitating their transfer to higher trophic levels via protozoan grazing. Dissolved organic carbon is released from members of the grazing food web (i.e. phytoplankton, zooplankton, and fish) and fuels bacterial production within the microbial loop. The bacteria in turn are ingested by other microorganisms such as ciliates as well as hetero- and mixotrophic flagellates. The microbial loop remains tightly linked to the

metazoan food web by trophic interactions, for example when copepods feed on ciliates (Fig. 1).

Prokaryotic as well as eukaryotic microbes rely on certain metals for structural and enzymatic functions. The alkali metals Ca and Mg are necessary for both functions, whereas the metals V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Mo, and W and the metalloid Se all participate in catalytic functions (Ehrlich 1997). Trace metals are required for such metabolic processes as photosynthesis, carbon fixation, nutrient assimilation, and respiration (Morel et al. 1991). Open ocean environments, such as the Equatorial Pacific, account for roughly 57.7% of the total marine primary productivity. At the same time these regions are characterized by very low concentrations of the essential micronutrients. It has become increasingly obvious in recent years that marine primary productivity is strongly affected by the availability of essential trace metals (Kirchman 2000). While necessary in small quantities for growth, some of these elements can also act as toxic inhibitors when they are present at higher concentrations (Tortell et al. 1999). In particular, much emphasis has been placed on studying the effects that Fe limitation exerts on the growth of the primary producers in regions where the major nutrients nitrogen and phosphorous are present in sufficient concentrations, as is the case in the Equatorial Pacific, the Southern Ocean and the North Pacific (Martin 1990; Martin et al. 1991). Iron, of course is also required by prokaryotes, where it is essential for the proper functioning of a variety of enzymes (Neilands 1981).

Due to their small cell size and large surface area-to-volume ratio, bacterial cells are well suited to effectively scavenge nutrients and trace elements, giving them an advantage over the larger-sized phytoplankton cells in competing for resources, especially in nutrient-limited regions (Azam et al. 1983). This is important because most of the essential trace elements are present in only very low concentrations in surface waters of the ocean and may limit the growth of planktonic organisms (Hudson and Morel 1993). Generally, the smallest planktonic cells exhibit the highest concentration factors of particle-reactive metals (Fisher and Reinfelder 1995). In addition to the advantage that their small cell size lends them, many microorganisms are also able to produce extracellular organic ligands that will chelate and increase the assimilation of

trace elements such as iron and zinc. Much emphasis has been given to a specific group of ligands, called siderophores, which are produced by marine heterotrophic bacteria and cyanobacteria in order to chelate iron (Wilhelm and Trick 1994; Tortell et al. 1999; Hirose and Tanoue 2001; Macrellis et al. 2001). It is now estimated that 20-45% of the Fe that is taken up by planktonic organisms in the subarctic Pacific region is by marine bacteria (Maldonado and Price 1999). Their ability to produce ligands enables them to influence the speciation and the solubility in seawater and reduces the bioavailability of Fe to other organisms (Tortell et al. 1996; Gledhill et al. 2004). A decrease in Fe supply and accumulation by planktonic organisms can lead to a decrease in the efficiency of the biological carbon pump, which is the mechanism by which carbon dioxide is consumed in surface waters and transported to depth in the form of sinking particulate organic carbon (fecal pellets, biogenic debris).

Prokaryotes assume a critical and major part in the biogeochemical cycles of various trace elements in the ocean, but of Fe in particular. Fe is considered to be one of the limiting factors of primary production in open ocean regions. In these areas, heterotrophic bacteria and phytoplankton (especially picoplanktonic cyanobacteria) compete with each other for access to this limiting nutrient (Tortell et al. 1996). The sorption of biologically nonessential metals to living cells also is important because it may lead to the bioaccumulation and trophic transfer of particle-reactive elements, such as lead, the actinides, and lanthanides. Interestingly, bacterial cells that are heavily pigmented are more resistant to potentially toxic metals such as zinc, mercury, and cadmium than their non-pigmented counterparts (Nair et al. 1992).

Along with predation by protists, viral lysis is an equally important factor that controls bacterial populations in natural waters (Proctor and Fuhrman 1990, Fuhrman and Suttle 1993). Both viral lysis and predation by protists establish the link between dissolved organic matter production and its recycling among planktonic organisms. Phagotrophic planktonic organisms do not directly rely on the production of ligands or the presence of dissolved metals for uptake. Instead, they accumulate essential metals through the ingestion of prey, such as bacteria. A study by Maranger et al. (1998) suggests that photosynthetic flagellates are also able to gain access to Fe by ingesting the

bacterial cells themselves, instead of accumulating Fe from the dissolved phase. Flagellates show an assimilation efficiency of about 30% for iron when they take it up by ingesting prey items instead of from the dissolved phase. Since bacteria are known to concentrate Fe very efficiently from marine waters, their flagellate predators can acquire the usually limited dissolved iron via grazing very effectively (Maranger et al. 1998). Bacteria also non-selectively sorb substances onto their cell surfaces that are not necessary for cell growth, and may thereby accumulate any element that is complexed within these substances. An example for this kind of interaction is the bioavailability and trophic transfer of humic-bound copper from bacteria to zooplankton (Lores and Pennock 1999).

While heterotrophic flagellates feed on bacteria, it is also known that some taxa of ciliates as well as naked amoebae (gymnamoeba) rely on bacterial cells as prey items. Therefore, these protists should also show metal assimilation to some extent when feeding on bacterial cultures. Although soil amoebae have been studied to some extent (Singh and Crump 1953), the exact role that amoebae play in marine systems is not well understood yet. However, this group of protists should not be underestimated because amoebae are often more abundant than other protists, such as ciliates. Naked amoebae are an important component of the planktonic community in terms of numbers, averaging 10^4 per liter in coastal waters that are nutrient- and particle-rich (Rogerson and Gwaltney 2000; Rogerson et al. 2003). More importantly, amoebae are known to feed on bacteria, in particular on bacteria that are attached to or loosely associated with particles in surface waters (Page 1980; Schuster and Levandowsky 1996; Mayes et al. 1998). The process of feeding on bacteria can therefore potentially remineralize major nutrients as well as trace metals, and also play a role in the cycling and of these elements.

Although viruses are most commonly thought of as causative agents of disease in higher organisms, their role in controlling microbial populations as well as their contribution to the process of nutrient cycling in the ocean is now well recognized. In addition to facilitating the transport of nutrients between the particulate and dissolved matter pools upon cell lysis (Suttle 2005), they also may serve as sorption sites to particle-reactive compounds themselves. In general, viruses are numerically more

abundant than bacteria in the marine environment, with average densities ranging between 10^4 to 10^8 viruses per ml (Mioni et al. 2005). Their abundance mirrors the pattern of the bacterial community in marine systems, with higher concentrations in coastal waters than in the oligotrophic open ocean, and a trend of decreasing numbers with depth in the water column (Cochlan et al. 1993; Fuhrman 1999). Because of their large numbers, viruses are an important factor in controlling the mortality of bacteria as well as cyanobacteria (Proctor and Fuhrman 1990). Approximately one quarter of marine bacteria are infected by viruses and it is estimated that at least 10% of the bacterial community is lysed on a daily basis. This is a number roughly equivalent to a loss of 2% of total primary productivity (Suttle 1994).

Given the fact that bacterial lysis is a common occurrence, viruses must be recognized for their role in the biogeochemical cycling, and their effect on the bioavailability of nutrients and trace metals in the marine environment. It has been demonstrated that viral lysis of phytoplankton cells significantly affects the partitioning and cycling of several macro- and micronutrients, including Fe, Se, C, N, and P (Gobler et al. 1997). Other studies have shown that iron associated with cell lysates is more bioavailable to heterotrophic bacteria than iron complexed into siderophores or artificial ligands (Poorvin et al. 2004; Mioni et al. 2005). In addition, viral particles themselves have been investigated for their potential to serve as nucleation sites for metals, e.g. iron, thereby affecting bioavailability of these elements as well (Daughney et al. 2004). These findings carry implications for the cycling of nutrients, as well as for the sinking flux of organic matter out of the surface waters. The nutrients and trace metals that are released from cell matter due to viral lysis most likely join the pool of recycled nutrients in the euphotic zone and are not included in the sinking flux of organic matter to the deep ocean. In addition to preventing organic matter from sinking, viral lysis also influences the efficiency of carbon transfer to higher trophic levels by eliminating prey items.

It is possible to classify different metals based on their affinities to bind with either oxygen or sulfur (Nieboer and Richardson 1980). According to this classification scheme, Class A metals exhibit a preference to bond with oxygen. These include the actinides, lanthanides, aluminum, and tin, among others. These elements are generally

particle reactive and tend to stick to the outside of cells. They do not have a biological function and therefore tend not to accumulate in the cytoplasm to a great extent (Fisher and Reinfelder 1995). Class B metals, on the other hand, show a strong affinity for sulfur and do accumulate in the cytoplasm, where they are mainly associated with proteins. Mercury and silver are included in this group. Based on their tendency to concentrate in the cytoplasm, these elements are more likely to be transferred to organisms in higher trophic levels. A large number of transition metals are also environmental contaminants, and these are grouped in the category of the "borderline metals". They exhibit characteristics of both metals classes, but do tend to associate with proteins as well and are therefore assimilated at least to some extent (Fisher and Reinfelder 1995). Their remineralization rates from biogenic material are comparable to those of organic carbon (Lee and Fisher 1992). Furthermore, they are recycled in surface waters, resulting in prolonged residence times.

In this dissertation I quantify the uptake of Mn, Fe, Zn, Cd, Am, and Cs by five different strains of prokaryotes. In addition, I examine the potential of trophic transfer of Fe, Zn, and Am upon ingestion of bacterial cells by a planktonic ciliate isolate, as well as the effects of viral lysis of bacterial cells on metal cycling and metal bioavailability of released metal. Metal quantification is expressed using volume concentration factors (VCFs), which compare a metal's enrichment in a volume of bacterial cell (or particle, in general) compared to that in an equivalent volume of seawater.

The prokaryotes used in the laboratory experiments include two *Vibrio* spp. (*V. natriegens* and a *Vibrio* sp. that was isolated from Long Island Sound, NY), *Halomonas aquamarina*, *Pseudoalteromonas atlantica*, and *Roseobacter litoralis*. All five strains are Gram-negative, heterotrophic bacteria. *H. atlantica* is a member of the family Halomonadaceae which typically consists of halophiles. However, most of these species are also able to grow in a range of temperatures and at different salinities (Akagawa and Yamasato 1989; Maruyama et al. 2000). *P. atlantica* is a member of the family Alteromonadaceae and is a pioneer colonist (Pernthaler et al. 2001). *P. atlantica* is frequently isolated from seaweed, as well as seawater, and tends to form biofilms on substrate surfaces (Yaphe 1957). *V. natriegens* and *Vibrio* sp. are commonly isolated

from coastal and estuarine waters (Oliver and Colwell 1973) and *V. natriegens* may display a generation time of approximately 10 minutes under optimal conditions (Eagon 1962). The genera *Vibrio* and *Pseudoalteromonas* (along with the genus *Shewanella*, which was not included in this study) are closely related to each other (Gauthier et al. 1995). *R. litoralis* is a pink-pigmented prokaryote that contains bacteriochlorophyll *a* and it is commonly isolated from seaweed and seawater (Shiba 1991). While the genera *Vibrio*, *Halomonas*, and *Alteromonas* are representatives of γ-Proteobacteria, the *Roseobacter* lineage consists of α-Proteobacteria, separating *R. litoralis* phylogenetically from the other four bacterial species used in the laboratory experiments.

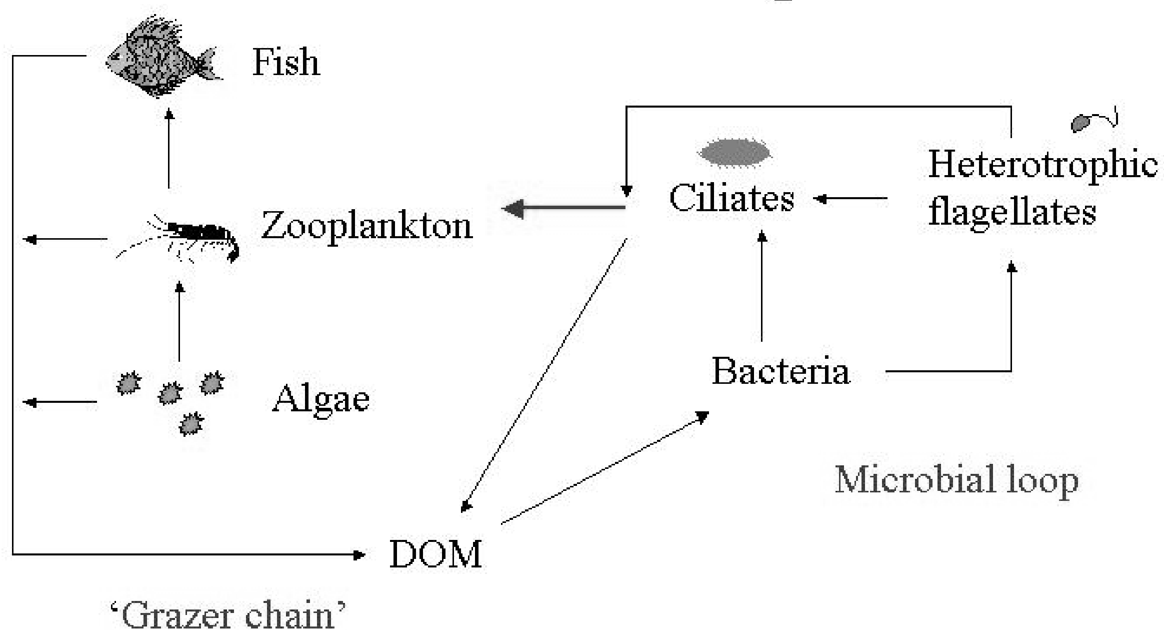

Fig. 1: Schematic illustrating the release and accumulation of dissolved organic matter (DOM) by organisms, and the link between the metazoan food web ('grazer chain') and the microbial loop. Figure is copied from http://www.esf.edu/efb/schulz/Limnology/Bacteria.html.

Chapter 2:

Metal accumulation by heterotrophic marine bacterioplankton

Abstract

The bioaccumulation of metals in marine bacterioplankton has largely remained unexplored, although these cells account for a substantial fraction of total particulate surface area suspended in marine surface waters. Radioisotopes of six metals (^{54}Mn, ^{55}Fe, ^{65}Zn, ^{109}Cd, ^{137}Cs, and ^{241}Am) were used to assess the accumulation of these metals by 5 species of heterotrophic marine bacteria in laboratory cultures exposed to environmentally realistic metal concentrations in natural seawater. Typically, uptake proceeded rapidly for the first 24 h and slowed down over the following 72-96 h. At steady-state, the rank order of bioconcentration of metals in cells was Fe > Am $\simeq$ Mn $\simeq$ Cd > Zn > Cs. Concentration factors were 0.6-2.8 x 10^6 for Fe, 0.6-3.5 x 10^4 for Mn and Am, 0.2-4.8 x 10^4 for Cd, 0.5-1.6 x 10^3 for Zn, and 1.9 x 10^2 for Cs. Rinsing with oxalate did not result in significantly lower Fe concentrations, but rinsing the cells with EDTA resulted in significantly lower concentration factors for Mn and Am in one species. Interspecific differences in metal uptake were small under the conditions tested and were proportional to surface:volume ratios of the cells. The Q_{10} of Zn uptake in two bacterial species examined was about 1, suggesting passive uptake. The Q_{10} of Mn uptake was in the range of 1.8 – 3.6, indicating active uptake and transport into the cells. Given typical bacterial biomass in surface waters, I calculate that <1% of most metals, but ~20% of Fe, should be associated with bacterial cells; they may serve as enriched sources of some metals for those organisms that consume them.

Introduction

Although much is known about how bacteria respond to metals (Beveridge and Doyle 1989; Fenchel et al. 1998), and how bacteria can be utilized for remediation purposes of metal-contaminated soils and groundwater systems (Francis 1994; Gillow et al. 2000; Valls and De Lorenzo 2002), the bioaccumulation of metals by heterotrophic marine bacterioplankton remains relatively little studied. Bacteria account for roughly half of the particulate organic matter in the world's oceans (Cho and Azam 1988; Fuhrman et al. 1989), and can represent an estimated 80 to 90% of bio-surface in the euphotic zone (Azam 1984; Fuhrman et al. 1989). Consequently, it may be expected that particle-reactive metals, including essential metals like iron and non-essential metals like lead, would associate with these cells.

Previous studies indicate that Cu (Rossi and Jamet 2008) and Cd (Dixon et al. 2006) become associated with bacterial cells by surface sorption and that the magnitude of association depends on the ambient metal concentrations in seawater. Certain metals, for example Zn, appear to enhance the growth of heterotrophic picoplankton in coastal marine systems, whereas toxic metals like Pb, reportedly exert a negative effect on picoplankton growth (Caroppo et al. 2006). While these studies illustrate that marine bacteria interact with metals, and may even be used for remediation purposes of polluted sediments (Iyer et al. 2005; Beolchini et al. 2009) there are relatively few measurements quantifying how much bacterial cells are enriched in metals relative to ambient seawater: Tortell et al. (1996) highlighted the importance of heterotrophic bacteria in the geochemical cycling of Fe by demonstrating that these cells contain more Fe per biomass than phytoplankton cells. Lee et al. (1993) reported that marine bacteria greatly concentrate Th due to the large surface area they present for metal sorption, and Chen et al. (2008) demonstrated that competitive interactions between Cd and Zn sorption on marine bacteria may exist under certain situations.

Once associated with bacteria, these cells can change the speciation of some metals, (e.g., via methylation, oxidation/reduction, and other processes) and thereby affect their subsequent behavior and biological interactions in aquatic systems. Experimental evidence suggests that Fe accumulated by bacteria may be more

bioavailable to planktonic organisms upon being released from the bacterial cells due to viral lysis than inorganic or artificially chelated forms in seawater (Poorvin et al. 2004; Mioni et al. 2005). It appears that these biological interactions may affect metal speciation and therefore lead to changes in the bioavailability of at least some metals. In addition, of course, bacterially mediated remineralization of organic matter can affect the geochemical cycling of metals associated with that organic matter, including sinking biogenic debris in the oceanic water column (Cho and Azam 1988). Furthermore, although phytoplankton are generally thought to serve as the base of most marine food webs, bacteria can also assume this position, including in the "microbial loop" of many surface waters, for example in oligotrophic ocean regions. Thus, bacteria may serve as highly enriched sources of such metals as Fe (Tortell et al. 1996; Tortell et al. 1999) for diverse organisms that feed on them (Sherr and Sherr 1994; Maranger et al. 1998; Vogel and Fisher 2009), and possibly for animals higher up in the food chain.

To better evaluate the extent to which metals can be bioaccumulated by bacterioplankton in the sea, I have quantified the uptake of six different metals (Fe, Mn, Cd, Zn, Cs, and Am) by cultures of Gram-negative, heterotrophic marine bacteria in controlled radiotracer experiments that were conducted in natural seawater. The metals were chosen to reflect different particle reactivity and to contrast essential and nonessential metals. The working hypothesis was that particle- reactive and essential metals should be accumulated to a greater extent than nonessential metals, or metals that are less particle-reactive in seawater (e.g., Cs). In addition, we expected to see a difference in the localization of the metals based on these characteristics as well, with essential metals being located intracellularly to a higher degree than the nonessential metals. The bacterial species chosen for this work commonly occur in coastal and open ocean regions.

Materials and methods

Five species of Gram-negative, heterotrophic marine bacteria were used to determine the accumulation of diverse metals from natural seawater that was not amended with any additional nutrients or chelators. The bacterial species and some of their properties, including strain designations and isolation sites, are listed in Table 1. The five species chosen have all been isolated as free-living cells from seawater, and many of them are also known to occur attached to surfaces such as seaweed and sediments. *Roseobacter litoralis*, *Pseudoalteromonas atlantica*, and *Halomonas aquamarina* were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), and cultures of *Vibrio natriegens* and *Vibrio* sp. were supplied by G. Taylor, Stony Brook University. All bacterial species were obtained and handled as pure cultures. *V. natriegens*, *Vibrio* sp, *H. aquamarina*, and *P. atlantica* are members of the gamma-proteobacteria, and *R. litoralis* is an alpha-proteobacterium. Members of the *Roseobacter* clade are ubiquitous and can comprise up to one quarter of naturally-occurring bacterial communities in some settings (Gonzalez and Moran 1997; Wagner-Dobler and Biebl 2006).

All seawater used for culturing and experimental purposes was collected from surface waters approximately 8 km off Southampton, New York. Stock bacterial cultures were stored on marine agar slants (1 L seawater, 5 g Bacto peptone, 1 g yeast extract) at 4°C for maintenance and were transferred periodically. In preparation for experiments, a small inoculum of cells was aseptically transferred into 10% liquid marine broth (1 L seawater, 0.5 g Bacto peptone, 0.1 g yeast extract) and the cells were allowed to grow in trace metal-cleaned glass flasks for several days until the cells were in log phase. The flasks were stirred periodically to prevent cells from aggregating and sinking. Liquid growth medium was microwave-sterilized before adding bacterial cells. Prior to inoculating experimental flasks with bacteria, aliquots of known cell density were centrifuged at 1,400 *g* for 10 min, and the resulting cell pellet was rinsed with sterile, 0.2-µm filtered seawater 3 times. Bacterial cell samples were fixed with 2% borate buffered formaldehyde and the filtered (0.2 µm) samples were processed according to the DAPI (4'6-diamidino-2-phenylidole) staining method described in Sherr et al. (2001), using

epifluorescence on an inverted Leica DM IRB microscope under UV light excitation. Cell counts were obtained in this manner for all inoculations and sample times.

The concentrations of Mn, Fe, Zn, Cd, and Cs in the 0.2 μm-filtered coastal surface seawater used for these experiments, measured by ICP-MS, were 5 nM for Mn, 14 nM for Fe, 134 nM for Zn, 1 nM for Cd, and 2 nM for Cs. Background concentrations of Am in the seawater used for these experiments were not measured but are expected to be approximately 10^{-21} M (Cochran et al. 1987).

The experiments were conducted using sterile 300-ml polycarbonate tissue culture flasks with screw caps. To trace the biological uptake and partitioning of metals, radioisotopes were used, including the beta emitter ^{55}Fe ($t_{½}$ = 996 d) obtained from NEN/Perkin Elmer Life Sciences, and the gamma emitters ^{241}Am ($t_{½}$ = 433 y) from Amersham, ^{65}Zn ($t_{½}$ = 245 d) from DOE Brookhaven Lab, ^{109}Cd ($t_{½}$ = 462 d) from DOE Los Alamos, ^{54}Mn ($t_{½}$ = 313 d) from Perkin Elmer Life Sciences, and ^{137}Cs ($t_{½}$ = 30 y) from Amersham. All radioisotopes were examined in separate experiments. After addition of the isotopes to the seawater, they were allowed to equilibrate for 12-24 h before adding the bacteria. Since no artificial chelators were used, the speciation of the metals, which was not determined, was assumed to be representative of that which would occur in this seawater containing naturally occurring organic ligands. The bioaccumulation of the metals therefore reflects that which would occur in this water regardless of speciation patterns, and makes no assumptions about which form of metal is most bioavailable.

Initial cell densities of bacteria were 1-6 x 10^6 cells ml^{-1} for *V. natriegens*, *Vibrio* sp., *H. aquamarina*, and *R. litoralis*, and 1-7 x 10^6 cells ml^{-1} for *P. atlantica*. The bacterial inocula were within the range of typical bacterial abundances in surface seawater (Meyer-Reil 1982; Ammerman et al. 1984). Simultaneously, control flasks containing filtered seawater and radioisotopes but without added bacterial cells were established to quantify metal sorption to flask walls and to the membrane filters used to harvest the bacteria after different periods of radionuclide exposure. The cultures, including controls, were incubated in darkness between removing samples at 17°C for 3-5 d and the experimental flasks were swirled periodically to ensure an even distribution

of cells and to prevent localized metal depletions. Samples were removed periodically to determine fractionation of the different metals between the dissolved (< 0.2 µm) and particulate phases (Fisher et al. 1983). Typically, 10 ml was removed from each flask at each sample time and passed through a 0.2 µm Nuclepore polycarbonate membrane to determine the radioactivity associated with the bacterial cells (particulate phase). The filtered samples were rinsed with 10 ml of unradioactive filtered seawater to remove any droplets containing radioactivity from the particles and filter. An additional 1-ml sample was removed at each time to determine cell growth, as described above. Another 1-ml of unfiltered water was pipetted from each flask into a separate radioactivity counting tube to determine the total radioactivity (dissolved plus particulate) in the "water column" of each flask. For each metal radioisotope, the metal associated with bacteria was determined by subtracting the radioactivity caught on filters from control flasks (containing filtered water but not bacteria) from the radioactivity on filters containing bacterial cells. The fraction of total "water column" metal that was associated with the cells was determined by dividing the radioactivity associated with bacteria in 1 ml of water by the total radioactivity (water plus particles) in the water. Concentration factors, expressed on a volume/volume basis for each combination of metal and bacterial species, were determined by dividing the radioactivity μm^{-3} of bacteria (taking into account the number of bacterial cells ml^{-1}) by the radioactivity μm^{-3} in the dissolved phase (< 0.2 µm) in ambient water (Fisher et al. 1983). The fraction of total metal bound to cells was, in turn, converted to moles of metal by using the specific activity of the radioisotope. It was assumed that the radioactive isotopes fractionated in the same manner as the respective stable metals in the seawater, and that both stable and radioactive isotopes were accumulated by the bacterial cells to the same extent. Metal sorption to the walls of the experimental vessels was determined for each isotope over the course of the experiments. Loss of radioisotope to the polycarbonate flask walls was measured as the decline in unfiltered water radioactivity over time; this was checked by acid washing the flask walls at the end of the experiments and measuring the radioactivity of the acid. Loss to the flask walls was taken into consideration when calculating metal concentrations in the bacterial cells.

Metal concentrations in different experiments differed somewhat for some metals, depending on the species. For Am, taken from a solution in 3N HNO_3, additions were 0.48 nmol L^{-1} (14.6 kBq L^{-1}) and 0.69 nmol L^{-1} (20.8 kBq L^{-1}), for Zn (in 0.1 N HCl), additions were 0.83 nmol L^{-1} (50 kBq L^{-1}) and 1.7 nmol L^{-1} (62.4 kBq L^{-1}), for Fe (in 0.5 N HCl), additions were 0.2 nmol L^{-1} (16.7 kBq L^{-1}), for Mn (in 0.5 N HCl), additions were 2.2 nmol L^{-1} (23.8 kBq L^{-1}) and 1.9 nmol L^{-1} (17.7 kBq L^{-1}) , for Cd (in 0.1 N HCl), additions were 0.17 nmol L^{-1} (20.1 kBq L^{-1}) and 0.1 (16.7 kBq L^{-1}), and for Cs (in CsCl), additions were 0.1 nmol L^{-1} (12.3 kBq L^{-1}). The seawater that received isotopes was first neutralized with an equimolar concentration of NaOH to offset the small amount of acid added with the radioisotopes. We confirmed that the pH in the flasks after radioisotope addition was 8.0 $\pm$ 0.1.

Taking the background metal concentrations in the seawater into account, the calculated specific activities (kBq $nmol^{-1}$) of the radioisotopes in the seawater to which the cells were exposed in individual experiments were 2.6-3.3 for Mn, 0.37-0.46 for Zn, 15.2-17.2 for Cd, 1.2 for Fe, 5.9 for Cd, and 30.7 for Am.

For Fe, an oxalate rinse (Tovar-Sanchez et al. 2003) was used to remove extracellular Fe from the bacteria. In this experiment, particulate samples were collected at each time with either a filtered seawater rinse or with the oxalate reagent in addition to the seawater rinse to remove sorbed Fe. The two particulate sample types were taken to represent total iron associated with cells and intracellular iron, respectively. For Zn, Am, and Cd an EDTA (ethylenediaminetetraacetic acid) rinse at 10^{-4} M concentration was used in the same manner as the oxalate rinse described above to remove loosely bound metals from cell surfaces (Fisher et al. 1984; Hassler et al. 2004).

For Zn and Mn, experiments were also run at 2°C, in addition to the regular treatment at 17°C. This "cold" treatment was used to estimate passive, rather than active uptake of metals by the cells by lowering the metabolic rate of the cells to a minimal level. I chose this treatment, rather than killing the bacteria with antibiotics or with heat, in order to preserve the cellular membrane structure as much as possible and to avoid metal complexation by antibiotic compounds. Based on differences in uptake rates

between the two temperatures, the temperature coefficient (Q_{10}) was calculated for both metals according to the following equation:

$$Q_{10} = (R2/R1)^{(10/(T2-T1))} .$$

For Mn, Fe, Zn, Cd, and Am uptake rates expressed as amol $cell^{-1}$ min^{-1} were calculated for the bacteria that were rinsed with either oxalate (Fe) or EDTA (Mn, Zn, Cd, and Am) to determine whether the rates differ among the metals tested. For Fe, the uptake rate was assessed over the first 4 h of the experiment, for Mn and Zn over the initial 2 h, and for Cd and Am over 12 h. Uptake rates were also normalized to cell weight and expressed as mol g^{-1} min^{-1}. Metal uptake rate constants were calculated for each metal by taking the total metal concentration that the bacterial cells were exposed to into consideration.

Samples containing gamma emitters were counted on an LKB Wallac 1282 Compugamma with a well-type NaI(Tl) detector, and a Packard Tri-Carb 2100 TR Liquid Scintillation Analyzer was used for samples containing the beta emitter ^{55}Fe. Counting times were adjusted to yield propagated counting errors < 5% and all samples were decay-corrected. Gamma emissions of ^{54}Mn were determined at 834 keV, ^{65}Zn at 1115 keV, ^{109}Cd at 88 keV, ^{137}Cs at 662 keV, and ^{241}Am at 60 keV.

Results

Metal sorption to the walls of the experimental vessels was undetectable for ^{137}Cs, 1.5 ± 0.2% of total ^{65}Zn, 4.1 ± 0.8% of ^{54}Mn, 6.7 ± 2.0% of ^{109}Cd, 21.6 ± 2.2% of ^{241}Am, and 22.5 ± 2.3% of ^{55}Fe.

For all metals and bacterial species analyzed, metal accumulation occurred relatively rapidly over the first several hours (0 to approx. 12 h), followed by a slower rate of accumulation during which an apparent steady-state was sometimes reached with respect to metal partitioning between dissolved and particulate phases (Figs. 2-8).

Fe uptake by *R. litoralis*, *V. natriegens*, and *H. aquamarina* resulted in a maximum percentage of Fe taken up by the cells of 50 to 80% of the total Fe added (Fig. 2). Except for *V. natriegens*, Fe uptake increased steadily over the time of the experiment. For *V. natriegens*, the maximum percentage of Fe associated with cells was measured between 4 and 10 h (approx. 60%), after which the percentage of Fe on the cells leveled off at approx. 40%. The same trend is reflected in the concentration of Fe $cell^{-1}$, expressed in amol of accumulated ^{55}Fe per cell. For *V. natriegens*, the final concentration of Fe was 0.55 ag $cell^{-1}$ (Table 2), with a sharp increase in Fe concentration in the first 4 h, and a subsequent release thereafter. Although a slight decrease in cellular Fe concentration after 12 to 24 h was observed for *R. litoralis* and *H. aquamarina* as well, it was much less pronounced than for *V. natriegens*. The final cellular Fe concentration was 0.56 amol for *H. aquamarina* and 1.04 amol for *R. litoralis* (Table 2).

The pattern of cellular Fe concentrations was the same when the cells were treated with an oxalate rinse that removed loosely adhering Fe from bacterial surfaces. Most of the Fe accumulated by the three species of bacteria was not removed after rinsing the cells with oxalate (Table 2), indicating that a large fraction of the Fe was located intracellularly. The largest fraction was removed from *V. natriegens* at 10 h, when the oxalate rinse resulted in approx. 18% less Fe on the cells (Fig. 2). However, in most cases the percentage of Fe removed using the rinse was $< 5\%$. Volume concentration factors (VCFs) of Fe for all treatments are reported as the average value calculated between 10 to 52 h, and were highest for *R. litoralis*, with and without oxalate rinsing (2.8×10^6 and 2.7×10^6, respectively). Concentration factors were slightly lower for *V. natriegens*, with a VCF of 9.9×10^5 for unrinsed cells and 6.0×10^5 for the oxalate rinsed cells. No difference in VCFs was observed between the two oxalate treatments for *H. aquamarina.* Final cellular Fe concentrations in the oxalate-rinsed cells were 0.48 amol for *V. natriegens*, 0.66 amol for *H. aquamarina*, and 0.91 amol for *R. litoralis* (Table 2). The VCF for Fe in *Vibrio* sp. in a separate experiment was 7.9×10^5 (Table 2).

Mn uptake, examined in the species *R. litoralis* and *P. atlantica* with and without an EDTA rinse (Fig. 3) showed that mean VCFs of Mn were 3.3×10^4 without the rinse and 2.7×10^4 with the rinse in *R. litoralis*, and 7.4×10^3 and 6.9×10^3 for unrinsed and

rinsed cells, respectively, for *P. atlantica* (Table 2). In a separate experiment, Mn uptake was monitored for 96 h in the species *R. litoralis*, *P. atlantica*, and *H. aquamarina* at 17°C and 2°C. At 17°C, the percentage of Mn associated with cells was approx. 3 % for *P. atlantica*, 6% for *R. litoralis*, and approx. 25% for *H. aquamarina*, after the initial rapid sorption of about 12 h (Fig. 4). *R. litoralis* and *P. atlantica* showed significantly ($P < 0.05$) higher Mn concentrations on a per cell basis at the end of the experiment than at the beginning, with an average of 1.98×10^{-2} amol Mn $cell^{-1}$ for *R. litoralis* and 2.76×10^{-2} amol Mn $cell^{-1}$ for *P. atlantica* at 96 h. VCFs of Mn were 3.8×10^4 and 2.2×10^4 for *R. litoralis* and *P. atlantica*, respectively. In comparison, VCFs for these two species grown at 2°C were 1.8×10^4 for *R. litoralis* and 1.4×10^3 for *P. atlantica* (Table 2). This trend is also reflected in the Mn concentration $cell^{-1}$, which was approx. 1.08×10^{-2} amol in *R. litoralis* and 0.07×10^{-2} amol in *P. atlantica* at the end of the experiment. The percentage of Mn associated with cells at the colder temperature was lower for all three species, at 2-3% for *R. litoralis*, 4-7% for *H. aquamarina*, and close to zero for *P. atlantica*. Uptake of ^{54}Mn over the first 24 hours at 17°C and 2°C indicated a Q_{10} for Mn accumulation of 1.8 for *R. litoralis* and 3.6 for *P. atlantica*. VCFs for *H. aquamarina* are not reported here because a large portion of the cells formed filaments of various lengths, rather than being distributed as single cells. The formation of filaments was only observed for this species, and only when exposed to Mn.

The accumulation of Am in *R. litoralis, V. natriegens* and *H. aquamarina* over 120 h with or without EDTA rinsing showed that the percentage of Am on cells not treated with EDTA ranged from approx. 6% for *V. natriegens* to 14% for *R. litoralis* and 20% in *H. aquamarina* at the end of the experiment (Fig. 5). Rinsing the cells with EDTA removed a relatively small fraction of Am in the cell samples taken before 48 h, but a large decrease in metal associated with the cells of *R. litoralis* and *H. aquamarina* was observed between 48 h and the end of the experiment. During this time interval, Am concentrations decreased by approx. 0.005 amol $cell^{-1}$. Cells that were not rinsed did not show this decrease in Am concentration over time. The volume concentration factor for Am in EDTA-rinsed cells was only significantly smaller for *R. litoralis* and did not change significantly for *V. natriegens* or *H. aquamarina* (Table 2). These values are

averages and do not include the large decrease in metal concentration observed in the EDTA rinsed cells of *H. aquamarina* and *R. litoralis*. The decrease in cellular Am concentrations over time, when the EDTA rinse was applied, coincided with a decrease in bacterial growth rate. Replicates of *H. aquamarina* exhibited a mean specific growth rate of 1.11 d^{-1} in the first 24 h and of 0.03 d^{-1} after 24 h in the experiment. For *R. litoralis*, the mean growth rates were 0.85 d^{-1} in the first 24 h, and 0.09 d^{-1} thereafter.

In a separate experiment, the average Am VCF for *Vibrio* sp. was 1.0×10^4, and 3.2×10^4 for *P. atlantica* (Table 2). Final cellular concentrations of Am varied from approx. 0.17×10^{-2} amol for *P. atlantica* and *Vibrio* sp. to 1.00×10^{-2} amol Am $cell^{-1}$ for *H. aquamarina* (Table 2).

Accumulation of Cd with and without EDTA rinses over 120 h for *H. aquamarina*, *R. litoralis*, and *V. natriegens* showed that approx. 17% of the added isotope was associated with cells of *H. aquamarina* whereas only 2% was found associated with *R. litoralis* and *V. natriegens* (Fig. 6). Similar to the pattern observed in Am accumulation, the EDTA-rinsed cells of *H. aquamarina* and *R. litoralis* displayed a large drop in Cd concentration after 48 h. Cellular Cd concentrations in the EDTA-rinsed cells decreased by 0.006 amol $cell^{-1}$ for *H. aquamarina* and by 0.001 amol $cell^{-1}$ for *R. litoralis* after 48 h. Prior to this decline, Cd VCFs for *H. aquamarina* were 4.6×10^4 for unrinsed cells and 4.8×10^4 for EDTA-rinsed cells. Similarly, VCFs for *R. litoralis* were 1.4×10^4 and 1.3×10^4 for unrinsed and rinsed cells, respectively. VCFs for *V. natriegens* were 2.0×10^3 for unrinsed cells and 2.1×10^3 for EDTA-rinsed cells (Table 2). In a separate experiment, the Cd VCF for *Vibrio* sp. was found to be 2.5×10^3 after 12 h (data not shown).

Experiments addressing Zn accumulation in *R. litoralis* and *P. atlantica*, with and without an EDTA rinse, showed that only a small fraction of the radioactive Zn was accumulated, with the maximum percentage of Zn on cells of approx. 0.4% in *P. atlantica*. The EDTA rinse resulted in a decline of 26% of cellular Zn (Table 2) in *P. atlantica*. This effect was more difficult to observe with *R. litoralis* because of the very small percentage of Zn accumulation, and relatively large errors. Zn concentration factors were 1.7×10^3 and 1.6×10^3 for *P. atlantica* in samples without and with the EDTA rinse,

respectively. Zn concentration factors for *R. litoralis* were 0.7 x 10^3 without the rinse and 0.5 x 10^3 with the EDTA rinse (Table 2). In a separate experiment, the percentage of Zn associated with cells ranged from approx. 1.2% for *P. atlantica* to 1.6% for *H. aquamarina* and 5% for *Vibrio* sp. (Fig. 7). The accumulation of Zn by the cells seemed to reach steady-state at 6 h, after which no significant increase in the particulate fraction was observed. Individual unrinsed cells were estimated to contain approx. 0.09 amol Zn $cell^{-1}$ for *Vibrio* sp., 0.13 amol $cell^{-1}$ for *H. aquamarina*, 0.03 amol $cell^{-1}$ for *P. atlantica* and 0.01 amol $cell^{-1}$ for *R. litoralis* at the final sample time (Table 2). Zn accumulation in *R. litoralis* and *P. atlantica* was also tested at 2°C and 17°C and was lower for both species at the colder temperature. Q_{10} values calculated over the first 24 hours were 0.8 and 1.3 for *R. litoralis* and *P. atlantica*, respectively. Concentration factors were 1.2 x 10^3 and 7.12 x 10^2 for *R. litoralis*, and were significantly different at 2.9 x 10^3 and 1.8 x 10^3 for *P. atlantica* for cells held at 17°C and 2°C, respectively (Table 2).

Cesium accumulation in *V. natriegens* and *Vibrio* sp. over a 96-h period was much less than that of other metals, with the maximum percentage of Cs associated with cells reaching only about 0.6% for both species (Fig. 8). The cellular ^{137}Cs concentrations were less than 7.21 x 10^{-5} amol $cell^{-1}$ and the average VCFs calculated at 24 h were 1.9 x 10^2 for both species.

Metal uptake rates that were calculated for four of the metals were highest for Fe, with a mean uptake rate of 0.011 amol $cell^{-1}$ min^{-1} for *V. natriegens* and 0.007 amol $cell^{-1}$ min^{-1} for *H. aquamarina* in the oxalate-rinsed cells. For Mn, uptake rates in the EDTA-rinsed cells ranged from 3.08 x 10^{-5} for *P. atlantica* to 9.35 x 10^{-5} amol $cell^{-1}$ min^{-1} for *R. litoralis*. For Zn, the rate was 2.22 x 10^{-5} for *R. litoralis* and 1.61 x 10^{-5} amol $cell^{-1}$ min^{-1} for *P. atlantica*. Results were lowest for Cd and Am in the bacteria rinsed with EDTA. Uptake rates for Cd ranged from 9.35 x 10^{-6} for *H. aquamarina* to 1.29 x 10^{-6} for *V. natriegens*, to 1.33 x 10^{-6} amol $cell^{-1}$ min^{-1} for *R. litoralis*. Uptake rates obtained for Am were similar, with 2.95 x 10^{-6} for *H. aquamarina*, 5.19 x 10^{-7} for *V. natriegens*, and 2.41 x 10^{-6} amol $cell^{-1}$ min^{-1} for *R. litoralis.* Uptake rates normalized to cell weights (mol g^{-1} min^{-1}) follow the same trend as the above rates expressed in amol $cell^{-1}$ min^{-1} and are listed in Table 3. Uptake rate constants (u) were considerably higher for Fe than for any

of the other metals included and follow the order of Fe >> Mn > Am ≥ Cd > Zn (Table 3). Within a given metal, uptake rate constants tend to be higher for the smaller bacterial cells, i.e. *R. litoralis*, except for Cd, for which *H. aquamarina* has the highest rate constant.

Discussion

Overall the affinity of metals for bacterial cells, as reflected by the concentration factors, followed the order: Fe > Am ≃ Mn ≃ Cd > Zn > Cs. Partition coefficients (K_d values) of these metals for marine sediments show generally comparable relative reactivities: Fe (3×10^8) > Am (2×10^6) = Mn (2×10^6) > Zn (7×10^4) > Cd (3×10^4) > Cs (4×10^3) (Iaea 2004), with Cd being the exception. The concentration factors of all metals varied somewhat between bacterial species, and this is most likely attributable to cell size. Indeed, the correlation coefficient of VCFs for Am (a surface-reactive metal) among bacterial species related to their cell surface to volume ratios (Table 1) gave a value of 0.88, consistent with the fact that this metal's uptake by cells is dominated by adsorption to cell surfaces. Generally, VCFs were greatest in *R. litoralis*, the smallest cell with the largest surface to volume ratio (Table 1). The exception to this trend was accumulation of Cd and Zn, where *H. aquamarina* was the species with the highest concentration factors. Generally, concentration factors for metal sorption to phytoplankton cells also correlate with surface to volume ratios (Fisher and Reinfelder 1995). The high particle reactivity of the metals observed here for bacterial cells, with the notable exception of Cs, is similar to observations for abiotic (sediment) particles and phytoplankton (Nyffeler et al. 1984; Fisher and Reinfelder 1995).

It has been shown that various marine bacteria and bacterial spores can bind and oxidize certain metals, including Mn (Nealson 1978; Rosson and Nealson 1982; Francis et al. 2001), Fe (Ghiorse 1984) and Co (Lee and Fisher 1993; Lee and Tebo 1994). While

we did not directly measure the oxidation or precipitation of Mn, we did observe a significant accumulation of this metal by the bacterial species we used in the current study. Bacterially precipitated Mn is often found associated with extracellular polysaccharides that are embedded in the outer cell membrane (Ghiorse and Hirsch 1979). However, not all cell-bound Mn is necessarily oxidized (Rosson and Nealson 1982). *P. atlantica* and *R. litoralis* accumulated significantly less Mn per cell at 2°C than at 17°C (Q_{10} values 1.8-3.6), suggesting that Mn accumulation by these species is at least in part an active uptake process requiring energy expenditure by the cells, as seen previously with other bacteria (Ehrlich 1983; Ehrlich and Salerno 1990). This is also supported by Lee and Fisher (1993) who demonstrated that microbial activity increased Mn and Co oxidation, and that oxidation is temperature-dependent. Previously published volume concentration factors for Mn are 10^3 for *Synechococcus* sp. (Fisher 1985), or 7 times less than the VCF for *P. atlantica* and 33 times less than the VCF for *R. litoralis*.

We are not aware of other published concentration factors for Am in marine bacterioplankton. As a highly particle-reactive, trivalent non-essential metal, Am can be used to illustrate the sorption of other particle-reactive metals, such as the lanthanides and other actinides, in seawater. Concentration factors for another particle-reactive, non-essential metal, Th, in natural marine bacterioplankton assemblages are on the order of 10^6 (Lee et al. 1993), and several species of Gram-negative marine bacteria are thought to produce ligands that aid in Th sequestration (Hirose and Tanoue 2001). The accumulation of Am by *Synechococcus* sp. resulted in a concentration factor of 6×10^5 (Fisher 1985), and $1 - 6.9 \times 10^5$ for a variety of phytoplankton cells (Fisher et al. 1983), both of which are higher than I observed for the bacterial cells exposed to the metal, despite the higher surface to volume ratios of the latter.

The uptake of Cd by marine bacteria has previously been investigated (Flatau et al. 1988; Iyer et al. 2005), and it has been shown that its accumulation is energy-dependent and that it occurs through the manganese transport system in at least some species (Laddaga et al. 1985). Phytoplankton and bacteria transport Cd into their cells where it sometimes replaces Zn when this metal is available in limiting amounts (Price and Morel 1990). Keung et al. (2008) demonstrated that the Gram-positive

Bacillus firmus tends to internalize most of its Cd, in contrast to Zn which is more prevalent than Cd on bacterial cell surfaces, as we showed as well. The pronounced decline (approx. half) in cellular Cd after 48 h in the EDTA-rinsed cells may indicate that the Cd was pumped out of the cells due to its toxic effects (Silver 1998). Previous studies (Flatau et al. 1988; Chen et al. 2008; Keung et al. 2008) investigated Cd uptake on much timescales of only hours, and this may influence the uptake and efflux kinetics observed. The growth stage of bacterial cells also may influence the degree of metal accumulation. Chang et al. (1997) and Daughney et al. (2001) noted that Cd uptake of *Pseudomonas aeruginosa* and *Bacillus subtilis* was greatest when cells were in exponential growth phase. Mean concentration factors of Cd for marine phytoplankton are about 10^3 (Fisher and Reinfelder 1995), 2-50 times lower than the values for the bacteria in our experiments.

Iyer et al. (2005) attributed Cd accumulation by the marine bacterium *Enterobacter cloaceae* to its binding by extracellular polysaccharides (also seen for Cu and Co). Binet et al. (2003) also showed that Cd (and Zn) has a tendency to attach to cellular proteins. Boyanov et al. (2003) and Beveridge and Murray (1980) noted that Cd binding on *B. subtilis* cells wall at pH values in the range of seawater occurs mostly with carboxyl and phosphoryl sites.

The apparent binding of Zn to bacterial cell surfaces was also seen in Gram-negative cells dominated by external lipopolysaccharides (Beveridge and Koval 1981; Guine et al. 2006). This is consistent with Q_{10} values near 1 for Zn accumulation in *R. litoralis* and *P. atlantica*, suggesting passive sorption to cell surfaces, possibly followed by transport into the cells. The cytological site of metal deposition can ultimately influence the extent to which that metal is transferred to the next higher trophic level, where assimilation efficiencies of ingested metals in consumers are generally directly proportional to the intracellular metal in the prey (Reinfelder and Fisher 1991), with some exceptions where it has been shown that the type of prey can determine the subcellular metal distribution in the consumer (Cheung et al. 2006). Previously published concentration factors for Zn in *Synechococcus* sp. and other phytoplankton are approx. 3×10^4 (Fisher 1985; Fisher and Reinfelder 1995), about two times higher than the highest

Zn concentration factor we measured in bacteria. The lower uptake of Zn than of Cd by bacteria is in contrast to results by Savvaidis et al. (2003) who reported that the Zn and Cd sorption to cells walls of *Pseudomonas cepacia* was comparable.

Fe is an essential metal that is also particle-reactive, thus explaining the relatively large percentage (> 50%) of this metal found associated with bacterial cells in our experiments and the intracellular incorporation of Fe (only a small fraction was removable with oxalate rinsing). The use of the oxalate rinse is supported by Tang and Morel (2006) who demonstrated that this reagent is effective in removing freshly precipitated Fe. This strong bacterial affinity for Fe is consistent with observations that about half of the Fe that is incorporated in organisms in the open ocean is associated with bacterial cells, and that heterotrophic species accumulate a major fraction (20-70%) of the available Fe in some ocean regions (Tortell et al. 1996; Maldonado and Price 1999; Tortell et al. 1999). Generally, Fe:C uptake rates are highest in the bacterial size class (Schmidt and Hutchins 1999) compared to other planktonic organisms. Many bacteria are able to produce siderophores, which are specialized Fe chelators that render them very efficient at scavenging this essential metal. Siderophore production is usually enhanced when the supply of biologically available Fe is low (Garibaldi and Neilands 1956; Neilands 1983), which further illustrates the competitive advantage that bacteria have in acquiring Fe.

Cs is a nonessential metal that can be enriched in fine-grain clay sediments (Hesslein et al. 1980; Charlesworth et al. 2006), but one that is not very reactive with respect to biological particles, particularly in seawater. Consistent with the low VCFs in bacteria, Heldal et al. (2001) found that VCFs of Cs in marine phytoplankton were $< 10^2$. Cs is accumulated to a somewhat greater extent in freshwater (Twiss and Campbell 1998) than in seawater, probably due to differences in K concentration.

Results from metal uptake rates calculated for Mn, Fe, Zn, Cd, and Am indicate that Fe is accumulated fastest, followed by Mn, Am, Cd, and Zn. Uptake rate constants for Fe were considerably higher than for the other metals, supporting the above described results of fast uptake by this metal. While these results could be interpreted in a way that essential metals (Zn being the exception) are characterized by higher uptake rates than

nonessential metals, they may have limited value and are to be used with caution. It is unclear why Zn was taken up at the lowest rate, particularly because it is an essential micronutrient and because it was the highest concentration of metal that the bacteria were exposed to during these experiments. While the rates for Fe, Mn, and Zn were calculated for a time span of 2 h (Mn, Zn) or 4 h (Fe) at the beginning of the experiments, the period of time used for Cd and Am was 12 h, which may be too long to obtain meaningful results.

By applying our mean concentration factors we calculated the fraction of total metal likely to be associated with bacterioplankton in surface waters. Assuming an average of $1x10^6$ bacterial cells ml^{-1} (Meyer-Reil 1982; Ammerman et al. 1984) and a mean volume of 0.25 μm^3 $cell^{-1}$, then about 20% of the bioavailable Fe would be associated with bacterial cells. This fraction may be larger in areas where the biomass of bacteria is higher, or where the smallest cell types dominate. For the other five metals studied here the association with bacterial cells would be < 1% with the assumptions made above. It is unlikely that bacteria-bound metals are transported out of the euphotic zone to any significant extent because planktonic bacterial cells are not dense enough to sink, unless they are packaged in fetal pellets or associated with organic aggregates. However, bacterial cells may serve as enriched sources of some metals for protozoa and other animals that consume bacteria, and metals that undergo trophic transfer would be expected to be recycled biologically in surface waters.

Table 1. Bacterial species used in metal accumulation experiments. Surface to volume ratios assume rod-shaped cells with "smooth" cell surfaces. "Proteo" is an abbreviation for γ-proteobacteria and α-proteobacteria.

Species	**Strain**	**Volume (μm^3)**	**Surface to Volume ($\mu m^2 \mu m^{-3}$)**	**Type**	**Original isolation from**	**Habitat designation**
Vibrio natriegens	Pwh3a	0.33	3.43	*γ*-proteo	coastal seawater	Coastal
Vibrio sp.		0.28	3.82	*γ*-proteo	coastal seawater	Coastal
Halomonas aquamarina	DSM4739	0.15	5.27	*γ*-proteo	seawater	oceanic, coastal
Pseudoalteromonas atlantica	DSM6839	0.12	6.08	*γ*-proteo	seawater	oceanic, coastal
Roseobacter litoralis	DSM6996	0.09	6.44	*α*-proteo	seaweed	oceanic, coastal

Table 2. Volume concentration factors (VCFs) and cellular metal concentrations (amol $cell^{-1}$ (x 10^{-2})) of six different metals associated with five bacterial species. Treatments include cells rinsed only with seawater, oxalate (for Fe), or EDTA (for Mn, Zn, Cd, and Am), and incubations at 17°C and 2°C (for Mn and Zn). VCFs for the radioisotopes are mean values of three (including standard errors) or two (standard errors not included) replicates calculated after a steady-state in metal accumulation had been approached. Cellular metal concentrations are mean values of the total metal concentration at the end of the uptake period. Data pairs in bold represent significant differences ($P < 0.05$) in VCFs between treatments within one bacterial species. nd: not determined.

	V. natriegens		*Vibrio* sp.		*H. aquamarina*		*P. atlantica*		*R. litoralis*	
	VCF	amol $cell^{-1}$	VCF	amol $cell^{-1}$	VCF	amol $cell^{-1}$	VCF	amol $cell^{-1}$	VCF	amol $cell^{-1}$
Fe (x10^6 for VCF)										
No rinse	1.0 ± 0.05	55.21 ± 4.71	0.8		0.9 ± 0.02	59.51 ± 8.70	nd		2.8 ± 0.5	103.74 ± 18.76
Oxalate rinse	0.6 ± 0.02	47.56 ± 3.12			1.1 ± 0.4	65.76 ± 14.24			2.7 ± 0.3	91.25 ± 10.26
Mn (x10^4 for VCF)	nd		nd		nd					
No rinse							0.7± 0.01	0.55 ± 0.03	**3.3 ± 0.6**	2.75 ± 0.39
EDTA rinse							0.7 ± 0.1	0.42 ± 0.05	**2.7 ± 0.7**	2.52 ± 0.44
17 °C							**2.2 ± 0.5**	2.67 ± 0.08	**3.8 ± 0.8**	1.98 ± 0.43
2 °C							**0.1 ± 0.6**	0.07 ± 0.03	**1.8 ± 0.6**	1.08 ± 0.27
Zn (x10^3 for VCF)	nd									
No rinse			2.7	9.38	13.1	13.11	1.7 ± 0.3	2.88 ± 0.43	0.7 ± 0.2	1.32 ± 0.34
EDTA rinse							1.6 ± 0.2	2.10 ± 0.10	0.5 ± 0.1	0.59 ± 0.27
17 °C							**2.9 ± 0.5**	3.02 ± 0.18	1.2 ± 0.4	0.35 ± 0.09
2 °C							**1.8 ± 0.5**	1.51 ± 0.47	0.7 ± 0.2	0.65 ± 0.34
Cd (x10^4 for VCF)										
No rinse	0.2 ± 0.02	0.10 ± <0.01	0.3	0.09	4.6 ± 0.7	1.85 ± 0.17	nd		1.4 ± 0.3	0.18 ± 0.07
EDTA rinse	0.2 ± 0.03	0.11 ± 0.02			4.8 ± 0.8	0.58 ± 0.24			1.3 ± 0.3	0.22 ± 0.06
Am (x10^4 for VCF)										
No rinse	0.5 ± 0.2	0.09 ± 0.03	1.0	0.17	6.3 ± 1.3	1.00 ± 0.43	3.2	0.17	**9.2 ± 1.0**	0.42 ± 0.21
EDTA rinse	0.5 ± 0.1	0.13 ± <0.01			6.2 ± 1.4	0.14 ± 0.07			**8.0 ± 0.6**	0.09 ± 0.07
Cs (x10^2 for VCF)					nd		nd		nd	
No rinse	1.9	<0.01	1.9	<0.01						

Table 3: Uptake rates normalized to bacterial cell weights (mol g^{-1} min^{-1}) and uptake rate constants (u) calculated for Mn, Fe, Zn, Cd, and Am and several bacterial strains. nd = not determined.

	R. litoralis		***P. atlantica***		***H. aquamarina***		***V. natriegens***	
	mol g^{-1} min^{-1}	u	mol g^{-1} min^{-1}	u	mol g^{-1} min^{-1}	u	mol g^{-1} min^{-1}	u
Mn	8.59×10^{-9}	1.24	2.12×10^{-9}	0.31	nd		nd	
Fe	nd		nd		3.64×10^{-7}	25.61	2.73×10^{-7}	19.22
Zn	3.54×10^{-9}	0.03	7.16×10^{-9}	0.01	nd		nd	
Cd	1.22×10^{-10}	0.10	nd		5.15×10^{-10}	0.43	3.23×10^{-11}	0.03
Am	2.21×10^{-10}	0.32	nd		1.63×10^{-10}	0.23	1.30×10^{-11}	0.02

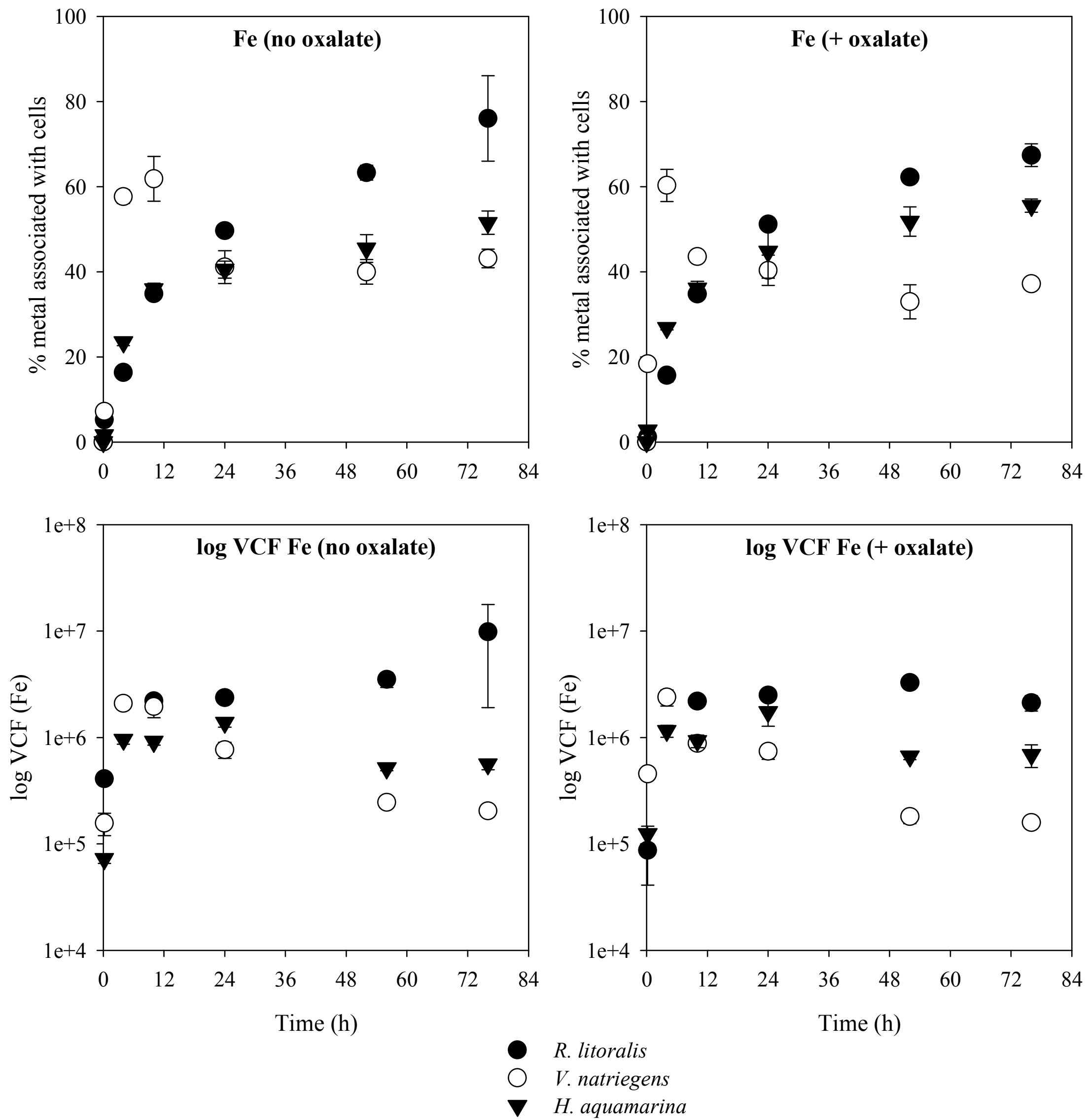

Fig. 2: Accumulation of ^{55}Fe by *R. litoralis*, *V. natriegens*, and *H. aquamarina* with and without an oxalate rinse. Panels in the top row display the percentage of ^{55}Fe associated with cells over time, panels in the bottom row display the VCF for ^{55}Fe on a log scale. Data points are means of three replicates ± standard errors.

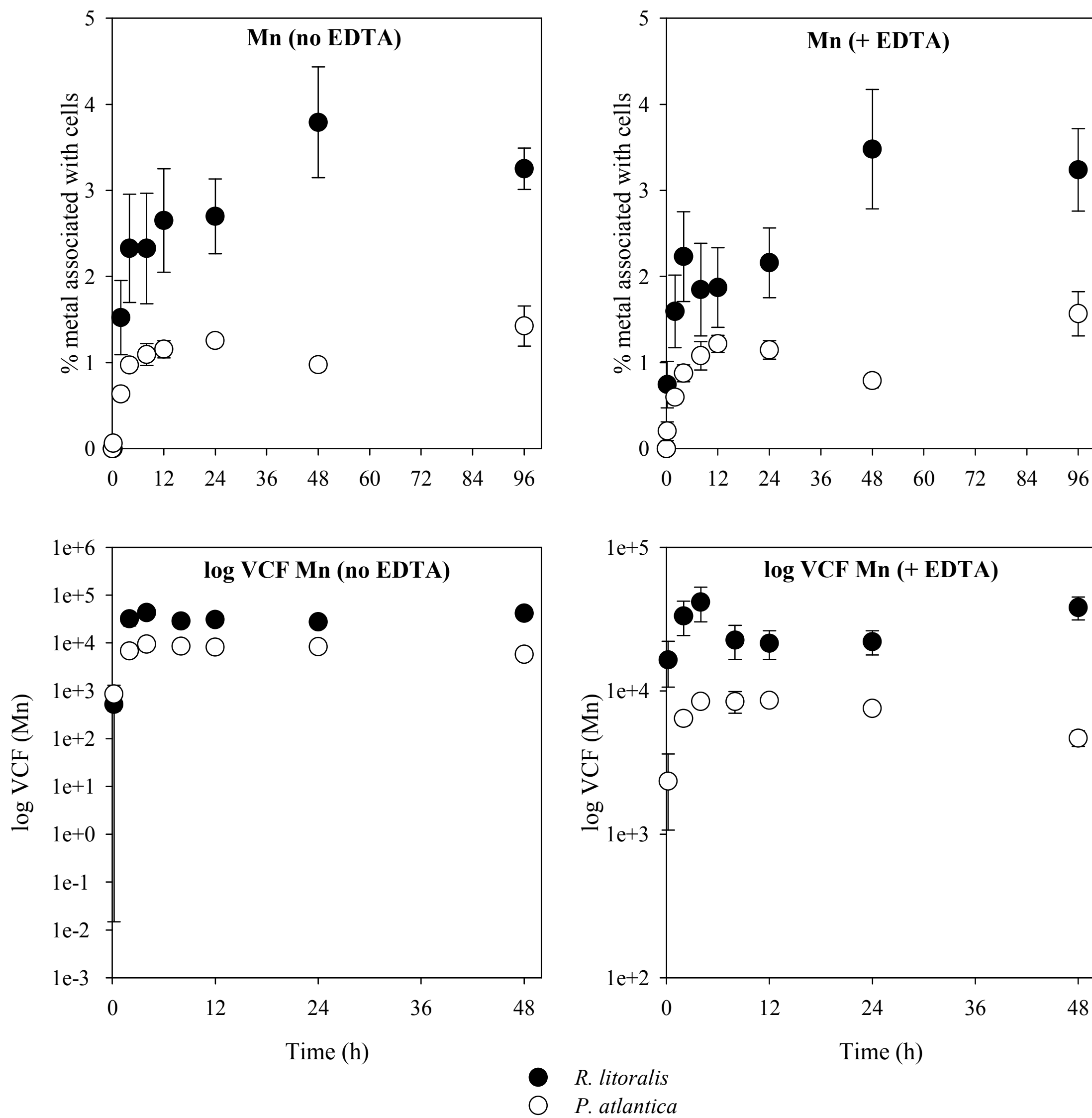

Fig. 3: Accumulation of ^{54}Mn by *R. litoralis* and *P. atlantica* with and without an EDTA rinse. Panels in the top row display the percentage of ^{54}Mn associated with cells over time. Panels in the bottom row display the VCF for ^{54}Mn on a log scale. Data points are means of three replicates ± standard errors.

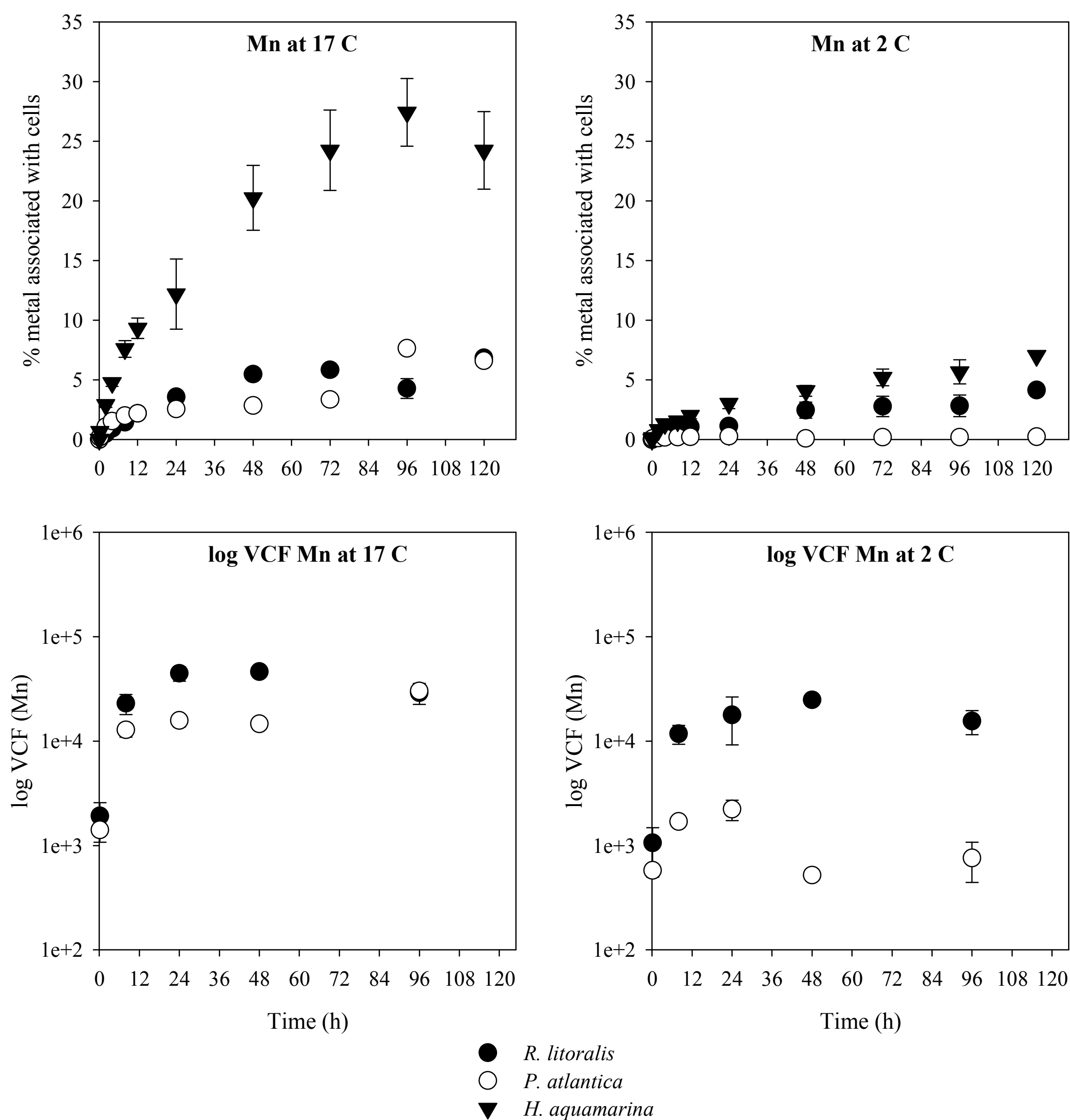

Fig. 4: Accumulation of ^{54}Mn by *R. litoralis, P. atlantica,* and *H. aquamarina* at 17°C and 2°C. Panels in the top row display the percentage of ^{54}Mn associated with cells over time. Panels in the bottom row display the VCF for ^{54}Mn on a log scale. Data points are means of three replicates ± standard errors.

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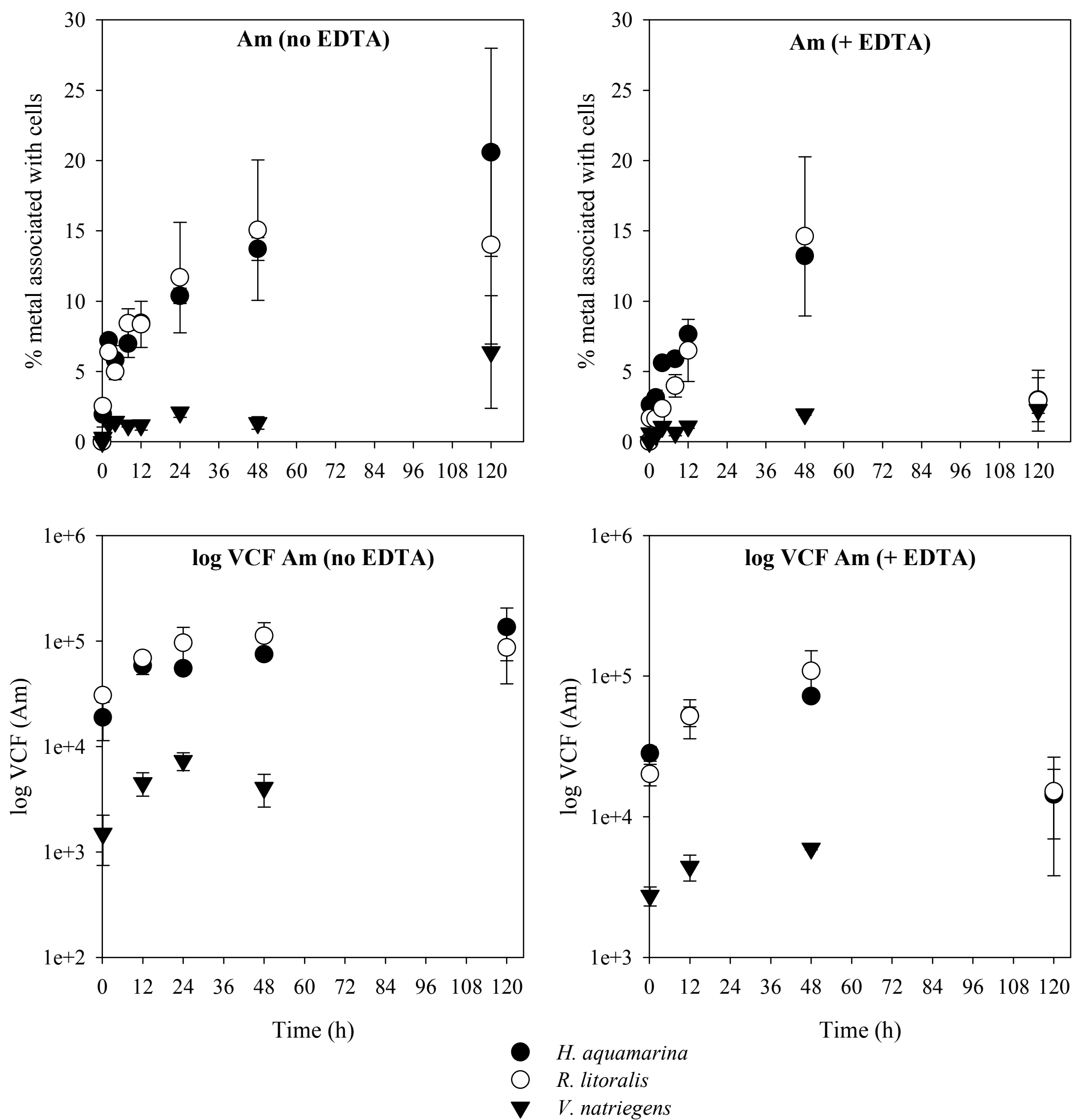

Fig. 5: Accumulation of ^{241}Am by *H. aquamarina*, *R. litoralis,* and *V. natriegens* with and without an EDTA rinse. Panels in the top row display the percentage of ^{241}Am associated with cells over time. Panels in the bottom row display the VCF for ^{241}Am on a log scale. Data points are means of three replicates ± standard errors.

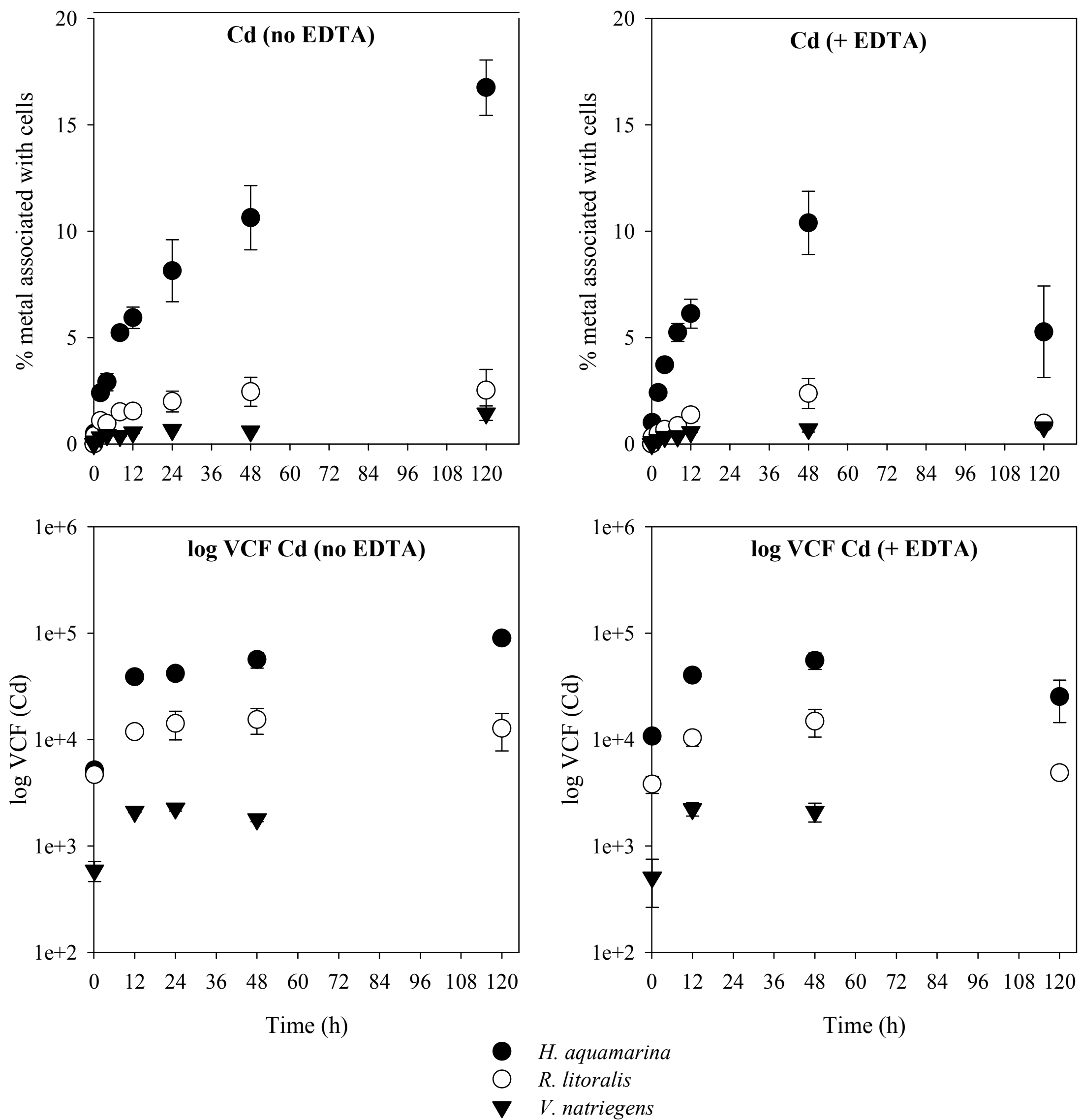

Fig. 6: Accumulation of ^{109}Cd by *H. aquamarina*, *R. litoralis,* and *V. natriegens* with and without an EDTA rinse. Panels in the top row display the percentage of ^{109}Cd associated with cells over time. Panels in the bottom row display the VCF for ^{109}Cd on a log scale. Data points are means of three replicates ± standard errors.

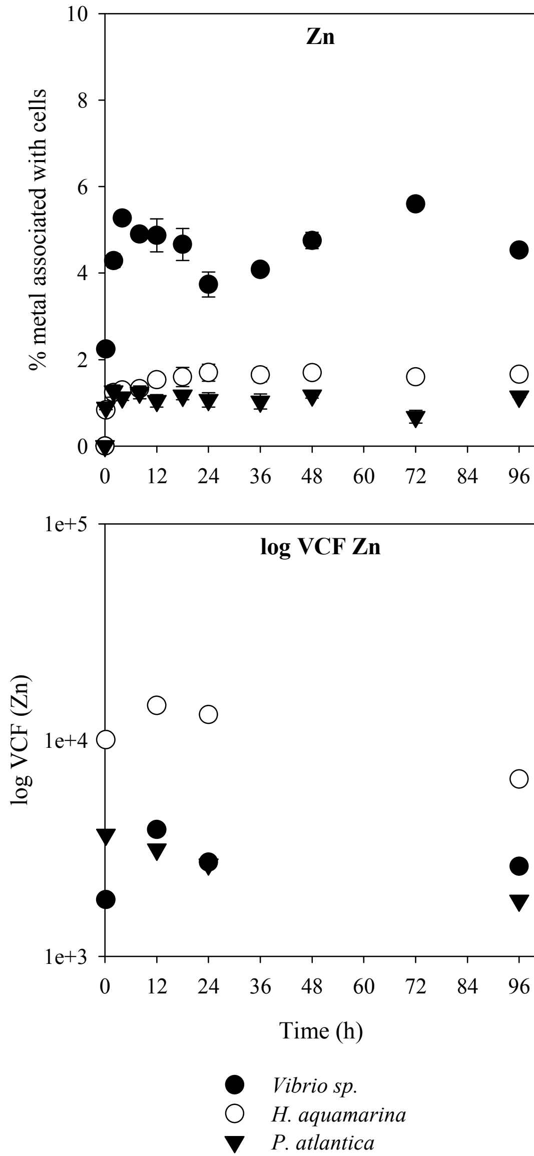

Fig. 7: Accumulation of ^{65}Zn by *Vibrio* sp., *H. aquamarina*, and *P. atlantica*. The top panel displays the percentage of ^{65}Zn associated with cells over time; the bottom panel displays the VCF for ^{65}Zn on a log scale. Data points are means of three replicates ± standard errors and show the mean percentage of ^{65}Zn associated with bacterial cells over time.

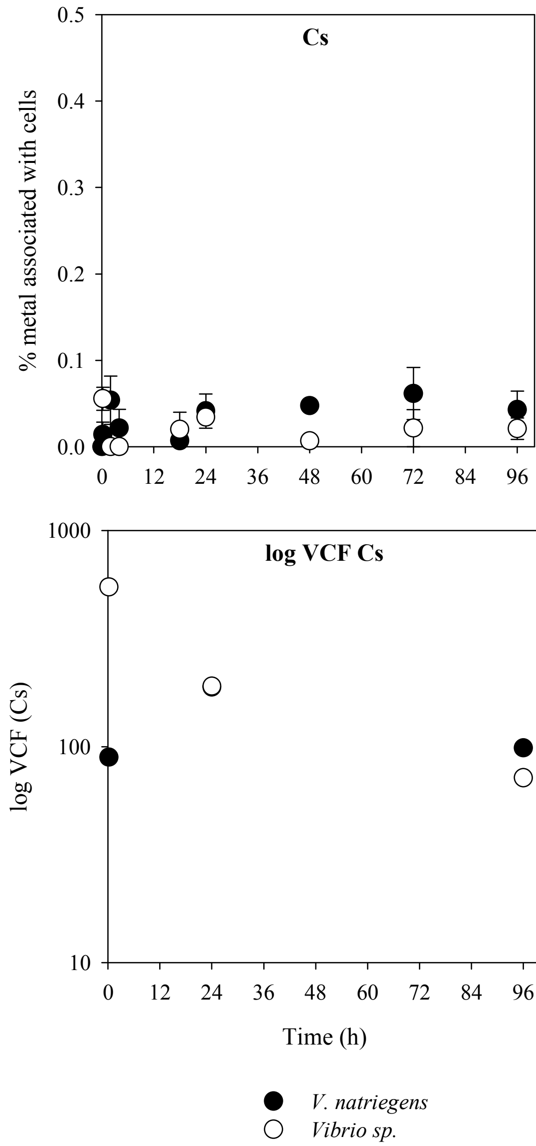

Fig. 8: Accumulation of ^{137}Cs by *V. natriegens* and *Vibrio* sp.. The top panel displays the percentage of ^{137}Cs associated with cells over time; the bottom panel displays the VCF for ^{137}Cs on a log scale.

Chapter 3:

Bioavailability of metals released from virally lysed bacterial cells

Abstract

Radioisotopes of 4 metals (^{55}Fe, ^{65}Zn, ^{109}Cd, and ^{241}Am) were used to assess the release of bacterially bound metals and their bioavailability from virally lysed and unlysed cells to another bacterial species and to diatoms in experiments conducted in natural seawater. Viral lysis of radiolabeled *Vibrio natriegens* cells released significantly more Cd into the dissolved phase (78%) than did unlysed cells (50%), but the other metals showed no such difference. The bacterium *Pseudoalteromonas atlantica* and the diatom *Thalassiosira pseudonana* were exposed to the metals released from both lysed and unlysed bacteria, and metal accumulation was measured over 3 d. For all metals in *T. pseudonana* and for Zn and Cd in *P. atlantica*, differences in accumulation of the released metals were negligible. However, *P. atlantica* concentrated 11 times more Fe and 3 times more Am when the metals were released from unlysed bacteria compared to the lysed cells. The data indicate that viral lysis can enhance the release of at least some metals from bacterial cells to ambient seawater, but no consistent influence of viral lysis is observed on the relative bioavailability of released metals to bacterioplankton or phytoplankton.

Introduction

A number of metals are essential for structural and enzymatic functions in both prokaryotic and eukaryotic organisms (Morel et al. 1991; Ehrlich 1997; Kirchman 2000) but in the surface of some ocean waters, their concentrations may be so low as to limit growth. This has been particularly noted for iron which can limit primary productivity in so called high nutrient low chlorophyll (HNLC) regions (Martin 1990; Martin et al. 1991). Small single-celled organisms with high cellular surface area to volume ratios, such as heterotrophic marine bacteria which are generally <1 μm in diameter, should therefore have a competitive advantage in acquiring these essential nutrients from waters exhibiting low concentrations. Heterotrophic bacterioplankton and the microbial loop have been recognized for their role in the remineralization of dissolved organic matter and the recycling of nutrients that can then be assimilated again by primary producers in the euphotic zone (Azam 1998; Tortell et al. 1999).

Given their ubiquity and abundance (on average 10^6 cells ml^{-1}), marine bacterioplankton present a major source of particle surface to which metals can bind. The only planktonic biological entity more abundant than bacteria are viruses, which occur at an average of 10^6 to 10^8 particles ml^{-1} (Suttle et al. 1990). The pattern for viral abundance is very similar to that for bacterial abundance, with higher densities in coastal regions than in oligotrophic open ocean waters, and with a decrease in numbers with water depth (Cochlan et al. 1993; Fuhrman 1999). Along with predation by protists, viral lysis is now considered the major factor leading to bacterial mortality in the marine environment (Bergh et al. 1989; Proctor and Fuhrman 1990), and viral infection of phytoplankton has also been demonstrated to have a negative effect on primary production (Suttle et al. 1990). Since viral lysis leads to rupture of the host's cell envelope, virus particles need to be recognized for their role in the biogeochemical cycling of elements, and their effect on the bioavailability of nutrients and trace metals needs to be investigated.

Gobler et al. (1997) showed that viral lysis of phytoplankton cells can significantly affect the partitioning and cycling of several macro- and micronutrients, including Fe, Se, C, N, and P. In addition, Poorvin et al. (2004) demonstrated that iron present in cell lysates is more bioavailable to heterotrophic bacteria than iron complexed

into siderophores or artificial ligands. Here I describe experiments designed to determine the effects of viral lysis of bacterial cells on the release of select metals from the bacteria and the bioavailability of these released metals to phytoplankton and bacteria. I hypothesized that metals that are released due to viral lysis would be organically-bound and might either be more readily accumulated by heterotrophic microorganisms that can acquire these organic compounds, or less accumulated by autotrophs that typically accumulate the free metal ions most effectively (Campbell 1995).

Materials and methods

The experiments described here were conducted in natural seawater that was not amended with any additional nutrients or chelators. We used radioisotopes of four different metals, Fe, Zn, Cd, Am, to enable us to rapidly and efficiently measure uptake/sorption and release of these metals while working with metal concentrations that were generally comparable to natural metal concentrations. Upon addition to seawater, the radioisotopes were allowed to equilibrate as they would in natural waters, thus the metals interacted with ligands that are naturally found in the water instead of artificial ones.

The heterotrophic bacterium *Vibrio natriegens* strain Pwh3a (provided by G. Taylor, Stony Brook University) was used in a series of radiotracer experiments in which it was allowed to accumulate each of 4 different metals (two essential metals: Fe and Zn, and two non-essential metals, Am and Cd). Stock cultures of this species were maintained on marine agar slants at 4°C and were transferred and grown out for 4 d in 10% marine broth (1 L seawater, 0.5 g Bacto peptone, 0.1 g yeast extract) at 17°C prior to the start of the experiments. *V. natriegens* cells used for metal uptake experiments were collected from their stock cultures by centrifugation at 1,400 *g* for 10 min and rinsed with sterile, 0.2 µm filtered seawater three times before inoculating experimental

flasks to yield initial bacterial cell densities were set at approx. 1 x 10^6 ml^{-1}. Each 2-L Erlenmeyer flask held 800 ml of sterile-filtered seawater (from surface waters collected 8 km off Southampton, NY) to which radioisotopes had been added 24 h prior to inoculation. Radioisotopes used in these experiments included ^{241}Am ($t_{½}$ = 433 y) from Amersham, ^{109}Cd ($t_{½}$ = 462 d) from DOE Los Alamos, ^{65}Zn ($t_{½}$ = 245 d) from DOE Brookhaven Lab, and ^{55}Fe ($t_{½}$ = 996 d) from NEN/Perkin Elmer Life Sciences. Each metal was assessed separately. The final activities and concentrations of radioisotopes added were 436.23 kBq ml^{-1} or 0.78 nM of ^{241}Am (taken from a solution in 3N HNO_3), 764.42 kBq ml^{-1} or 0.20 nM of ^{109}Cd (in 0.1 N HCl), 518 kBq ml^{-1} or 0.58 nM of ^{55}Fe (in 0.5 N HCl), and 2664 kBq ml^{-1} or 3.89 nM of ^{65}Zn (in 0.1 N HCl). Each volume of seawater that was radiolabeled was first neutralized with an equimolar concentration of NaOH to offset the small amount of acid added with the radioisotopes.

After 4 d exposure to the radioisotopes, the radiolabeled bacteria were filtered onto Nuclepore polycarbonate membranes (0.2 µm) and resuspended in two separate flasks each containing 400 ml sterile-filtered seawater, after which they were incubated in the dark at 17°C for 2 d. In one flask nothing was added while in the second flask the bacteriophage Pwh3a-P1 (provided by G. Taylor) was added at a volume of 5ml per flask. This bacteriophage is in the group of the Myoviridae, and is characterized by an icosahedral head (diameter of ca. 83 nm), and a rigid tail measuring approximately 104 nm and was first isolated from the Gulf of Mexico by Suttle and Chen (1992). Both flasks were swirled periodically. Samples were taken at the beginning and at the end of the 48-h period to determine the partitioning of each metal bound to cells, using methods described in Fisher et al. (1983). Therefore, it was possible to determine the degree of metal lost from the cells due to desorption (in the absence of added viruses), as well as the metal released into the water due to viral lysis of the bacteria. Samples of bacteria were inspected by epifluorescent microscopy to verify cell lysis in the viral treatment; we estimate that 80% of the bacteria underwent lysis by the viruses, whereas none of the bacteria in the culture containing no added viruses appeared to have lysed.

The bioavailability of the metals released from *V. natriegens* cells by viral lysis was assessed for the heterotrophic bacteria *Pseudoalteromonas atlantica* (DSM 4739)

and for the centric diatom *Thalassiosira pseudonana* (clone 3H). *P. atlantica* was cultured identically to the *V. natriegens* cells. The *T. pseudonana* cells were maintained and grown axenically for 1 wk in f/2 medium (Guillard and Ryther 1962) without the addition of EDTA at 17°C in a 14:10 h light:dark cycle at 70 µEin m^{-2} s^{-1} prior to the start of each experiment.

Following the 2-d incubation, the radiolabeled *V. natriegens* cells (without viruses) and cell debris (with viruses) were gently filtered through a 0.2 µm polycarbonate membrane and the resulting radioactive filtrates split into three separate treatments. One treatment consisted of a control, containing only the radioactive filtrate. A second treatment consisted of the filtrate and an inoculum of fresh, uninfected bacterial cells of *P. atlantica* at a cell density of 0.9 – 1.0 x 10^7 cells ml^{-1}. *P. atlantica* was tested in preliminary experiments to verify that it is not a suitable host for the bacteriophage that was added to the virus treatment and would therefore not be infected and undergo lysis during the remainder of the experiment. The bacterial cells were grown out in 10% marine broth and triple-rinsed with sterile, filtered seawater prior to inoculation. A third treatment consisted of the filtrate plus an inoculum of *T. pseudonana*. Initial cell densities of *T. pseudonana* ranged between 2.1 – 4.4 x 10^5 cells ml^{-1}. Cultures were incubated for 3 d, during which samples were taken periodically to determine the metal accumulation from the two types of filtrate (i.e., with and without viruses) by both the bacterial and the phytoplankton cells. Samples for cell counts were also taken at various times throughout the experiment.

For bacterial cells counts, bacteria were fixed with 2% final volume borate-buffered formaldehyde and counted by 4'6-diamidino-2-phenylidole (DAPI) staining (Sherr et al. (2001)) and epifluorescence microscopy using a Leica DM IRB inverted microscope in all experiments. Diatom cells were fixed with 2% Lugol's solution and counted using a hemacytometer or a Coulter Counter (Beckman Coulter Multisizer 3).

Growth rates were obtained using the following equation:

$\mu = [(\ln Nt - \ln Nt_0) \times 24] / [(t - t_0) \times (\ln 2)]$, where μ = divisions per day, and Nt and Nt_0 = cell counts at times t and t_0, respectively.

To measure radioactivity of the samples, the gamma emitting radioisotopes ^{241}Am, ^{109}Cd, or ^{65}Zn were counted on a LKB Wallac 1282 Compugamma equipped with a NaI(Tl) well detector. Gamma emissions of ^{241}Am were measured at 59.5 keV, of ^{109}Cd at 22 keV, and of ^{65}Zn at 1115 keV. A Packard Tri-Carb 2100 TR Liquid Scintillation Analyzer was used for samples containing the beta emitter ^{55}Fe, taking into consideration a quench correction curve. Two kinds of samples were collected at each sample time: one 10-ml filtered sample to determine the activity associated with particles (cells) and one 1-ml unfiltered sample. Typically, samples were counted for long enough periods to achieve propagated counting errors <5%.

For all organisms, concentration factors for each metal were determined by dividing the radioactivity of each isotope in the cells by the radioactivity in the ambient seawater at time of equilibrium with respect to metal partitioning between dissolved and particulate phases. Concentration factors were calculated on a volume basis, thus the radioactivity μm^{-3} of cell was divided by the radioactivity μm^{-3} of water.

Results

At the end of the initial labeling period, cell densities of *V. natriegens* ranged from 5.0 to 18 x 10^6 cells ml^{-1}, depending on the experiment. The concentration factors of the metals were 9.4 x 10^3 for Cd, 4.3 x 10^4 for Am, 9.7 x 10^4 for Zn, and 7.9 x 10^5 for Fe, measured at the point before the bacteria were filtered and resuspended in fresh seawater. After resuspension, the bacterial cells released radioisotopes to varying degrees, ranging from 24% for Am to 79% for Fe (Fig. 9). Except for Cd, there was no significant difference in the fraction of metal released from the cells between lysed and unlysed cells (Fig. 9). For Cd, the release was higher in the treatment that contained viral particles (77.9% lost from cells) than in the treatment without viral particles (50.1%).

For all experiments, phytoplankton cells and bacterial cells grew in both types of filtrate provided to them as their growth medium. For the bacteria, there was no detectable difference in growth rate between the filtrates (with or without viral lysis). However, the growth rate of the diatom *T. pseudonana* (1.1 divisions d^{-1}) was significantly slower following lysis of bacteria exposed to ^{55}Fe than in filtrate from ^{55}Fe-labeled bacteria that did not contain viruses (1.7 divisions d^{-1}) (Fig. 10).

The average percentage of Fe that was associated with the *T. pseudonana* cells over the 72-h exposure period was unaffected by the presence or absence of viral particles (Fig. 11). In the bacterial samples, however, the percentage of Fe associated with cells was consistently higher for those cells that had not been exposed to the viral particles (Fig. 12). Average volume concentration factors for cells grown in the viral lysate were 5.3×10^5 for *T. pseudonana* and 2.0×10^6 for *P. atlantica*. Concentration factors for cells that were incubated in the filtrate not produced by viral lysis were 5.2×10^5 for *T. pseudonana* and 2.2×10^7 for *P. atlantica* (Table 4). The Fe concentration factors in *P. atlantica* from the filtrates with and without viruses were significantly different from each other ($P < 0.05$) and both values are higher than for Fe uptake by *V. natriegens*.

The percentage of Zn on *T. pseudonana* after 12 h was approximately 30 to 44% (Fig. 11) and there was no significant difference in metal uptake by the cells between the two types of filtrates. The average concentration factor for Zn on *T. pseudonana* was 8.9×10^3 for cells that were exposed to the viral filtrate and 9.2×10^3 for *T. pseudonana* that were incubated in the filtrate resulting from metal desorption from intact bacteria (Table 4). For the bacterial cells, average concentration factors were calculated to be 4.7×10^3 for cells exposed to the viral lysate and 5.5×10^3 for *P. atlantica* that had grown in the filtrate resulting from metal desorption from intact bacteria (Table 4), with approximately 2% of the metal associated with bacterial cells (Fig. 12). These values are about 20-fold lower than for Zn uptake in *V. natriegens.*

The percentage of Cd associated with *T. pseudonana* cells was somewhat higher when the cells were exposed to the filtrate not produced by viral lysis, while no difference could be seen in the bacterial samples (Figs. 11, 12). Average concentration

factors were 0.8×10^3 for the diatom in the lysis induced filtrate compared to 1.6×10^3 in the filtrate that did not contain viral particles. Concentration factors for *P. atlantica* were 5.4×10^3 in the viral filtrate and 3.5×10^3 in the treatment without viral particles once a steady-state had been approached after 12 h (Table 4), which is similar to Cd uptake in *V. natriegens.*

The average percentage of Am on the bacterial cells was significantly higher ($P < 0.05$) for the treatment that did not contain viral particles (Fig. 12), and was also slightly higher for *T. pseudonana* (Fig. 11). Concentration factors for Am in *P. atlantica* were 3.7×10^4 for cells in the filtrate that resulted from viral lysate and 9.9×10^4 in the filtrate that resulted from metal desorption from intact cells; this difference was significant. For the diatom, the average concentration factor was 4.6×10^5 in the presence of viruses and 5.8×10^5 in the absence of viral particles (Table 4).

Discussion

Previous research (Gobler et al. 1997) demonstrated that a possible effect of viral lysis of phytoplankton is a change in the availability of major nutrients and some minor nutrients to uninfected planktonic cells by releasing elements and compounds into the surrounding water. They showed that viral lysis of the chrysophyte *Aureococcus anophagefferens* increased the fraction of C and Se that was released from the cells, but that the release of N, P, and Fe remained largely unaffected by viral lysis. Our results agree with their observation that viral lysis does not increase the release of Fe into the dissolved phase and, in fact, Fe displayed the least variation between lysed and unlysed bacterial cells. Fe is a highly particle-reactive metal and one that can limit growth of planktonic organisms. Iron in ocean surface waters is recycled rapidly and efficiently (Hutchins et al. 1993) and any Fe that is released will likely be assimilated by or sorb to another cell (or abiotic particle) very rapidly. Heterotrophic bacteria and cyanobacteria

possess siderophores which are especially efficient at acquiring iron (Haygood et al. 1993; Wilhelm and Trick 1994). It may therefore be difficult to measure a difference in Fe release from lysed versus intact bacterial cells because any available Fe would be complexed again quickly. While our results agree with those of Gobler et al. (1997), Poorvin et al. (2004) reported a release of Fe of approx. 70% from lysed, and of approx. 40% from unlysed *V. natriegens* cells. Differences between my results and those of Poorvin et al. (2004), who worked with the same bacterial strain (*V. natriegens* Pwh3a) that was used in my experiments, are not readily explained. It is possible to speculate that my results of high Fe release from unlysed cells may be the result of damaged cells. Radiolabeled *V. natriegens* cells were filtered (0.2 μm) and resuspended into fresh seawater in order to measure metal release from virally lysed and unlysed cells. It is possible that the cells were damaged during the filtration and resuspension (by experiencing too much pressure or due to desiccation), thereby resulting in a higher than normal release of Fe from the unlysed bacteria.

Metal release from intact bacterial cells due to desorption processes occurs in two distinct phases: a rapid initial release, followed by a slower loss. Recent findings for Am and Zn (C. Vogel and N. S. Fisher, unpublished data) showed that the rapid initial release occurs over the first 8 to 9 hours after radiolabeled bacteria are resuspended in seawater, and that after 15 hours release slows considerably. This suggests that the 48 hour period over which metal release was allowed to occur in the experiments described above was sufficiently long to reach equilibrium. This length of time was sufficient for viral lysis of the cells as well, and falls into the range of 24-96 hours that previous researchers used for lytic periods in their experiments (Middelboe et al. 1996; Poorvin et al. 2004).

One difference between the two types of metal release studied here is that during viral lysis, surface-bound as well as intracellular metals in bacteria are brought into contact with the surrounding medium and can ultimately become available for uptake by other organisms. In contrast, only metals bound to the surface of cells are in direct contact with the water in the absence of bacteriophages. Hence, we expected that viral lysis would lead to a larger release of those metals that are significantly localized inside cells, namely Fe, Zn, and Cd (Reinfelder and Fisher 1991; Hutchins et al. 1995).

However, we did not see a significant effect of viral lysis on the release of bacterial Zn during viral lysis. According to Guiné et al. (2006) Zn binds dominantly to extracellular polymers in Gram negative bacteria, while only a small fraction is transported into the cells. This might explain why we did not observe a large difference between Zn release from lysis compared to the fraction that was released from unlysed cells. Similarly, loss of bacterial Am, which was lowest for all the metals examined and which has no biological function, also showed no difference between lysed and unlysed cells. Americium is most likely not transported into bacterial cells but is bound to cell surfaces instead (Fisher et al. 1983). Am is a highly particle-reactive non-essential metal whose behavior in marine systems with respect to particle reactivity and adsorption/desorption are likely similar to other +3 valence, particle-reactive, nonessential metals, such as the lanthanides.

We found that bacterial cells that underwent viral lysis tended to release more Cd than unlysed cells. This finding is supported by Feung et al. (2008) who demonstrated that only a minor fraction of Cd is removable from surfaces of *Bacillus firmus* cells using EDTA rinses, indicating that the metal was probably located inside the cells. Laddaga and Silver (1985), Laddaga et al. (1985), and Flatau et al. (1988) also showed that Cd is transported into bacterial cells by energy-dependent metabolic pathways. Although a release of 50.1% of Cd from unlysed bacterial cells may seem high for a healthy cell, Chen et al. (2008) have reported a release of up to approx. 40% of Cd from Gram-positive bacterial cells upon resuspension into fresh medium, further supporting my results.

Work by Daughney et al. (2004) indicated that the marine bacteriophage Pwh3a-P1 is able to sorb Fe to its protein coat, and it is possible that virus particles in general have the potential to sorb and precipitate metals other than Fe, such as Cd and Zn which have a tendency to attach to proteins in live cells (Binet et al. 2003), and Am, a particle-reactive element. The smallest pore size that was used in my filtrations was 0.2 µm, which should allow passage of viral particles the size of Pwh3a-P1 and any metal that adheres to them during this process. Viral particles were therefore most likely present in the viral filtrates, and the lower degree of metal association with prokaryotes and

diatoms that was measured in most cases during the final uptake phase of the experiments can be explained by metal sorption to the bacteriophage. This is further supported by the fact that the differences in metal accumulation were largest for Fe and Am when accumulated by *P. atlantica*. In both cases, accumulation was significantly lower when the metals were released from lysed bacterial cells, which is plausible because Fe and Am are the most particle-reactive elements that were included in this study and were probably sorbed to the virus particles more than the less particle-reactive metals Zn and Cd. The difference in growth rates observed for *T. pseudonana* when the cells were exposed to ^{55}Fe in the two types of filtrates is another probable result of metal sorption by the viral particles. *T. pseudonana* showed a significantly higher growth rate in the absence of viral particles (Fig. 10). Since Fe is an essential micronutrient, it is possible that enough of it was bound to the particles to render the cells slightly nutrient-limited.

Poorvin et al. (2004) demonstrated that viral lysis of bacterial cells led to a large (approximately 60%) release of Fe in the size fraction <3 kDa, while Fe that was released from uninfected cells was more evenly divided between the size classes of <3 kDa and >30 kDa. Poorvin et al. (2004) and Mioni et al. (2005) found that the Fe in lysates of bacterial cells was accumulated faster by bacteria and phytoplankton than was Fe that was complexed by EDTA or desorbed from bacterial cells, indicating that Fe in the <3 kDa size fraction was most readily accumulated. We did not see a significant difference in the bioavailability of any of the four metals released due to viral lysis when their subsequent uptake was measured in *T. pseudonana*, supporting results by Gobler et al. (1997) for Fe, the only metal that was included in both studies. Their work demonstrated that virally released Fe was not significantly more bioavailable to uninfected diatoms than Fe that had been added to the growth medium in its inorganic form, as $FeCl_3$. The experimental protocols of Poorvin et al. (2004) and those described in our study differed mainly in the water that was used in the experiments. Poorvin et al. (2004) used carbon-supplemented artificial seawater with EDTA where our study used natural seawater without EDTA or other amendments. It is not clear whether this difference can account for the divergent results observed for Fe between the studies.

In comparing our metal concentration factors in *T. pseudonana*, concentration factors for Am are about one order of magnitude lower in our study than previous findings for diatoms, and Zn and Cd concentration factors are about 5 fold lower in our study than previous findings (Fisher and Reinfelder 1995). Reasons for these differences are not obvious, although previous studies evaluated the uptake of these metals added to seawater in an inorganic form, and it is possible that at least some of the metals released from bacterial cells was bound to organic compounds and hence less bioavailable (Campbell 1995). The concentration factor for Fe was comparable to earlier estimates for this metal in diatoms (Iaea 2004).

Table 4. Mean volume concentration factors (VCFs) of metals for bacteria and phytoplankton cells exposed to metals released from bacteria by desorption (-virus) or following viral lysis (+virus). Mean values (± 1 standard deviation) were calculated following 12, 24, and 48 h exposures (at which time an apparent equilibrium in metal partitioning had been reached). Significant differences (*P* < 0.05) are denoted by *.

		Fe (x 10^6)	**Zn (x 10^3)**	**Cd (x 10^3)**	**Am (x 10^4)**
T. pseudonana	+ virus	0.5 ± 0.6	8.9 ± 0.8	0.8 ± 0.2	4.6 ± 0.4
	-virus	0.5 ± 0.7	9.2 ± 1.1	1.6 ± 0.2	5.8 ± 1.3
P. atlantica	+ virus	2.0 ± 0.8	4.7 ± 0.9	5.4 ± 0.6	3.7 ± 0.2
	- virus	22 ± 0.6 *	5.5 ± 1.0	3.5 ± 0.9	9.9 ± 0.6 *

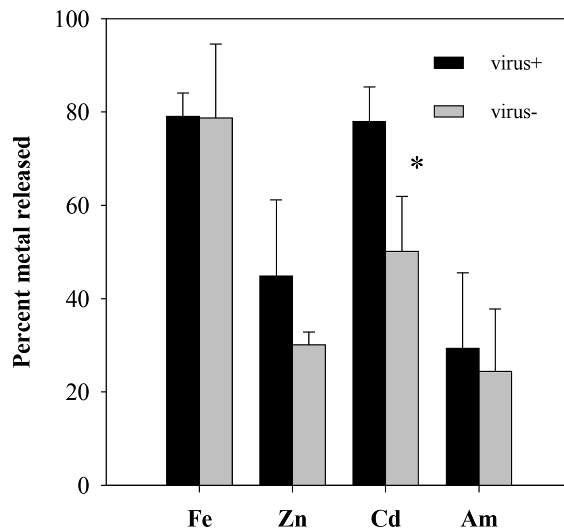

Fig. 9: Mean percentage (n = 2) of metals released from *V. natriegens* Pwh3a after a period of 48 h in the presence or absence of the added bacteriophage Pwh3a-P1 (virus+, virus-). Error bars represent 1 standard deviation. An asterisk (*) denotes a significant difference ($P < 0.05$) between values for metal released from lysed vs. unlysed cells.

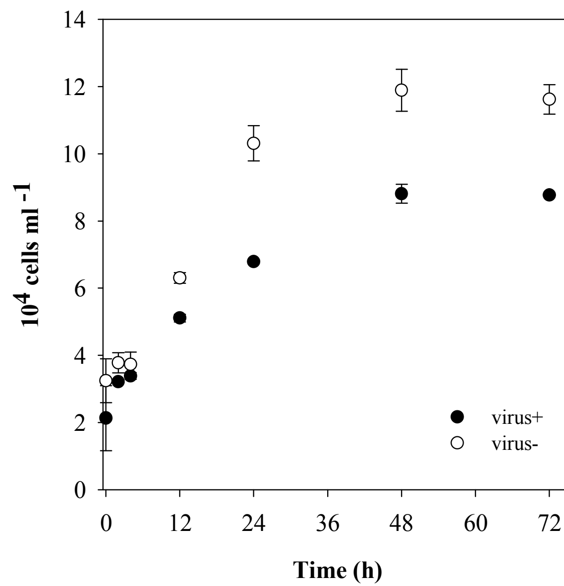

Fig. 10: Growth of *Thalassiosira pseudonana* in response to bacterially released Fe in the presence (virus+) or absence (virus-) of viral particles; growth rates (divisions d $^{-1}$) calculated for the period from 0 to 24 hrs are 1.1 and 1.7, respectively. Error bars represent 1 standard deviation.

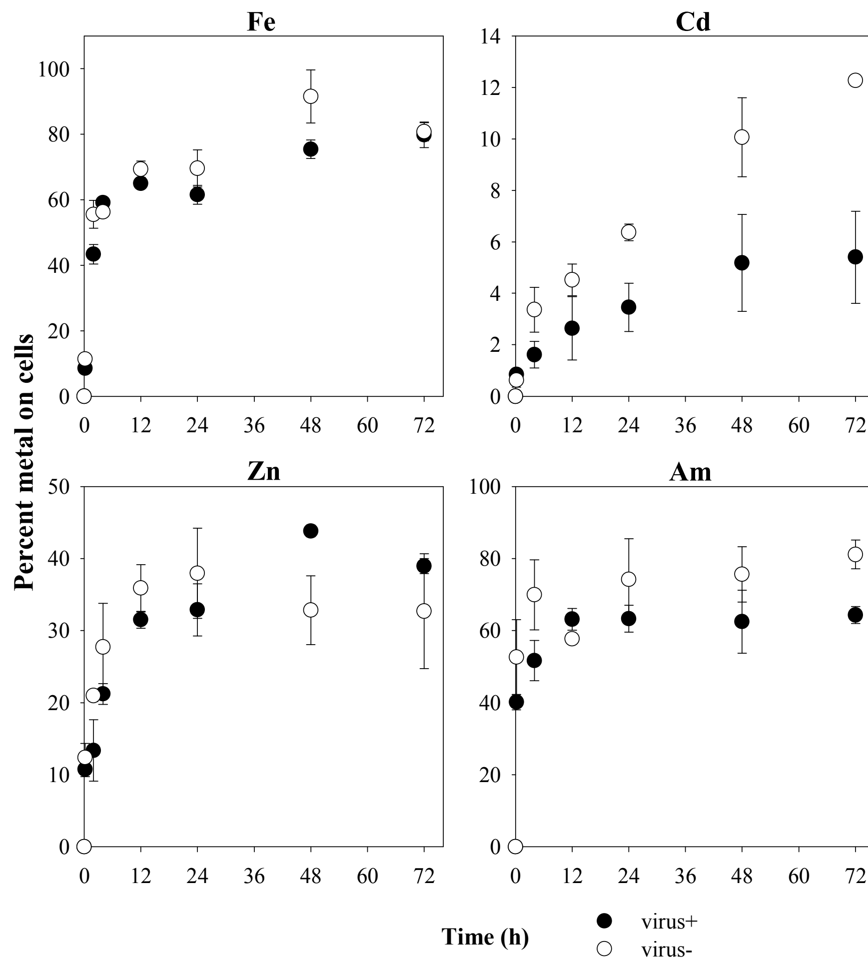

Fig. 11: Mean percentage of metals associated with *Thalassiosira pseudonana* cells growing in either seawater containing viral lysate (virus+) or in seawater containing metals that had desorbed from unlysed bacterial cells (virus-). Data points are means from 3 replicate cultures; error bars denote 1 standard deviation.

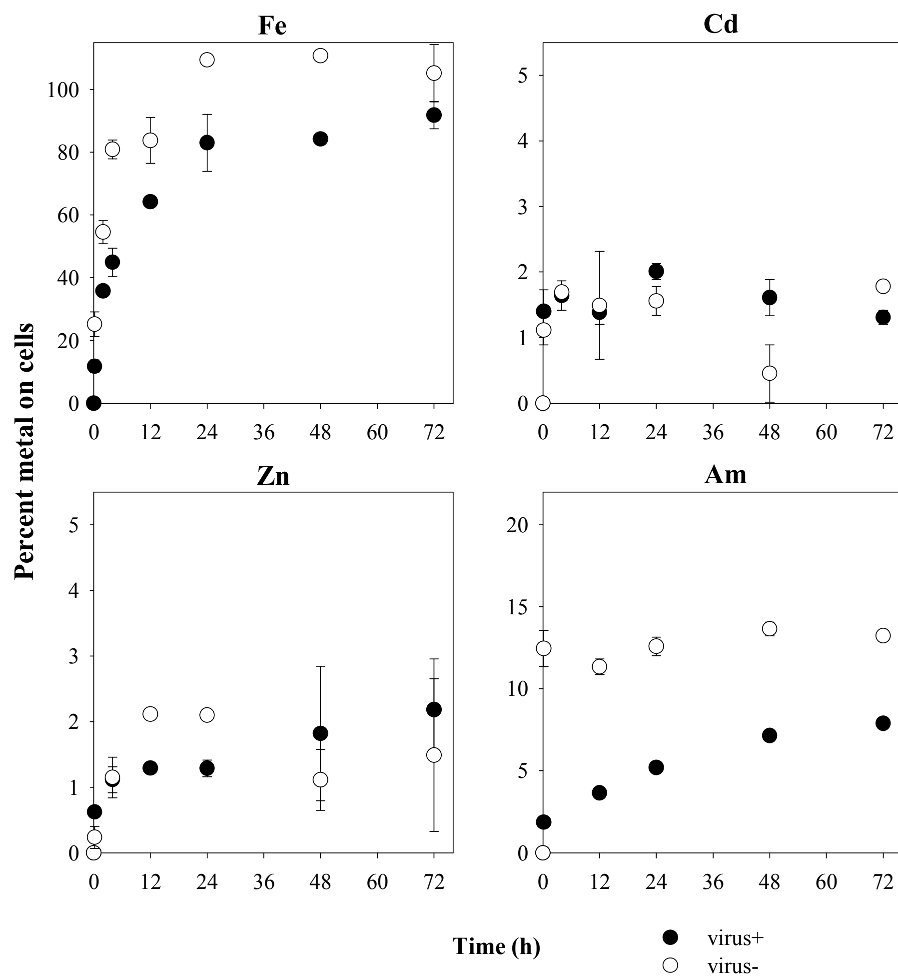

Fig. 12: Mean percentage of metals associated with *Pseudoalteromonas atlantica* cells growing in either seawater containing viral lysate (virus+) or in seawater containing metals that had desorbed from unlysed bacterial cells (virus-). Data points are means from 3 replicate cultures; error bars denote 1 standard deviation.

Chapter 4:

Trophic transfer of Fe, Zn, and Am from marine bacteria to a planktonic ciliate

Abstract

Marine bacteria may serve as an enriched source of essential and non-essential metals for organisms that eat them. We evaluated this trophic transfer by measuring the accumulation of Fe, Zn, and Am by a planktonic ciliate (*Uronema* sp.) isolate following the ingestion of radiolabeled heterotrophic bacteria (*Vibrio natriegens*) in laboratory experiments. *V. natriegens* cells were allowed to accumulate ^{55}Fe, ^{65}Zn, and ^{241}Am from seawater for 5 d and were subsequently fed to ciliate cultures. Uptake and depuration of the metals was monitored in the ciliates over time. Measured assimilation efficiencies were 79% for Fe, 29% for Zn, and 24% for Am; corresponding efflux rates were 0.1 d^{-1} for Fe, 0.9 d^{-1} for Zn, and 0.7 d^{-1} for Am. These results suggest that bacteria present an alternate pathway for some metals (especially Fe) to enter metazoan food webs that does not directly involve phytoplankton.

Introduction

Trophic transfer studies in the marine environment most often rely on phytoplankton to represent the base of the food web under examination. The ability of microalgal cells to bioconcentrate metals of varying properties (Fisher 1986) and to transfer them to the organisms grazing on phytoplankton has been documented (Fisher et al. 1983; Fisher et al. 1984; Reinfelder and Fisher 1991). Fewer studies have examined the transfer of metals from bacterial cells to their consumers in aquatic environments (Chase and Price 1997; Maranger et al. 1998; Barbeau et al. 2001), particularly involving ciliates (Berk and Colwell 1981; Lores et al. 1999). Half of the particulate organic carbon in the world's oceans is contained in heterotrophic bacteria (Cho and Azam 1988; Fuhrman et al. 1989), illustrating their potential importance in influencing the geochemical cycling of metals and nutrients in general. The accumulation of diverse metals by bacterial cells from ambient seawater has been quantified experimentally (Vogel & Fisher, in review). Here I examine the extent to which bacterially associated metals can be transferred to protozoans that graze upon them.

Elements that are accumulated by bacteria become incorporated into the microbial loop, a highly efficient system at regenerating nutrients (Azam et al. 1983). However, the microbial loop is not a system that exists in isolation, but is also connected to the "traditional" phytoplankton-based metazoan food web. Therefore, bacterially bound metals may eventually enter into the larger oceanic food web. One of these links is represented by planktonic ciliates, many of which are bacterivores (Porter et al. 1985; Sherr and Sherr 1987) and which are ubiquitous in most aquatic systems (Beers and Stewart 1969; Fenchel 1986). Through excretion, protozoans can play a significant role in the regeneration of nutrients in the aquatic environment (Johannes 1965; Taylor 1982; Gast and Horstmann 1983; Porter et al. 1985) and ciliates in particular are suitable prey items for zooplankton such as copepods (Berk et al. 1977; Rieper 1985). Evidence suggests that they may be a better dietary source for some copepods than phytoplankton based on egg development and hatching success (Heinle et al. 1977). Further, copepods assimilate certain metals (e.g., Zn) more efficiently from ciliates than from algal cells (Twining and Fisher 2004).

Different types of metals are accumulated by bacteria, and some metals are bound mostly to external cell surfaces while others may also move across the membranes and accumulate inside the cells. The assimilation of a metal from algal food by herbivores is tightly correlated with that metal's cytoplasmic distribution in the food (Reinfelder and Fisher 1991; Reinfelder and Fisher 1994; Hutchins et al. 1995). A similar pattern was observed in the trophic transfer of Ag and Pb to the estuarine ciliate *Fabrea salina* feeding on the naked prymnesiophyte *Isochrysis galbana* (Fisher et al. 1995). In this study we have chosen three different metals (Fe, Zn, and Am) to investigate the trophic transfer to planktonic ciliates feeding on heterotrophic bacteria. Zn and particularly Fe are particle-reactive and essential trace metals that are known to be transported into bacterial cells (Tortell et al. 1996; Keung et al. 2008) and could therefore be expected to be assimilated to a relatively large extent by the ciliates. In contrast, Am is a particle-reactive element with no biological function. However, there is interest in this element from a radiological protection perspective (IAEA 2004) and its geochemical behavior is much like that of the lanthanides and some other actinides which also are particle-reactive, +3 valence metals. As with algal cells (Fisher et al. 1983), Am is expected to remain attached to the cell surfaces of bacteria and be less assimilated by the ciliates.

Materials and methods

The experiments described here were conducted in filtered natural seawater that was collected in trace-metal clean fashion from surface waters 8 km offshore of Southampton, New York. Cultures of the heterotrophic marine bacterium *Vibrio natriegens,* strain Pwh3a (provided by G. Taylor) were maintained on marine agar slants (1 L seawater, 5 g Bacto peptone, 1 g yeast extract) at 4°C and were subcultured periodically. In preparation for experiments, *V. natriegens* was cultured in 10% marine broth (1 L seawater, 0.5 g Bacto peptone, 0.1 g yeast extract) for 4 d before an inoculum

was centrifuged at 1,400 *g* for 10 min and rinsed with sterile 0.2 µm-filtered seawater before resuspending the cells.

Cultures of the planktonic ciliate *Uronema* sp. (provided by A. Hartz, and isolated from coastal seawater in Oregon) were maintained at 17°C in sterile-filtered seawater that was supplemented with a sterile rice grain and *V. natriegens*, and were subcultured periodically. Prior to each experiment, batches of the ciliates were transferred to fresh seawater containing an inoculum of rinsed *V. natriegens*, but no rice grain, for 4 d. Preliminary experiments established that *V. natriegens* serves as an adequate food source for the ciliates. *Uronema sp*. are small-celled, bacterivorous ciliates in the order Scuticociliatida that are abundant in coastal regions (Sherr and Sherr 1987). The average cell volume of the ciliates was 559 μm^3, based on the shape of a prolate ellipsoid and was calculated from length and width measurements obtained by light microscopy of 50 individual cells.

For the trophic transfer experiments, rinsed *V. natriegens* cells (10^6 cells ml^{-1}) were suspended in 800 ml of 0.2 µm-filtered seawater to which radioisotope had been added 12 h before the bacterial inoculation. Radioisotopes used in these experiments included ^{55}Fe ($t_{½}$ = 996 d; dissolved in 0.5 N HCl) in the form of $FeCl_3$ from NEN/Perkin Elmer Life Sciences, ^{65}Zn ($t_{½}$ = 245 d; dissolved in 0.1 N HCl), as $ZnCl_2$ from Brookhaven National Laboratory, and ^{241}Am ($t_{½}$ = 433 y; dissolved in 3 N HNO_3) as $Am(NO_3)_3$ from Amersham. The trophic transfer of Fe was tested separately, whereas Zn and Am were tested simultaneously. The concentrations (and activities) of the radioisotopes were 0.9 nmol L^{-1} (19.98 kBq) for ^{55}Fe, 1.94 nmol L^{-1} (39.96 kBq) for ^{65}Zn, and 1.14 nmol L^{-1} (23.68 kBq) for ^{241}Am. Each volume of seawater that received radioisotopes also received a small amount (microliter quantities) of 0.5 N NaOH first in order to offset the small amount (microliter quantities) of dilute acid added along with the radioisotopes.

To determine the accumulation of the isotopes in the ciliates from bacterial food, the bacteria were exposed to radioisotopes for 5 d, after which they were collected on 0.2 µm Nuclepore polycarbonate membranes, rinsed with sterile seawater, and resuspended in triplicate flasks, each containing 250 ml seawater and approx. 2 – 4 x 10^3 *Uronema* sp.

ml^{-1}. A separate set of flasks containing 250 ml seawater but no ciliates also received an inoculum of radiolabeled *V. natriegens* to determine the loss via desorption of the radioisotopes from bacterial cells to the water. Cellular and dissolved samples were taken periodically over a period of 4 h for Fe and 15 h for Am/Zn. At each sample time, a 1-ml unfiltered sample was taken to determine the total activity of the metal in the aqueous and the particulate phases, and a 10-ml sample was collected on 5.0 µm Nuclepore polycarbonate membranes (and rinsed 2 times with 5 ml of filtered seawater) to determine the radioactivity associated with the ciliates. In other studies (Vogel and Fisher, in review), bacterial clumps that might be caught on these filters were not apparent. A 10-ml sample was also filtered (0.2 µm) and rinsed with 10 ml seawater to determine the activity of the metals associated with *V. natriegens* in the flasks containing only the bacterial cells.

To measure metal retention in ciliates, radioactive *Uronema* were removed from their radioactive bacterial food and fed non-radioactive bacteria to purge any undigested bacteria and unassimilated metal. Thus, at the end of the radioactive feeding, the radiolabeled ciliates that had been feeding on radiolabeled *V. natriegens* were gravity filtered (5.0 µm), rinsed with seawater, and resuspended into 3 flasks each containing 160 ml of seawater and 5×10^6 cells ml^{-1} of unlabeled *V. natriegens* food. Filtered (10 ml) and unfiltered (1 ml) samples were taken periodically from these flasks to determine the loss of metals from the ciliates for 28 h in the Fe experiment and 15 h in the Am/Zn experiment.

In addition, the contents of the flasks that contained only radiolabeled *V. natriegens* (described above) were collected on a 0.2 µm polycarbonate filter, and the resulting radiolabeled filtrate was used to measure the accumulation of desorbed metals by unlabeled *Uronema* (added at $1.0 – 1.4 \times 10^3$ cells ml^{-1}) from the dissolved phase.

Radioactive samples were measured using an LKB Wallac 1282 Compugamma equipped with a NaI(Tl) well detector for the gamma emitting radioisotopes ^{65}Zn and ^{241}Am. Gamma emissions of ^{65}Zn were measured at 1115 keV, and at 59.5 keV for ^{241}Am. Samples containing the beta emitter ^{55}Fe were measured in a Packard Tri-Carb

2100 TR Liquid Scintillation Analyzer, taking a quench curve into consideration. In general, samples were counted so that propagated counting errors were <5%.

At various times throughout the experiments, samples were taken to determine the cell densities of ciliates and bacteria. *V. natriegens* samples were filtered onto 0.2 µm black polycarbonate filter membranes for this purpose, and 5.0 µm filters were used for *Uronema* sp. Both types of cells were fixed with 2% final volume borate-buffered formaldehyde and counted by DAPI (4'6-diamidino-2-phenylidole) staining (Sherr et al. 2001) and epifluorescence microscopy using a Leica DMB IRB inverted microscope in all experiments.

For *V. natriegens*, concentration factors for each metal were determined by dividing the radioactivity of each radioisotope in the cells by the radioactivity in the ambient seawater at the time of equilibrium with respect to metal partitioning between dissolved and particulate phases. Concentration factors were calculated on a volume/volume basis, assessed by dividing the radioactivity μm^{-3} of bacterial cells by the radioactivity μm^{-3} dissolved in the ambient seawater (Fisher et al. 1983). The cell volume for the ciliates was calculated based on the shape of a prolate ellipsoid, with the length and width dimensions measured using light microscopy; the cell volume for the bacterial cells was calculated based on the shape of a rod.

Assimilation efficiencies (AE) of the metals in *Uronema* sp. were calculated based on the filtered and unfiltered samples taken during the depuration phase of the experiments. Depuration data were plotted showing the percentage of each metal retained by the ciliates upon resuspension into unlabeled seawater. Assimilation efficiencies were obtained by fitting an exponential regression to the depuration data from 2 h after the beginning of the depuration period to the last sample time. The assimilation efficiency is represented by the y-intercept of this curve and the slope of the curve represents the efflux rate constant (k_e) (Wang and Fisher 1999). This method of determining a metal's assimilation efficiency is appropriate for organisms for which the time required for digestion is difficult to measure. Typically, marine invertebrates, including zooplankton, display a biphasic depuration pattern, with an initial rapid loss representing egestion of unassimilated material, followed by a slower loss that represents loss of assimilated metal

due to metabolic processes. I chose the 2 h timepoint as the beginning of the second phase of depuration because 2 h is a sufficiently long enough time for egestion to take place in *Uronema* sp. (Sherr et al. 1988). Essentially, the *y*-intercept of the slowly exchanging pool is equal to (the ingested metal – the egested metal)/(ingested metal).

Results

The initial 5 d exposure period of *V. natriegens* to the different radioisotopes resulted in expected volume concentration factors of 1.5×10^6 for ^{55}Fe, 8.4×10^4 for ^{65}Zn, and 4.1×10^4 for ^{241}Am associated with the bacterial cells (Table 5). The radiolabeled *V. natriegens* retained most of the Fe that they had accumulated during the initial 5 d labeling period. After 4 h of resuspension in seawater without ciliates, the cells still retained approx. 88% of the Fe (Fig. 13). *V. natriegens* cells lost Zn to the surrounding water relatively rapidly over the course of 1 h, after which approx. 50% of this metal remained bound to the bacteria. The bacterial cells lost Zn at a slower rate from this point onward and retained 30% of this metal by the final sample time. Americium was also lost relatively rapidly from the bacteria over the first 1 h, at which time 40% of it remained associated with bacterial cells. After 1 h, Am also desorbed from the cells at a slower rate, until 23% of Am remained cell-bound at the final sample time (Fig. 14).

Once the radiolabeled bacteria were available for consumption by the ciliates, approx. 65% of the Fe bound to bacterial cells was transferred to the ciliates due to ingestion of the bacteria (Fig. 15). Of the ^{55}Fe found in the ciliates, up to 15% may have been taken up from the aqueous phase resulting from desorption from bacteria and the remainder from ingestion of the radiolabeled bacteria. The initial dietary uptake of Fe by *Uronema* sp. occurred rapidly and leveled off after 1 h. At the end of the dietary uptake phase, the radioactivity associated with the ciliates had decreased slightly (to 52%), indicating a release due to cycling of the metal. Upon ingestion of *V. natriegens*, roughly

20% of the bacterially-bound Zn was transferred to the ciliates, and approx. 20 to 25% of Am (Fig. 16). For Zn, uptake of desorbed metal may have accounted for up to 9%, and for Am up to 8% (Fig. 16). The assimilation efficiencies of the 3 metals in *Uronema* sp. from ingested bacteria were about 79% for Fe, 29% for Zn, and 24% for Am (Table 5). The mean concentration of each metal μm^{-3} of cell was determined for *V. natriegens* and *Uronema* sp cells using the specific activity of each radioisotope. Biovolume-specific concentrations in bacterial cells were 2-3 times those in the ciliates (Table 5).

At the end of the depuration period (28 h for the Fe experiment, 15 h for the Am and Zn experiments), a mean value of 75.4% of the Fe that the *Uronema* sp. had accumulated by feeding on *V. natriegens* remained in or bound to the ciliates (Fig. 17), whereas only about 20% of the Zn and Am remained associated with the ciliates (Fig. 18). Depuration occurred in two phases; an initial phase characterized by a rapid loss rate of the metal lasting about 1 h, followed by a slower loss phase. Efflux rates (d^{-1}) of assimilated metals from *Uronema* sp. were only calculated for the second, slower loss phase and were 0.1 for Fe, 0.9 for Zn, and 0.7 for Am (Table 5). Efflux rate constants were calculated for the slower (second) loss phase only as this phase represents metal loss due to metabolic activities.

Discussion

Ciliated protozoa have been noted for their potential to be used as indicator organisms to assess the pollution of sediments and water in a variety of systems (Stebbing et al. 1990; Foissner 1999), including the monitoring of toxic effects of heavy metals in sewage sludge (Madoni et al. 1996; Martin-Gonzalez et al. 2005). In addition to their role as bioindicator organisms, ciliates are abundant and ubiquitous in benthic and pelagic environments where they can serve as a link between microbial loop organisms and animals in metazoan food webs. Ciliates provide copepod predators with similar

amounts of C as phytoplankton cells (Rieper 1985) and may even increase their egg hatching success (Heinle et al. 1977). They exhibit growth efficiencies of up to 40% when consuming bacterial cells (Ducklow 1983), indicating a relatively efficient transfer of energy and nutrients between the two trophic levels.

Uronema sp. can ingest about 700 bacterial cells h^{-1}, and the process of digestion occurs within about 25 minutes (Sherr et al. 1988), which accounts for the sharp increase in the percentage of all three metals found associated with the ciliates within only 1 h of feeding on the radiolabeled *V. natriegens*. The fluctuation of radioactivity in the ciliates between sample times during the dietary uptake (seen for Am and Zn) can probably be attributed to cycling of these elements through the cells. Individual protozoan digestive vacuoles have a life span of 20 to 60 min from the time of formation to the point where their remaining contents are expelled (Fok et al. 1982). If ingestion and digestion occur within such short time scales, then it can be expected that the ciliates in our experiments were repeatedly ingesting bacterial cells and excreting waste products over the course of the dietary uptake phase.

When assessing assimilation efficiencies in higher (multicellular) animals, gut passage time is often taken into account as a factor that can have an influence on this process (Wang and Fisher 1999). This is based on the fact that organisms with longer gut passage times generally display high assimilation of contaminants due to the extended time period the digestive enzymes have to act on the food items (Sibly and Calow 1986). Ciliates, on the other hand, produce food vacuoles in which prey items are contained and processed, a process which usually occurs on shorter timescales than it does for higher animals. The microenvironment of the food vacuoles is acidic in comparison to the ciliates' external environment. A pH range between 2 to 4 during active digestion (Howland 1929; Fok et al. 1982) may not only serve to kill ingested cells (Fok et al. 1982), but also create a tendency for metals to occur in soluble form (Millero 1998), and possibly to be transported into the ciliate cytoplasm rather than being egested. This is of interest especially for a metal such as Fe, which is not very soluble in seawater and readily forms colloidal precipitates or oxides that display reduced bioavailability for microorganisms (Rich and Morel 1990). However, the acidity of the food vacuole leads

to a dissolution of this colloidal Fe and therefore an increase of its bioavailable form (Barbeau et al. 1996; Barbeau and Moffett 1998). Once these complexes have dissolved, it is possible that the now soluble metal is transported across the vacuole membrane and into the cytoplasm of the cell leading to its accumulation inside the organism. It is possible that these conditions account, at least partially, for the high assimilation efficiency of Fe that was measured in the current study (79%). In addition, Fe is mostly stored inside the cells, and therefore less likely to be egested by the organisms. Since it is required as an enzyme cofactor for a variety of metabolic reactions, Fe should be retained by the cells rather efficiently as was illustrated by its low efflux rate from the ciliates.

The assimilation efficiency of Fe in the ciliates in our study (79%) was higher than we calculate from Chase & Price's (1997) study of 36% and 25% in the microflagellate *Paraphysomonas imperforata* under high and low Fe exposures, respectively. They found that 58-75% of the ingested Fe was excreted, whereas the ciliates in our study retained 75% of the Fe at the end of the depuration period. Chase & Price (1997) also exposed their bacterial food to greater Fe concentrations, 12.5 nM (low Fe treatment) and 8.4 μM (high Fe treatment), than we used (0.9 nM). Thus, the flagellates in their study ingested substantially greater amounts of Fe than the ciliates in our study, and, together with physiological differences between the two protozoa, this may contribute to the differences between the two sets of assimilation efficiencies.

Of the three metals analyzed here, Fe was also accumulated by heterotrophic marine bacteria to the greatest extent (VCF of 1.5×10^6 in this study). It can be assumed that at least 20% of the bioavailable Fe in surface ocean waters is bound to bacterial cells, and that the majority of it is located inside the cells (Vogel & Fisher, in review). Given the high assimilation efficiency of Fe in *Uronema* sp. in this study, a significant portion of the bacterially-bound Fe is transferred to planktonic ciliates, which may then in turn be consumed by zooplankton, and so on. The relatively low efflux rate of Fe from ciliates ($k_e = 0.1$ d^{-1}) further strengthens the potential of Fe transfer to the next trophic level. Copepods have displayed an Fe assimilation efficiency of 32% when their food consists of ciliates (Twining and Fisher 2004). The assimilation efficiency of Am in ciliates feeding on bacteria was the lowest of the 3 metals analyzed, as might be expected given

that Am is a nonessential metal that mainly remains bound to cell surfaces (Fisher et al. 1983). When bacteria are exposed to Zn over a range of ambient concentrations, less than half of the cellular Zn is transported into the cytoplasm (Keung et al. 2008), consistent with the assimilation efficiency observed for Zn in the ciliates.

We can compare the assimilation efficiencies of metals ingested by ciliates from bacteria to those in copepods feeding on ciliates and phytoplankton cells. Copepods feeding on ciliates assimilated about 32% of Fe and 77% of Zn (Twining and Fisher 2004). Copepods feeding on log-phase diatoms assimilated about 5-16% of ingested Fe (Hutchins et al. 1995), 27% of Zn, and 1% of Am (Reinfelder and Fisher 1991). The greater assimilation of Fe in ciliates than in copepods may be due to physiological differences or requirements between protozoans and crustaceans. Differences in the pH of the digestive organs in ciliates and copepods may also account for these differences. Actively feeding copepods have a gut pH > 7 (Pond et al. 1995), which is significantly higher than that reported by Howland (1929) and Fok et al. (1982) for ciliate digestive vacuoles (pH 2 to 4). As noted above, the higher acidity of the ciliate vacuoles may explain the higher assimilation efficiency for Fe in ciliates than in their predators.

While metals can be taken up by ciliates from the aqueous phase and from diet, the results shown here indicate that the latter predominates. Thus, metals that are assimilated by a predator have the greatest potential to be transferred to the next higher trophic level. Generally, ingested material that is not assimilated is either egested in the form of discrete fecal pellets (e.g., in copepods), or excreted in dissolved form or egested as small amorphous particles, as is the case for ciliates (Elliott and Clemmons 1966; Stoecker 1984). The fecal material produced by ciliates is not densely packaged and tends to break apart into fine particulate matter upon egestion (Elliott and Clemmons 1966; Sieburth et al. 1978). It is therefore unlikely that metals incorporated in these small particles will enter the particulate flux of organic matter that sinks out of the photic zone, as is the case for copepod fecal pellets. Instead, metals not assimilated by ciliates are most likely recycled in surface waters where they, like excreted nutrients, can become available again for accumulation by other planktonic organisms, including bacteria (Prast et al. 2007) and phytoplankton (Ota and Taniguchi 2003). Finally, the lower

concentration of metals observed in ciliates than in their bacterial food is consistent with the general observation that most metals do not display biomagnification in marine food chains, methylmercury and cesium being notable exceptions (Iaea 2004; Mathews and Fisher 2008).

Table 5. Calculated volume concentration factors (VCF) in *V. natriegens* during the initial 5 d labeling period, assimilation efficiencies (AE, in percent) of the 3 metals in *Uronema* sp., daily efflux rates of the metals (k_e) from the ciliates, and concentrations of the 3 metals μm^{-3} in each of the two study organisms at the end of the metal uptake phase (bacterial cells and ciliates).

	Bacterial VCF	**Ciliate AE (%)**	**Ciliate k_e (d^{-1})**	**amol μm^{-3} bacteria**	**amol μm^{-3} ciliate**
Fe	1.50×10^6	79 ± 3	0.1	0.55	0.17
Zn	8.43×10^4	29 ± 2	0.9	0.14	0.07
Am	4.14×10^4	24 ± 1	0.7	0.04	0.02

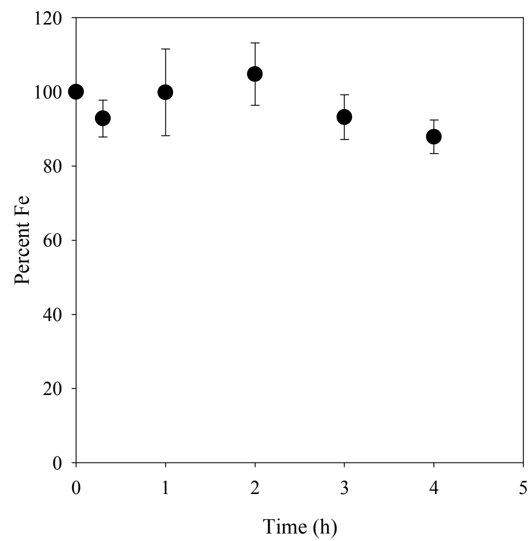

Fig. 13: Percentage of ^{55}Fe retained by *V. natriegens* over time after resuspending the cells into sterile-filtered, unlabeled seawater. Data points are means of 3 replicates with standard errors.

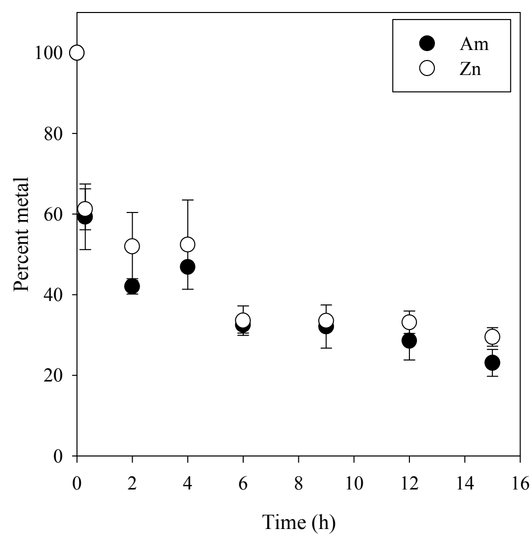

Fig. 14: Percentage of ^{241}Am and ^{65}Zn retained by *V. natriegens* over time after resuspending the cells into sterile-filtered, unlabeled seawater. Data points are means of 3 replicates with standard errors.

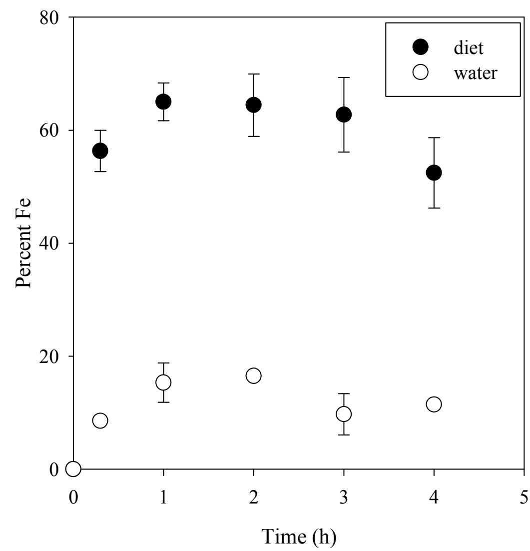

Fig. 15: Percentage of total ^{55}Fe in the culture that was associated with *Uronema* sp. over time while ingesting radiolabeled *V. natriegens* (i.e. "diet"), and accumulation of ^{55}Fe by *Uronema* sp. that had desorbed from *V. natriegens* cells (i.e. "water"). Data points are means of 3 replicates with standard errors.

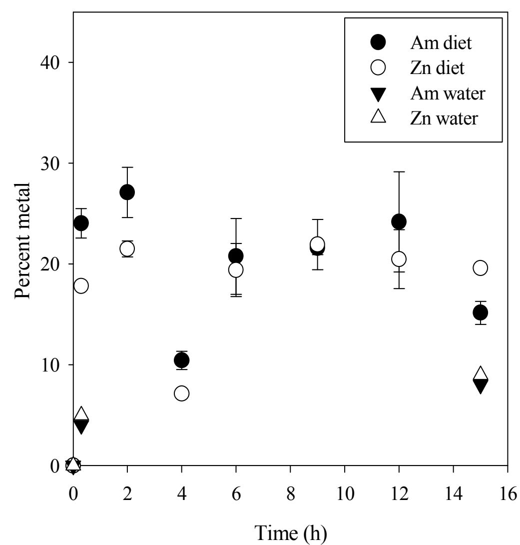

Fig. 16: Percentage of total ^{241}Am and ^{65}Zn in the culture that was associated with *Uronema* sp. over time while ingesting radiolabeled *V. natriegens* (i.e. “Am diet”, “Zn diet”), and accumulation of ^{241}Am and ^{65}Zn by *Uronema* sp. that had desorbed from *V. natriegens* cells (i.e. “Am water”, “Zn water”). Data points are means of 3 replicates with standard errors for dietary uptake.

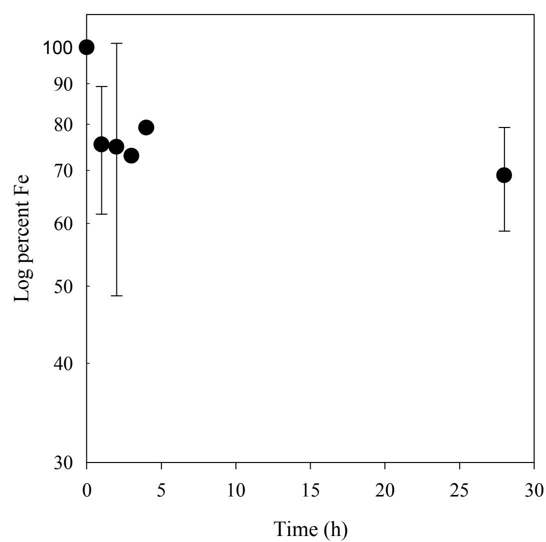

Fig. 17: Percentage of ^{55}Fe retained by *Uronema* sp. over time during the depuration period in unlabeled seawater. Data points are means of 2 replicates with standard errors.

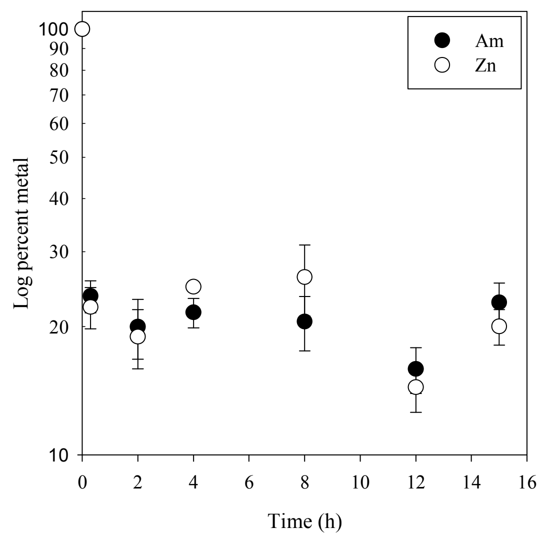

Fig. 18: Percentage of ^{241}Am and ^{65}Zn retained by *Uronema* sp. over time during the depuration period in unlabeled seawater. Data points are means of 3 replicates with standard errors.

Chapter 5:

Accumulation of Fe by heterotrophic marine bacteria and picoplankton cells

Abstract

The accumulation of ^{55}Fe by three species of cultured heterotrophic marine bacteria (*Roseobacter litoralis*, *Vibrio natriegens*, and *Halomonas aquamarina*) was assessed in laboratory experiments using natural seawater collected from the Sargasso Sea and environmentally realistic levels of iron (0.2 nM). An oxalate rinse was used to determine the fraction of intracellular Fe. Fe accumulation proceeded rapidly over the first 10 h of the experiments, after which it seemed to reach a steady-state for all three bacterial species. Volume concentration factors ranged from 1.3×10^6 to 7.4×10^7, with the highest values obtained for the smallest bacterial cells. The maximum fraction of cellular Fe removable with the oxalate rinse was 11%, indicating efficient Fe transport into the cells. Cellular Fe concentrations of the bacteria ranged from 0.07 to 0.10 amol cell^{-1}, which is approx. 7 to 10 times lower when compared to Fe concentrations of the same bacterial species grown in coastal seawater. However, the results fall within the range calculated for a natural assemblage of picoplankton cells collected from the Equatorial Pacific Ocean (0.02 to 0.19 amol Fe cell^{-1}).

Introduction

Iron has been a metal of great interest in the context of oceanic productivity, especially since the recognition that iron can limit primary production in large ocean regions (Martin 1990; Martin et al. 1991). Adding excess Fe to HNLC (high-nutrient low-chlorophyll) regions can lead to increases in algal biomass and possibly growth rates, and may change the community structure of the phytoplankton assemblage by favoring larger cells, in particular diatoms (Martin 1992; Coale et al. 1996). The total bacterial biomass also increases following these nutrient additions (Cochlan 2001), but compared to the species shift observed in the phytoplankton assemblage, these Fe additions seem to have only little effect on the dominant bacterial species in these areas (Hutchins et al. 2001).

Most intracellular Fe is used as a redox catalyst in the respiratory electron transport chain, where it is associated with reductases and cytochrome complexes (Tortell et al. 1999). The biological removal of Fe contributes, along with low solubility and, in some regions, limited input, to its extremely low dissolved concentrations in surface waters. Fe concentrations increase below the euphotic zone where primary producers are absent and biogenic debris decomposes, leading to a remineralization of cellular contents.

Although Fe is abundant in the Earth's crust, it is highly insoluble in oxic seawater where it readily forms thermodynamically stable hydroxides (Stumm and Morgan 1996; Millero 1998), contributing to its low bioavailability to marine organisms. In order to cope with the stress associated with low dissolved Fe concentrations, prokaryotes (Haygood et al. 1993; Wilhelm and Trick 1994; Neilands 1995; Martinez et al. 2000; Macrellis et al. 2001) and some eukaryotes (Trick et al. 1983) have responded by producing specific Fe ligands (siderophores) which have a high affinity for this metal and serve to solubilize it, enabling cells to accumulate it (Butler 1998). The ligand-bound Fe is generally not available for assimilation by phytoplankton (Hutchins et al. 1999). However, some mixotrophic phytoplankters are known to ingest bacterial cells when experiencing Fe limiting conditions, at least on a temporary basis (Maranger et al. 1998). It is partially because of their ability to produce siderophores (Neilands 1983) that marine bacteria have a competitive advantage over other cells to accumulate the bioavailable Fe

within a system. In oligotrophic areas like the Sargasso Sea, bacteria can account for more than 70% of the microbial C and N, and most of the biological surface area (Fuhrman et al. 1989).

In this study, I measured the accumulation of Fe from oligotrophic seawater by several cultures of heterotrophic marine bacteria in laboratory experiments, and compared the total Fe accumulated to that transported into the cells. The results are also compared to Fe accumulation results obtained for the same bacterial species when they were exposed to Fe in coastal seawater. In addition, results from shipboard radiotracer experiments conducted in Fe-limited Equatorial Pacific waters that investigated the Fe uptake by naturally occurring picoplankton communities are included.

Materials and methods

For laboratory experiments, cultures of *Roseobacter litoralis*, *Vibrio natriegens*, and *Halomonas aquamarina* (see Table 6) were maintained on marine agar (1 L seawater, 5 g Bacto peptone, 1 g yeast extract) slants stored at 4 °C that were transferred periodically. In preparation for experiments, a small inoculum of each culture was transferred to 10% marine broth (1 L seawater, 0.5 g Bacto peptone, 0.1 g yeast extract) and the bacteria were allowed to grow for several days until the cells reached log phase.

The seawater used for culturing and experimental purposes was collected in trace metal clean fashion from the Sargasso Sea (25°38.023'N, 77°26.804'W), in the vicinity of the Bahamas Islands, and for the purpose of this study will be referred to as Bahamas seawater (BSW). The water was stored in acid-washed containers from the time of collection until it was used for experiments. The experiments were conducted without amending the water with additional nutrients or synthetic metal chelators. The Fe concentration in BSW was 1.0 nmol L^{-1} (B.S. Twining, Bigelow Laboratory for Ocean Sciences, personal communication).

Bacterial cell counts were performed by fixing cell samples with 2% borate buffered formaldehyde and staining the samples with 4'6-diamidino-2-phenylidole (DAPI). The stained cells were collected on 0.2 μm black polycarbonate filters and processed using epifluorescence microscopy according to the protocol outlined by Sherr et al. (2001). Slides were analyzed under UV excitation using an inverted Leica DM IRB microscope. All cell count samples were obtained and processed in this manner for the experiments described here.

Sterile 300-ml polycarbonate tissue culture flasks with screw caps, containing the BSW as growth medium, were incubated at 17°C for the duration of the experiments. Each flask received an addition of the beta emitter ^{55}Fe ($t_{½}$ = 996 d) in the form of $FeCl_3$, obtained from NEN/Perkin Elmer Life Sciences, to yield a final concentration of 0.2 nmol L^{-1} (16.7 kBq L^{-1}). In order to account for the small amount of acid introduced with the radioisotope addition, an equimolar amount (microliter volumes) of NaOH was also added to each volume of seawater. The radioisotope was allowed to equilibrate for 24 h with the seawater before the bacteria were added to the flasks at a starting density of 1.5 x 10^6 cells ml^{-1}. For this inoculation, the cells were collected by centrifugation (10 minutes at 1,400 *g*), and rinsed with sterile, 0.2 μm seawater before resuspending the cells into sterile-filtered (0.2 μm) seawater in the experimental flasks. One set of flasks contained only seawater and the radioisotope (no bacterial cells) to assess the sorption of ^{55}Fe to the vessel walls and filter membranes in the absence of cells.

All experimental flasks were swirled periodically to ensure that the cells remained distributed evenly and to avoid localized Fe depletions. Samples were removed from the flasks periodically to determine the fractionation of the added ^{55}Fe over time, as well as to obtain bacterial cell counts. For this purpose, different kinds of samples were removed at each timepoint: a 1 ml unfiltered sample containing ^{55}Fe that was associated with the cells as well as ^{55}Fe that remained in the dissolved phase (< 0.2 μm), and a 10 ml filtered (0.2 μm, Nuclepore polycarbonate filters) sample that contained only ^{55}Fe that was associated with the bacterial cells. Filtered bacteria were rinsed with 10 ml of filtered seawater to remove any unbound Fe from cell and filter surfaces (Fisher et al. 1983). An additional 10 ml filtered sample was washed with an oxalate reagent (Tovar-Sanchez et

al. 2003) to remove surface-bound Fe from the bacteria. The two types of filtered samples were used to determine the fraction of intracellular Fe compared to the total Fe associated with the bacteria. All radioactive samples were counted on a Packard Tri-Carb 2100 TR Liquid Scintillation Analyzer.

After subtracting the radioactivity associated with the filter membranes from the control flasks, the association of the radioisotope with the bacteria was expressed as a concentration factor on a volume/volume basis. These volume concentration factors (VCFs) were calculated by dividing the radioactivity μm^{-3} of bacterial cells by the radioactivity μm^{-3} in the dissolved phase (< 0.2 μm) in the ambient seawater, as described in Fisher et al. (1983).

Field data from the Pacific was collected on an Equatorial Biocomplexity cruise in September 2005 (lead scientist: D. M. Nelson). The cruise track consisted of two transects; the first transect was located along 140°W (from approx. 3°N to 3°S) and the second transect was located along the Equator from approx. 135°W to 110°W. The locations of water collection for the data included were Stations 9, 15, 18, 22, and 23 (Table 7). At each station, water was collected from water depths of 20-30 m using a trace metal clean rosette and transferred into 3 trace metal clean polycarbonate bottles that were stored in flow-through deck incubators at 25 to 30% incident light, approximating the light level at the depth of water collection. Experimental bottles received additions of ^{14}C and ^{55}Fe in order to measure Fe:C uptake rates in picoplankton (measuring between 0.2-3.0 μm) cells. Table 7 provides information on sample station locations, ambient dissolved Fe concentrations (C. Measures, personal communication) and added ^{55}Fe concentrations. ^{55}Fe was added at tracer levels without EDTA to control for Fe complexation.

After 4 d, the picoplankton cells contained in the sample bottles were filtered and collected on 0.2 μm filters. Filters were rinsed either with filtered seawater or with oxalate in order to remove externally sorbed Fe from the cells. The filtration volumes consisted of 2 replicates of 50 ml each for unrinsed and rinsed picoplankton (0.2 μm filters), and 2 replicates of 250 ml each for unrinsed and rinsed nanoplankton (3.0 μm filters) per sample bottle. Results for the nanoplankton size fraction are not included as

part of this study. Unfiltered samples (3 replicates of 4 ml each) were also removed, and all samples were analyzed using a liquid scintillation counter.

^{14}C uptake data collected from the same experiments was used here to estimate the average biomass of picoplankton cells collected in the filtration steps described above at each station. For this purpose, an average carbon content of 110 fg C cell^{-1} was assumed. The reasoning was that a large portion of the picoplankton collected consisted of heterotrophic bacteria and cyanobacteria (M. Landry, personal communication). I arrived at this estimate by using average reported values of 20 fg C cell^{-1} for heterotrophic bacteria (Lee and Fuhrman 1987) and 200 fg C cell^{-1} for cyanobacteria (Caron et al. 1995) combined with abundance assessments for each cell type. Cellular Fe concentrations in the picoplankton fraction were calculated in a similar fashion, assuming again a C content of 110 fg cell^{-1} and using the ^{14}C:^{55}Fe obtained from the incubation experiments at each station.

Results

For the laboratory experiments, bacterial cultures in all replicate flasks grew steadily throughout the experiment and without a noticeable difference in growth pattern between the three species (Fig. 19 and Table 8). Figure 2 shows that the accumulation of ^{55}Fe by the bacterial cultures can be divided into two phases: an initial, comparatively fast accumulation rate spanning the time period from 0 to approx. 10 h, and a period of slower accumulation after this point. Fe accumulation increased steadily up to 10 h for *R. litoralis* and *H. aquamarina*, after which it leveled off (for *R. litoralis*) or continued to increase only slightly (for *H. aquamarina*). *V. natriegens*, on the other hand, showed a rapid accumulation of Fe for the first 4 h, after which the cells released a small fraction of the metal before approaching a steady-state in metal accumulation. The average percentage of ^{55}Fe associated with the cells after the 10 h timepoint to the end of the

experiment was 89-97% for *R. litoralis*, 62-71% for *V. natriegens*, and 68-89% for *H. aquamarina* (Fig. 20). Rinsing the filtered samples with the oxalate reagent resulted in only minimal removal of ^{55}Fe from bacterial surfaces (Fig. 20). The maximum percentage of Fe removed from cells was 11% for *V. natriegens* at one sampling point, and remained below 10% in all other cases. No significant difference in the concentration of Fe ($P > 0.05$) between unrinsed and rinsed cells in any of the three bacterial species was noted.

Volume concentration factors (VCFs) were calculated for the time period from 10 to 52 h and the average values were 7.4×10^7 for *R. litoralis*, 1.8×10^6 for *V. natriegens*, and 8.2×10^6 for *H. aquamarina* for the unrinsed bacterial cells. Upon rinsing the cells with oxalate, average VCFs were 6.1×10^7 for *R. litoralis*, 1.3×10^6 for *V. natriegens*, and 2.5×10^7 for *H. aquamarina* (Table 9). As reported above for the percentage of Fe accumulation by the cells, VCFs for any one species did not differ significantly between rinsed and unrinsed cells. However, *R. litoralis* accumulated significantly more Fe than both *V. natriegens* (for rinsed and unrinsed cells) and *H. aquamarina* (unrinsed cells only) ($P < 0.01$). Fe accumulation expressed as VCF was also significantly higher ($P < 0.01$) for *H. aquamarina* than for unrinsed *V. natriegens*. Average Fe concentrations per cell were calculated for the final timepoint. Values were 0.10 and 0.09 amol cell^{-1} for unrinsed and rinsed cells of *R. litoralis*, respectively, 0.07 amol cell^{-1} for *V. natriegens*, and 0.09 amol cell^{-1} for *H. aquamarina* in both unrinsed and rinsed treatments (Table 10).

Fe sorption to filter membranes was measured in the control flasks and accounted for 2-10% of Fe in the particulate phase when the filters were rinsed only with sterile seawater, and ranged between 1-2% when the filters were rinsed with oxalate (data not shown).

For the experiments conducted in the Equatorial Pacific, estimates (using the conversion factors described above) of picoplankton cell counts at the different stations ranged from 2.10×10^5 to 8.76×10^5 cells ml^{-1} (Table 7). Calculated mean cellular Fe concentrations for the picoplankton cells ranged between 0.02 to 0.19 amol Fe cell^{-1} (Table 7).

Discussion

A key finding of the laboratory experiments conducted in BSW was that the fraction of bioavailable Fe (expressed as percent Fe accumulated by the bacteria) was relatively high, possibly indicating a low concentration of natural ligands contained in the seawater. Cells of *V. natriegens* demonstrated the lowest Fe accumulation out of the three bacterial cultures, but still accumulated 62 to 71% of the total Fe (Fig. 20). Prior to inoculating the water used in the experiments, the radioactive Fe was allowed to equilibrate for 24 h with the seawater and whatever natural ligands were present. Any complexation of the added Fe with natural ligands contained in the water would have been expected to have occurred during this time (Wu and Luther 1995). Cells of *R. litoralis* accumulated between 89 to 97% of the Fe, which was the highest observed among the three species included here, a result which is at least partially due to its cell size. Compared to *H. aquamarina* and *V. natriegens*, *R. litoralis* is characterized by the smallest cell size and cell volume. This same trend also emerged when we measured Fe accumulation of the same bacterial species grown in Southampton seawater (SHSW) from off the coast of Long Island, NY. Similarly, for phytoplankton, smaller cells with higher surface-to-volume ratios are often characterized by higher Fe:C ratios (Twining et al. 2004) and become more enriched in many other particle-reactive metals than larger cells (Fisher 1985).

Detailed results from the Fe uptake experiments conducted in SHSW are described in Vogel and Fisher (in review). These experiments employed identical incubation conditions, bacterial cultures, initial cell inocula, sampling procedures, and concentration of ^{55}Fe added to the experimental flasks as with BSW experiments. Therefore, it is possible to make a direct comparison of Fe uptake dynamics between BSW and SHSW.

For all three bacterial species, and including oxalate-rinsed and unrinsed cells, VCFs for Fe are consistently and considerably higher for the cells maintained in BSW than for those in SHSW (Table 9). The differences are significant ($P < 0.05$) for *R. litoralis* when comparing Fe accumulation from the two water types in rinsed and unrinsed cells, as well as for *V. natriegens* in the oxalate rinsed cells, and *H. aquamarina*

in the unrinsed cells. Since the ^{55}Fe additions were identical for both BSW and SHSW, there must be a difference in the naturally available Fe that was present in the seawater already, or the bioavailability might be differentially affected by ligands. However, the most likely explanation for the differences in VCFs between the two water types is that SHSW has a 14-fold higher background Fe concentrations than BSW (14 nM Fe in SHSW (see Vogel and Fisher, in review) vs. 1 nM in BSW (B. S. Twining, personal communication)). It is therefore likely that the bacteria in SHSW did not need to accumulate as high a fraction of the added radioactive Fe as the cells in BSW in order to function, resulting in lower VCFs. This result also emerges when comparing the percentage of ^{55}Fe associated with bacterial cells in BSW and SHSW (Fig. 21). This is a valid comparison because the initial cell densities in both sets of experiments were identical and the growth rates were comparable as well.

The cells of *R. litoralis* were by far the most enriched in Fe compared to the other two species, regardless of water type or treatment (with or without oxalate rinse), probably due to its small cell size, as described above. *V. natriegens* had the lowest Fe accumulation. It is a commonly occurring marine bacterium, more representative of coastal, or nutrient-rich, environments (Oliver and Colwell 1973). In addition to its relatively larger cell size, it is also possible that its Fe uptake pathway is slightly less efficient as a result because of an adaptation to higher ambient Fe levels and a lowered requirement for competitive Fe uptake or ligand production. The *Roseobacter* clade, on the other hand, often includes members of open-ocean communities, including the Sargasso Sea (Selje et al. 2004; Venter et al. 2004) and in addition constitutes one of the major lineages of marine bacteria that is cultivable in the laboratory (Wagner-Dobler and Biebl 2006), suggesting that *R. litoralis* may be a good representative of naturally-occurring prokaryotes.

Comparing our results from the BSW experiments to those obtained by growing the bacteria in SHSW also indicates that differences in the natural nutrient concentrations in the two water types did not have an effect on bacterial growth rates (Table 8). While BSW is representative of oligotrotrophic ocean water, SHSW represents more nutrient rich coastal waters and it would have been reasonable to expect the bacteria grown in

BSW to exhibit slower growth than in SHSW. However, cell divisions occurred at a rate of 0.9 to 1 per day (Table 8) for all three cultures and in both water types. Fuhrman et al. (1989) measured bacterial doubling rates of 5 to 12 d in oligotrophic ocean waters that are characterized by a lower availability of C sources due to lower phytoplankton concentrations, and Landry et al. (1996) reported generation times of 3 to 14 d in oligotrophic Pacific waters. These samples also represent the impact of grazing on bacterial abundances, and it is therefore not surprising that growth rates reported from the current experiments are higher than those reported from the above mentioned field studies. In contrast to open ocean populations, bacteria can divide about once per day in more eutrophic coastal regions (Fuhrman and Azam 1980; Williams 1981).

Although the Fe uptake experiments in the Equatorial Pacific did not investigate heterotrophic bacteria as a separate entity, they were most likely present in the picoplankton size fraction to a substantial degree. Landry et al. (1996) describe that picoplankton are the dominant planktonic group in the central Equatorial Pacific and that the heterotrophic bacteria are the predominant cells within this group, especially in the upper euphotic zone. In the Sargasso Sea as well, heterotrophic bacteria account for over 90% of the biotic surface areas and are the predominantly occurring microbes to great depths (Fuhrman et al. 1989). The other members included in the oceanic picoplankton group are the autotrophic *Prochlorococcus* spp., *Synechococcus* spp., and some small autotrophic eukaryotes (Landry et al. 1996). Most of these ocean regions are characterized by low Fe concentrations, and it has previously been demonstrated that this Fe limitation tends to select for smaller cell types with high surface area to volume ratios (Morel et al. 1991; Sunda and Huntsman 1995), providing the smaller cells with a competitive advantage in gaining access to limiting nutrients such as Fe.

We can compare cellular Fe concentrations from our laboratory and field experiments to those of previous studies. Using a bacterial biomass conversion factor of 20 fg C $cell^{-1}$ (Lee and Fuhrman 1987) Tortell et al. (1996) measured an average concentration of 0.02 amol Fe $cell^{-1}$ for heterotrophic bacteria collected in the subarctic ocean, a result that is only slightly lower than the values reported for bacteria grown in BSW in our laboratory experiments (0.07 to 0.09 amol Fe $cell^{-1}$ in oxalate rinsed cells,

Table 10). In contrast, the cellular Fe values calculated for bacteria grown in SHSW were measurably higher, at 0.48 to 1.04 amol Fe $cell^{-1}$ (Table 10). This difference is probably due to the higher ambient Fe concentration in SHSW. If the same biomass conversion factor of 20 fg C $cell^{-1}$ is applied to picoplankton samples collected in the Southern Ocean (Twining et al. 2004), an average cellular Fe concentration of 0.03 to 0.06 amol Fe results. In the Twining et al. (2004) study, the cellular assemblage sampled consisted of cells sized between 0.2 to 1.0 µm, which of course not only included heterotrophic bacteria, but also cyanobacteria and possibly some picoeukaryotic phytoplankton cells. The same is true for the data collected from the Equatorial Pacific, in that the cells included in the picoplankton size class represent different types of organisms, including heterotrophic and cyanobacteria. The Fe concentrations calculated for the picoplankton cells from the Equatorial Pacific span the range of values obtained from the bacterial cultures grown in BSW, as well as the results reported in Tortell et al. (1996) and Twining et al. (2004).

It is difficult to make direct comparisons between the laboratory and field components of this study. One important common characteristic is that any ^{55}Fe that was added to the sample flasks in the field and laboratory experiments was allowed to equilibrate with the natural seawater and the ligands contained in it. However, the laboratory experiments analyzed Fe uptake in monocultures of bacteria whereas the field experiments not only included different species of heterotrophic and cyanobacteria, but grazers as well during the initial 4 d incubation. In addition, depending on the contribution of heterotrophic bacteria vs. cyanobacteria at any one station, the above calculated cellular Fe concentrations may be an under- or overestimate. If the entire picoplankton assemblage consisted of only heterotrophic bacteria, cellular Fe concentrations would be lower than reported here. Similarly, Fe concentrations would be higher if the picoplankters consisted solely of cyanobacteria.

The current laboratory experiments also demonstrate that most bacterially-associated Fe is located intracellularly and not removable by rinsing with oxalate. The maximum percentage of Fe removed with the oxalate rinse was 11%, which is on the lower end of reported ranges for externally bound (and removable) Fe. Ho et al. (2003)

reported that approx. 20 to 30% of Fe in cultured phytoplankton cells is externally bound, however field results from the Equatorial Pacific indicate that surface-bound Fe may account for as little as 10 to 30% of the total Fe accumulated in planktonic cells after applying an oxalate rinse (S. B. Baines, personal communication). Over 90% of the Fe contained in heterotrophic cells is known to be utilized in the respiratory electron transport chain, where it is associated with reductases and cytochrome complexes (Tortell et al. 1999). Although Fe is a highly particle-reactive element, only a small fraction (< 5%) of the bioavailable Fe is accounted for by abiotic sorption (Maldonado and Price 1999), illustrating the efficiency of cellular Fe uptake systems. Much of this accumulation is accomplished by marine bacteria (heterotrophic bacteria and cyanobacteria), many of which have the ability to produce siderophores (Neilands 1993; Wilhelm and Trick 1994; Martinez et al. 2000), providing them with a competitive advantage in Fe acquisition.

Whereas adding Fe to natural waters usually results in changes in the phytoplankton assemblage by favoring larger cells, the main response of the bacterial community is an increase in biomass and is not necessarily accompanied with a shift in species composition (Hutchins et al. 1999; Boyd et al. 2000; Cochlan 2001). It is somewhat ambiguous whether the increased bacterial abundance is a direct result of the release of the Fe limitation (Pakulski et al. 1996), or whether the bacterial response is due to the increase in DOC available due to the phytoplankton bloom (Church et al. 2000; Kirchman et al. 2000). The lack of a change in dominant bacterial species upon Fe fertilization justifies using bacterial strains that are commonly isolated from the marine environment for the laboratory experiments instead of working with a natural bacterial assemblage.

Free-living marine bacteria are abundant and ubiquitous, present at approximately 10^6 cells ml^{-1} in surface waters. In addition, marine bacteria account for about half of the POC in the oceans and represent at least 80% of biotic surfaces in the euphotic zone (Cho and Azam 1988; Fuhrman et al. 1989). Because of their small cell size, bacteria are characterized by large surface area to volume ratios. It is therefore not surprising that bacteria accumulate a variety of metals, including Fe, to very high levels. In the case of

Fe accumulation, many bacteria of course have the added advantage over other planktonic organisms of effectively sequestering this essential trace metal due to their ability to produce Fe-binding ligands (Trick 1989; Granger and Price 1999). Among other things, the laboratory experiments described above enabled us to determine whether Fe is accumulated by cultured marine bacteria differently when the cells are grown in different water types, particularly in waters characterized by different ambient Fe concentrations. Metals that are accumulated by bacteria can be reintroduced into the water column in dissolved form upon lytic events (Gobler et al. 1997; Poorvin et al. 2004; Mioni et al. 2005), or can be introduced into the grazing food chain upon ingestion by bacterivorous protists (Chase and Price 1997; Vogel and Fisher 2009). Fe is a metal that is not only of interest because of its status as an essential, and often limiting, micronutrient for microorganisms, but also because it has been shown that it is accumulated to a large extent by prokaryotes and that it is cycled effectively through the surface water column by these above mentioned processes.

Table 6. Cell characteristics of bacterial species utilized in ^{55}Fe accumulation.

Species	**Strain**	**Volume (μm^3)**	**Surface to volume (μm^2 μm^{-3})**
Vibrio natriegens	Pwh3a	0.33	3.43
Halomonas aquamarina	DSM 4739	0.15	6.44
Roseobacter litoralis	DSM 6996	0.09	5.27

Table 7. Information on data collected from field experiments conducted in the Equatorial Pacific, locations of water collections, ambient dissolved Fe concentrations (dFe), additions of ^{55}Fe, estimated cell counts based on C data (assuming 110 fg C $cell^{-1}$) and calculated cellular Fe concentrations.

Station #	Location	dFe (nmol L^{-1})	^{55}Fe (pmol L^{-1})	Cells ml^{-1} ($x10^5$)	amol Fe $cell^{-1}$
9	0.5°N 140°W	0.500	14.61 ± 0.98	3.58 ± 1.36	0.19 ± 0.01
15	2.5°S 140°W	0.344	9.38 ± 0.54	2.10 ± 0.07	0.08 ± 0.02
18	0°N 132.5°W	0.400	38.64 ± 1.20	4.54 ± 0.36	0.04 ± <0.01
22	0°N 123.5°W	0.135	15.40 ± 4.33	8.76 ± 1.96	0.02 ± <0.01
23	0°N 125°W	0.259	19.17 ± 1.00	6.48 ± 1.64	0.07 ± 0.02

Table 8. Growth rates (μ) in divisions d^{-1} for bacterial cultures during experiments conducted in Bahamas seawater (BSW) and Southampton seawater (SHSW). Growth rates are mean values of three replicates for each culture and are calculated spanning the time period from t_0 to t_{76}.

	BSW	**SHSW**
R. litoralis	0.9	0.9
V. natriegens	1.0	0.9
H. aquamarina	0.9	1.0

Table 9. Volume concentration factors (VCFs) for *R. litoralis*, *V. natriegens*, and *H. aquamarina* with respect to ^{55}Fe accumulation. Values represent the means of three replicates with standard errors over the time between 10 to 56 h during the experiment. VCFs listed are for unrinsed cells and oxalate-rinsed cells for each bacterial species. Values marked (*) are adapted from Vogel and Fisher (in review).

	BSW		**SHSW ***	
	VCF, no rinse	**VCF, oxalate**	**VCF, no rinse**	**VCF, oxalate**
R. litoralis	$7.4 \times 10^7 \pm 1.5$	$6.1 \times 10^7 \pm 1.2$	$2.8 \times 10^6 \pm 0.9$	$2.7 \times 10^6 \pm 0.5$
V. natriegens	$1.8 \times 10^6 \pm 0.3$	$1.3 \times 10^6 \pm 0.3$	$9.9 \times 10^5 \pm 0.9$	$6.0 \times 10^5 \pm 0.3$
H. aquamarina	$8.2 \times 10^6 \pm 1.7$	$2.5 \times 10^7 \pm 1.2$	$9.3 \times 10^5 \pm 0.4$	$1.1 \times 10^6 \pm 0.6$

Table 10. Bacterial Fe concentration expressed as amol Fe $cell^{-1}$ at the end of experiments in Bahamas seawater (BSW) and Southampton seawater (SHSW) with and without an oxalate rinse. Data points are means of three replicates per bacterial culture, including standard errors. Values for cells grown in SHSW (*) are adapted from Vogel and Fisher (in review).

	BSW		**SHSW***	
	no rinse	**oxalate**	**no rinse**	**Oxalate**
R. litoralis	0.10 ± 0.02	0.09 ± 0.02	1.04 ± 0.12	0.91 ± 0.10
V. natriegens	0.07 ± 0.01	0.07 ± 0.01	0.55 ± 0.04	0.48 ± 0.03
H. aquamarina	0.09 ± 0.002	0.09 ± 0.001	0.60 ± 0.09	0.66 ± 0.14

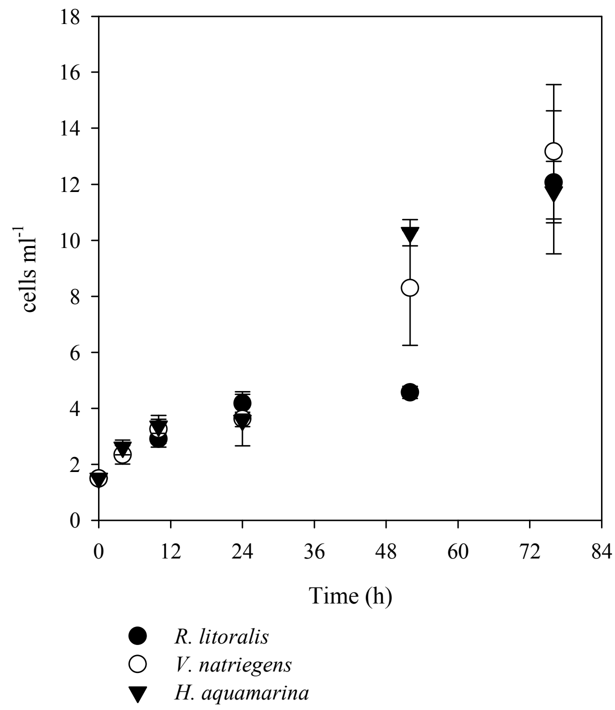

Fig. 19: Cell counts of the cultured bacteria *R. litoralis*, *V. natriegens*, and *H. aquamarina* in Bahamas seawater (BSW). Data points are means of three replicates for each bacterial species, including standard errors.

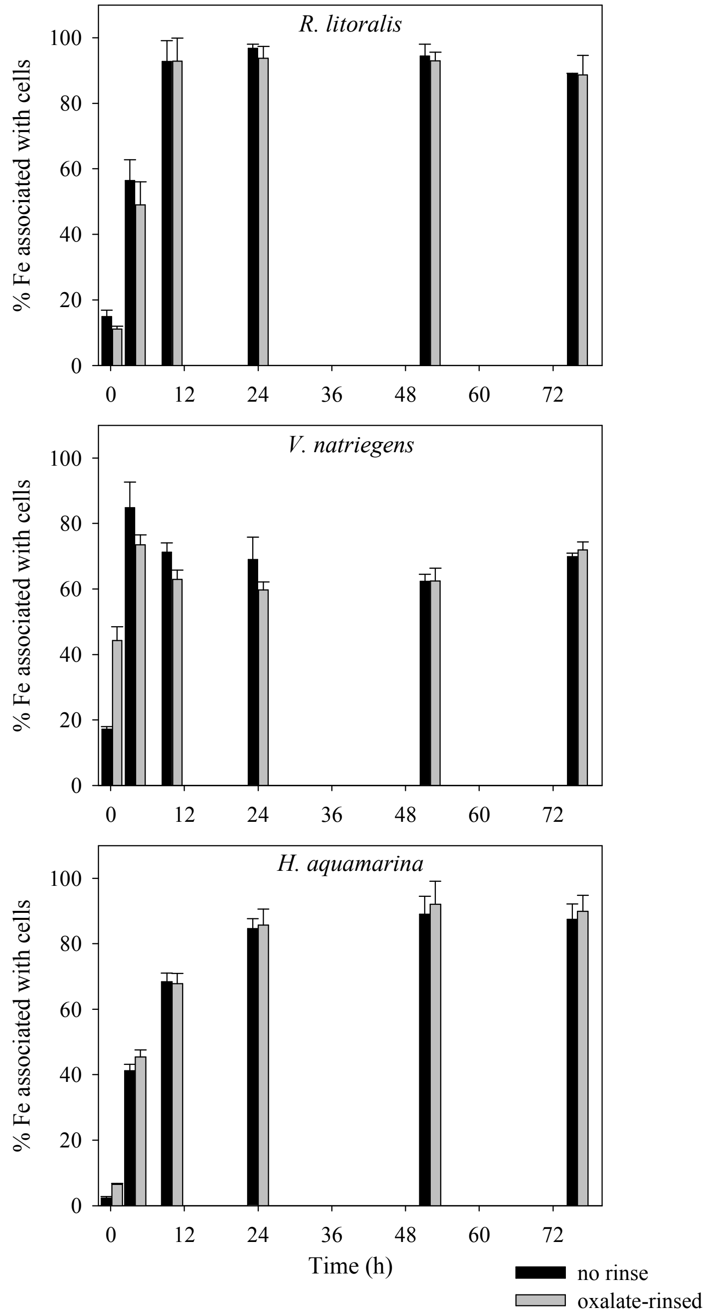

Fig. 20: Percentage of ^{55}Fe associated with unrinsed (dark bars) and oxalate-rinsed bacterial cells (lighter bars) of *R. litoralis*, *V. natriegens*, and *H. aquamarina* in BSW. Values are means of three replicates with standard errors.

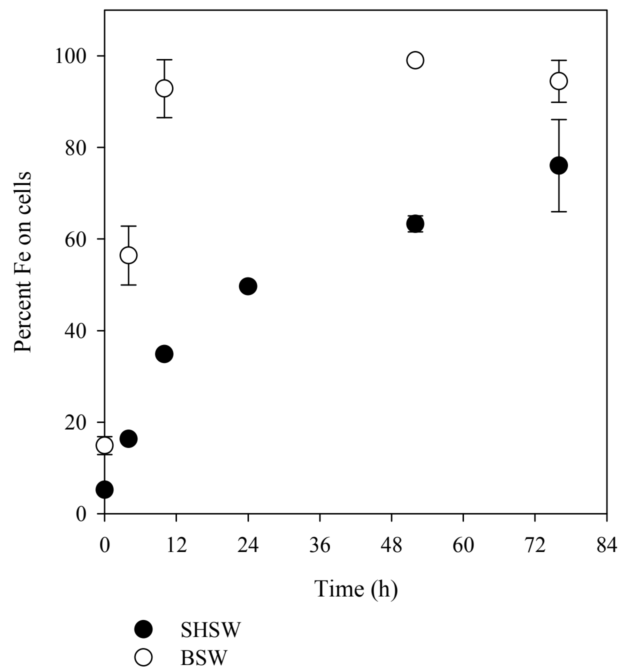

Fig. 21: Percentage of ^{55}Fe associated with cells of *R. litoralis* over time in Southampton seawater (SHSW) and Bahamas seawater (BSW). Data points are means of three replicates with standard errors.

Chapter 6:

Summary and conclusions

The general purpose of this study was to elucidate the role of planktonic, Gram-negative heterotrophic bacteria in the accumulation and cycling of several metals that are found in the oceans in dissolved form. Some metals fulfill a function as micronutrients and are therefore of interest in terms of plankton productivity, biogeochemical cycling and trophic transfer. Other metals may be toxic contaminants and are of interest with regard to biomagnification as they move through food webs, potentially to humans where they may exert public health impacts. Sparse information is available on this topic in the scientific literature with respect to heterotrophic bacteria in the surface ocean. While much is known about the mechanics of metal uptake by cultured bacterial cells (Beveridge and Doyle 1989, Beveridge and Murray 1980, Ehrlich 1997), relatively little information is available on the biomagnification of those metals in marine bacteria. For this purpose, I exposed five bacterial cultures to radioisotopes of six different metals: ^{54}Mn, ^{55}Fe, ^{65}Zn, ^{109}Cd, ^{137}Cs, and ^{241}Am. The metals were added at trace level concentrations to nutritionally unamended, natural seawater. The metals were chosen to represent different chemical and biological properties. Fe and Am were included because they are both highly particle-reactive elements, with Fe at the same time also representing a biologically essential micronutrient. In contrast, Am, a trivalent cation, was included as a nonessential metal but one that is similar in its geochemical behavior to most lanthanides and some actinides. Mn and Zn were also included for their status as essential metals. Cd does not have a known biological function except as a possible replacement for Zn in the enzyme carbonic anhydrase in some phytoplankton taxa. Cs was included in this study to serve as a contrast to the other metals; it has no biological function and does not associate with particles to a great extent in seawater, but has been shown to biomagnify in marine food webs. The bacterial species tested for their metal accumulation potential included *V. natriegens*, *Vibrio* sp., *R. litoralis*, *P. alteromonas*, and *H. aquamarina*. All of these prokaryotes are commonly isolated from either open ocean or coastal environments, and especially *R. litoralis* is known to be a common and major constituent of many naturally occurring bacterial assemblages (Gonzalez and Moran 1997; Wagner-Dobler and Biebl 2006).

The main finding of this set of this dissertation was that the six metals are accumulated to varying degrees by these bacterial isolates, but that interspecific differences among the bacteria tested for any one metal are relatively small and most likely related to cell size. The order of association of the metals with these bacteria with respect to the magnitude of their volume concentration factors was Fe > Am ≥ Mn ≥ Cd > Zn > Cs. Mean volume concentration factors were 0.6-2.8 x 10^6 for Fe, 0.6-3.5 x 10^4 for Mn and Am, 0.2-4.8 x 10^4 for Cd, 0.5-1.6 x 10^3 for Zn, and 1.9 x 10^2 for Cs. This order of metal accumulation is somewhat similar to what is reported for phytoplankton in general, however, there are some differences between the two planktonic groups. For example, compared to autotrophic picoplankton (Fisher 1985), heterotrophic bacteria accumulate Mn by about one order of magnitude more, and Zn by about one order of magnitude less, with the two metals' position in the rank order of accumulation reversed. This result illustrates that the magnitude and the order of metal accumulation is not necessarily the same for all planktonic groups, even if cells of similar sizes are compared.

Exposing bacterial cells to an oxalate rinse did not result in a significant removal of Fe for any of the bacterial species tested, indicating that most of the Fe is transported into the cells, and not merely sorbed to their surfaces. In contrast, rinsing cells with EDTA resulted in significantly lower concentration factors of Mn and Am in at least one species. Metal accumulation by the bacteria followed a general pattern of rapid initial accumulation over mostly 10 to sometimes 24 h, followed by a much slower phase of accumulation during the reminder of the experiments during which the cells often seemed to approach a steady state in terms of metal concentration. Even though the seawater used in all of the experiments described herein was not amended with nutrients, it should be noted that the bacteria exhibited normal growth patterns which was verified by performing cell counts periodically. In addition to quantifying metal uptake, the experiments also demonstrated that certain metals are taken up passively (i.e. Zn), while others follow active uptake pathways (i.e. Mn), as is indicated by responses to incubation temperatures (Q_{10} effect).

In addition to the results noted above, it would be very interesting to measure the metal uptake rates by these prokaryotes in more detail. One of the limitations of my

experimental design with respect to obtaining uptake rates is that the time between samples was too long, since samples were generally taken 2 to 4 h apart at the beginning of some experiments, while the time for Cd and Am was 12 h. In order to obtain meaningful and clearer results it may be necessary to design an experiment where samples (including samples for cell counts) are taken in intervals on the order of minutes, rather than hours. By so doing, it would be possible to determine whether metal uptake rates are different for essential elements such as Fe, Mn, and Zn, compared to the nonessential metals Cd, Am, and Cs. It is likely that an essential micronutrient will be taken up and transported into the cells at a faster rate than a nonessential metal, especially when the micronutrient is present in low, even limiting, concentrations.

These results demonstrate that heterotrophic bacteria should not be overlooked when biogeochemical cycling of metals in the oceans is considered. While these prokaryotes may not accumulate a significant fraction of every metal dissolved in seawater, their accumulation potential of at least some metals, for example Fe, is considerable. In that respect, bacterial metal accumulation needs to be included in considerations of metal cycling in the ocean. Metals associated with bacteria tend to remain suspended in surface waters, unless the cells get incorporated into fecal pellets or other dense aggregates that are prone to sinking out of surface waters. Therefore, metals that are associated with planktonic prokaryotes are less likely to be involved in metal export to the deep ocean, but are more likely to remain in the surface layers where they are recycled repeatedly through biological activity. Given that approximately half of the particulate organic carbon in the oceans is present in the form of marine bacteria (Cho and Azam 1988), and that these cells account for a large fraction of biological surface areas in the surface ocean (Azam 1984, Fuhrman et al. 1989), bacterial metal accumulation is of interest for a variety of reasons.

Viruses comprise the only entity of biological particles that is numerically more abundant than bacteria in the surface ocean. In addition to their high abundance, viral particles are also exceedingly diverse in their genetic makeup and with respect to their host organisms, which includes bacteria (Suttle 2005). One way in which viral particles may have an effect on metal cycling in surface waters is by infecting and lysing bacterial

cells, which in turn may release the bacterially-bound metals into the water where they can become biologically available to other uninfected planktonic organisms, including bacteria and phytoplankton. The two main questions addressed with the viral lysis experiments were whether virally lysed bacteria release a larger or different fraction of metals than unlysed bacteria, and whether the metals that are released from lysed and unlysed bacteria are differentially bioavailable to uninfected bacteria and diatoms. For this purpose, the radioisotopes ^{55}Fe, ^{65}Zn, ^{109}Cd, and ^{241}Am were tested. Out of the four metals, Fe was released to the greatest extent (by about 80%) and there was no difference between the release from lysed and unlysed bacterial cells. The release of Am also did not differ between lysed and unlysed bacteria, and was the smallest out of the metals tested. In contrast, Cd and Zn were released to a greater degree by lysed bacterial cells, and in the case of Cd this difference was statistically significant.

Exposing diatoms (*T. pseudonana*) to the dissolved fraction of the metals released from virally lysed and unlysed cells resulted in no difference in bioavailability for any of the metals. The bioavailability of the released Cd and Zn was also not different for bacteria (*P. atlantica*), but there was significantly higher accumulation of Fe and Am when those two metals had been released from unlysed bacterial cells. These results indicate that viral lysis of bacterial cells may not have any effect on metal bioavailability to phytoplankton (at least not to diatoms). However, the results are not as clear when the bioavailability to bacteria is considered and warrant further research addressing this question. These findings agree with some previous research and disagree with other published studies. Results by Gobler et al. (1997) are in agreement in that viral lysis did not result in an increased release of Fe from the chrysophytes in their study. On the other hand, Mioni et al. (2005) and Poorvin et al. (2004) demonstrated that virally released Fe is more bioavailable to planktonic organisms.

The fate of metals during trophic interactions has been studied extensively with phytoplankton representing the base of the food web (Mathews and Fisher 2008, Reinfelder and Fisher 1994, Wang et al. 1996). However, much less information on the fate of metals is available when heterotrophic bacteria occupy this position (Chase and Price 1997, Maranger et al. 1998). Since I was able to illustrate the accumulation of

several metals by planktonic bacteria, it was a reasonable assumption that at least some metals may also be assimilated by bacterivorous organisms in turn. A ciliate isolate of the genus *Uronema* was chosen as a predator for *V. natriegens* in experiments designed to test for the extent of transfer of the radioisotopes ^{55}Fe, ^{65}Zn, and ^{241}Am from the bacteria to the protozoans. I discovered that Fe was transferred most effectively, with an assimilation efficiency of 79%. This value for Fe assimilation efficiency is substantially higher than was measured for flagellates feeding on bacteria (Chase and Price 1997), as well as for copepods feeding on ciliates (Twining and Fisher 2004), both of which are reported as maximally 36%. Furthermore, the assimilation efficiency of Fe for copepods feeding on diatoms is approximately 16% (Hutchins et al. 1995). The high Fe assimilation efficiency measured for the ciliates was mirrored by a low Fe efflux rate of 0.1 d^{-1}, which indicates that these ciliates have a large requirement for Fe and excrete very little of it.

In contrast, the assimilation efficiency for Zn in my experiments (29%) was similar to that measured for copepods feeding on diatoms (Reinfelder and Fisher 1991), which they reported at 27%. At higher trophic levels Zn assimilation can be significantly elevated and reach 77% in the case of copepods ingesting ciliates (Twining and Fisher 2004). The assimilation efficiency for Am measured in my experiments was surprisingly high at 24%, but can be explained by the ciliate feeding pattern which includes a rapid ingestion and digestion time, resulting in multiple ingestion events over the period of one experiment. Copepods feeding on diatoms, for example, result in an Am assimilation of only 1% (Reinfelder and Fisher 1991). This result makes sense when considering that Am is a particle-reactive, but nonessential metal, which remains primarily attached to external cell surfaces and should therefore not be transferred to consumers to any great extent. All of these results illustrate the importance of conducting research on metal transfer that involves a variety of different types of organisms since the degree of assimilation of any one metal may vary depending on the trophic status and the physiology of the organism in question. The results also confirmed that Fe, Zn, and Am (similar to most metals) do not exhibit biomagnification in marine organisms as they are moved through the food chain which was illustrated by the lower metal concentrations measured in the ciliates

than in the bacterial cells. Most importantly however, my results document the potential of trophic transfer, (in particular of Fe) upon ingesting bacterial cells and demonstrate that bacteria present an alternate source of introducing at least certain metals into metazoan food chains.

We now know that bacteria accumulate and internalize some metals to a large extent, for example Fe. Similar to the trophic transfer experiments it should therefore also be possible to use radiolabeled bacteria (labeled with ^{55}Fe, for example) and introduce them to an assemblage of organisms that may be bacterivores. This might be accomplished by setting up mesocosm experiments that include different kinds of organisms and sediments. It might be feasible to study multiple food web interactions in this manner by measuring which organisms accumulate the ^{55}Fe that was originally bound by the bacterial cells.

The accumulation of Fe by three bacterial species was also compared between two different water types, one meant to represent a more coastal/eutrophic environment, the other an open ocean/oligotrophic region. Fe was highly concentrated by all bacterial cultures but to the highest degree by cells of *R. litoralis* in both water types. The results illustrate that the majority of bacterially-associated Fe is located intracellularly, as no more than 11% of the Fe was removable using an oxalate rinse. Fe volume concentration factors among the three bacterial species ranged from 1.3×10^6 to 7.4×10^7. Results from these laboratory experiments are similar to results obtained from field work samples addressing Fe uptake by natural picoplankton cells in the Equatorial Pacific. The field samples were analyzed based on size classes, and results obtained from the picoplankton size group are used to compare to the laboratory experiments with Fe. Laboratory and field results agree that only a minor fraction of the added Fe was loosely sorbed to cell surfaces. In addition, both sets of results demonstrated an increased Fe quota in the smallest cells.

Most of the work described in this dissertation was conducted with cultured marine bacteria in natural seawater, which presents some advantages as well as disadvantages. An advantage was that I was able to compare the potential of several different known bacterial species to accumulate metals, as this setup allowed me to work

with monocultures instead of the mixed microbial assemblages that are characteristic of natural samples. By analyzing the cultures separately, it was possible to determine that interspecific differences in metal accumulation are generally small and mostly determined by cell size (specifically, surface to volume ratios of cells). A downside of this approach is that it is unclear if, and how, different bacterial species might affect the accumulation or the cycling of metals by interacting with each other, for example by competitive exclusion or interspecific competition for access to Fe. This might be especially interesting in a situation where Fe is available in limiting concentrations, and when some bacteria might exhibit an increased production of siderophores in order to successfully compete with other species for the limiting resource. It would be interesting to determine whether the bioaccumulation of Fe varies among bacterial species in such a situation compared to what it is when the bacteria are tested separately.

It should be noted that a large number of marine bacteria cannot be cultured successfully in the laboratory, and that a substantial fraction of species currently remains undescribed. Therefore, I think it is essential to conduct more metal accumulation studies in natural waters and with natural bacterioplankton assemblages, in addition to studies involving cultured species. With the advances in molecular methodologies it is possible to identify the genotypes/phylotypes of the most frequently occurring species in a particular marine system. By combining the radiotracer approach with molecular analysis and working with naturally occurring bacteria, the true accumulation potential of metals by marine bacteria in their natural setting can be investigated.

While the experiments described here were conducted in the laboratory, all of them were conducted with natural seawater as the growth medium. This means that any radioisotopes that were added to the seawater could interact with the ligands that are naturally present in the seawater, and that they interacted with them as they would in a natural setting. Furthermore, using radioisotopes as tracers of metal accumulation allowed me to work with environmentally realistic metal concentrations, at least in most cases (concentrations for Am being an exception).

Undoubtedly, this project could be expanded significantly by including more metals to assess for their potential to be accumulated by marine bacteria, as well as by

including a larger variety of bacterial species. Of particular interest would be metals such as methylmercury, which is of special interest because of its impact on human health. Methylmercury is assimilated very efficiently by a variety of organisms when it is introduced into the food web through phytoplankton, and bacteria should present an alternate source to phytoplankton. Metals that are similar to Fe should also be of interest, especially metals that are mostly transported into bacterial cells, as this is the metal fraction that is transferred most efficiently to higher trophic levels.

An aspect that was not addressed as part of this study was the metabolic status of the bacterial cells. It would have been interesting to include fluorescent stains to indicate the fraction of bacteria that were metabolically active during the experiments. This is particularly interesting because the seawater used was never amended with any nutrients in form of carbon sources, and it is possible that the water became sufficiently depleted in dissolved organic carbon or other essential nutrients by the end of the experiments. In addition, it would be interesting to determine whether there is a significant difference in the accumulation of metals when the majority of the bacterial cells analyzed are metabolically active or passive. The fluorochrome 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) could possibly be used for this purpose since it only becomes fluorescent when it is reduced by actively respiring bacteria (Rodriguez et al. 1992).

While at present it is not quite feasible because of technological limitations, in the near future it might well be possible to analyze even individual heterotrophic bacteria directly for cellular metal (and other elemental concentrations) by using synchrotron-based X-ray fluorescence microscopy (Twining et al. 2003, Twining et al. 2004). This approach has proved to be very effective in analyzing elemental concentrations of metals, as well as providing maps the individual algal cells that indicate the location of the metals. For heterotrophic bacteria, an elemental map would be of interest in determining what fraction of a given metal is sorbed to the cell envelope, compared to the fraction that is located intracellularly, as well as showing any "hotspots," for example of Fe. At present, it is possible to use this methodology to analyze cellular metal concentrations of individual phytoplankton cells, as well as some cyanobacterial taxa. While the sample analysis itself is restricted to synchrotron sites, it is relatively easy to collect and prepare

cell samples. By developing probes with finer resolution than are available presently, it should be possible to include individual heterotrophic bacterial cells in these analyses. The SXRF approach is especially useful in comparing the stoichiometries of cells collected from different ocean regions, and to address the question of nutrient sequestration by certain groups of cells in cases of nutrient limitation.

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