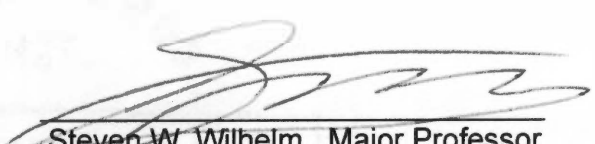
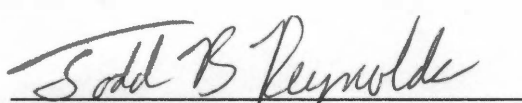
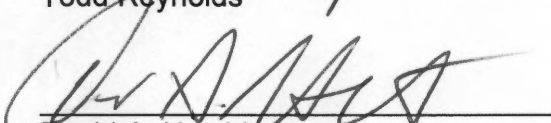


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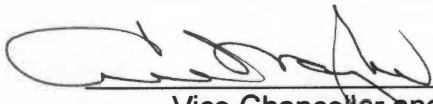
I am submitting herewith a dissertation written by Leo Poorvin entitled "The role of viruses in Fe recycling in the world's oceans." I have examined the final paper copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.


Steven W. Wilhelm., Major Professor

We have read this dissertation
and recommend its acceptance:


Gary Sayler
Lee Cooper
Todd Reynolds
David A. Hutchins

Acceptance for the Council:


Vice Chancellor and
Dean of Graduate Studies

Thesis
2005b
.P6b

The role of viruses in Fe recycling in the world's oceans.

A Dissertation Presented

for the Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Leo Poorvin

December 2005

Dedication

This is dedicated to my parents and Shawn, for making this possible, and to the Little Guy, for making it worthwhile.

Abstract

Viral lysis is responsible for a significant fraction of bacterioplankton mortality in marine systems. This work shows that viral lysis of both heterotrophic bacterial and autotrophic cyanobacterial plankton releases iron (Fe) at a greater rate than is released from unlysed cells. These studies also show that the Fe released is bound to organic ligands, these ligands are not siderophores, and that these ligands have Fe binding stability constants similar to organic ligands found in seawater. Further, these studies have shown Fe released via viral lysis to be highly bioavailable to a range of model marine plankton and may potentially satisfy the demands of primary producers in Fe limited marine systems.

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Table of contents

Part I	1
Introduction	1
Primary production in the oceans and the microbial loop	2
The case for iron	5
Complexation of Fe.....	6
The importance of viruses	9
Objectives	12
Literature Cited	13
Part II	19
Viral release of iron and its bioavailability to marine plankton	19
Abstract	20
Introduction	21
Material and methods	23
Results.....	27
Discussion	35
Acknowledgements.....	45
Literature Cited	45
Part III	49
Virus and siderophore-mediated transfer of available Fe between heterotrophic bacteria: characterization using a Fe-specific bioreporter	49
Abstract	50
Introduction	51

Material and Methods.....	54
Results	62
Discussion.....	74
Conclusions	85
Acknowledgements	86
Literature Cited.....	86
Part IV	92
Comparative bioavailability of a catecholate siderophore and virus-mediated lysates from the marine bacterium <i>Vibrio natriegens</i> PWH3a.....	92
Abstract.....	93
Introduction	94
Material and Methods.....	96
Results	100
Discussion	101
Acknowledgements	109
Literature Cited.....	109
Part V	114
Summary and Conclusions	114
Literature Cited.....	118
Vita	119

List of tables

Table I.1. Classes of siderophores and representative examples from marine organisms.	8
Table II.1. Virus-mediated recycling of dissolved organic nutrients.....	34
Table III.1. Chemical speciation of Fe in EDTA buffered BESAW medium.....	57
Table III.2. Concentrations of the Fe ligands isolated from the supernatants of <i>V. natriegens</i> and <i>P. putida</i> FeLux.	69
Table III.3. Evaluation of the impact of DFB addition on the bioluminescent signal of <i>P. putida</i> FeLux.....	73
Table IV.1. Comparison of biochemical analyses of siderophores and virus released organics produced by <i>Vibrio natriegens</i> PWH3a.....	102
Table IV.2. Fe assimilation rates by model organisms. ($\text{mol Fe g C}^{-1} \text{ day}^{-1}$)	103

List of figures

Figure I.1. Net primary production in the oceans.....	3
Figure I.2. Diagram of the marine food web.	4
Figure I.3. Diagram of siderophore-mediated Fe transport in aerobic microorganisms.	7
Figure II.1. Release of ^{55}Fe by heterotrophic bacteria.....	29
Figure II.2. Release of ^{55}Fe by cyanobacteria.	30
Figure II.3. Uptake of various Fe sources ($\text{mol Fe cell}^{-1} \text{d}^{-1}$).....	31
Figure II.4. Uptake of Fe released by viral lysis ($\text{mol Fe (g carbon)}^{-1} \text{d}^{-1}$).	32
Figure II.5. Flow diagram of the major pathways for the steady-state biological cycling of iron in marine systems.	42
Figure III.1. Dose-response characterization of the Fe bioreporter <i>P. putida</i> FeLux in BESAW medium.....	63
Figure III.2. Dose response of <i>P. putida</i> FeLux in both Fe-EDTA- and lysate- supplemented BESAW medium.	65
Figure III.3. Uptake of Fe from <i>V. natriegens</i> PWH3a lysate by <i>P. putida</i> FeLux.	67
Figure III.4. Comparison of the bioavailability of Fe in presence of various ligands, including the siderophores produced by <i>V. natriegens</i> and <i>P. putida</i> FeLux.....	71
Figure III.5. Determination of the impact of DFB on bioavailable Fe pool.....	72
Figure IV.1. Comparison of the bioavailability of lysates and siderophores to three model organisms.	106

Part I

Introduction

Primary production in the oceans and the microbial loop

Oceans extend over more than 70% of the surface of the earth (Costanza et al. 1997). In addition to geographic significance, the oceans play an enormous role in global carbon cycles. It has been long acknowledged that phytoplankton (microscopic photosynthetic organisms in the water column) form the base of the marine food web (Ryther 1969). Marine photosynthetic organisms may be responsible for up to 50% of the earth's total primary production (Field et al. 1998) (Figure I.1.), although they make up only 0.2% of total primary producer biomass (Field et al. 1998). This disparity in production versus biomass is the result of rapid turnover of the ocean's biomass which is estimated at an average turnover time of 2-6 days (in comparison, the average terrestrial biomass turnover time is 13-16 years) (Behrenfeld and Falkowski 1997).

While it was once assumed that carbon flow involved a fairly linear "food chain" in the oceans, it is now believed that carbon cycling is more complex (Figure I.2.). Azam et al. (1983) were the first to coin the phrase "microbial loop", in which dissolved organic carbon (DOC) released by phytoplankton is taken up by bacteria. A portion of the carbon is released, particularly via viral lysis (Bratbak et al. 1994), by bacteria as DOC which may then be available to other bacteria. In each cycle of the loop there is loss of energy in the form of respiration, which results in less eventually transferred up to higher trophic levels.

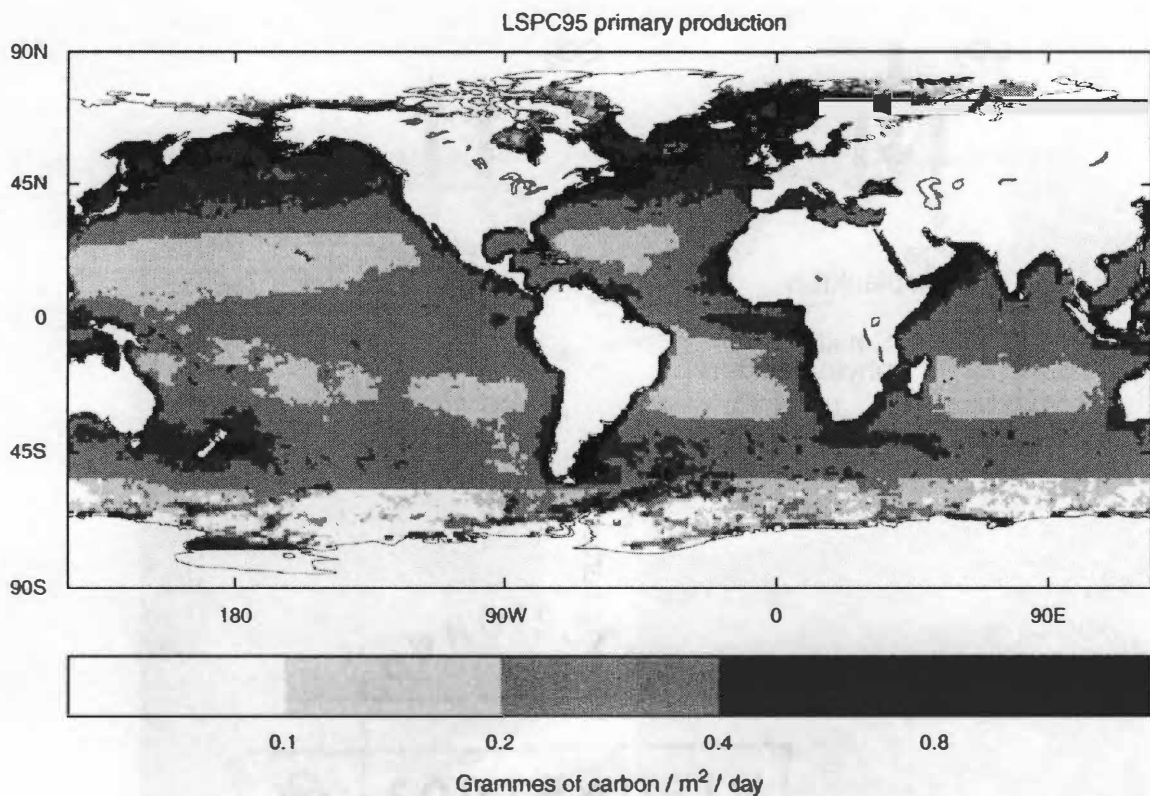


Figure I.1. Net primary production in the oceans. Inferred color data demonstrating of primary production in the oceans. Values given are daily net production [(gross primary production) – (respiration)] rates averaged over an annual cycle. Taken from Palmer and Totterdell 2001.

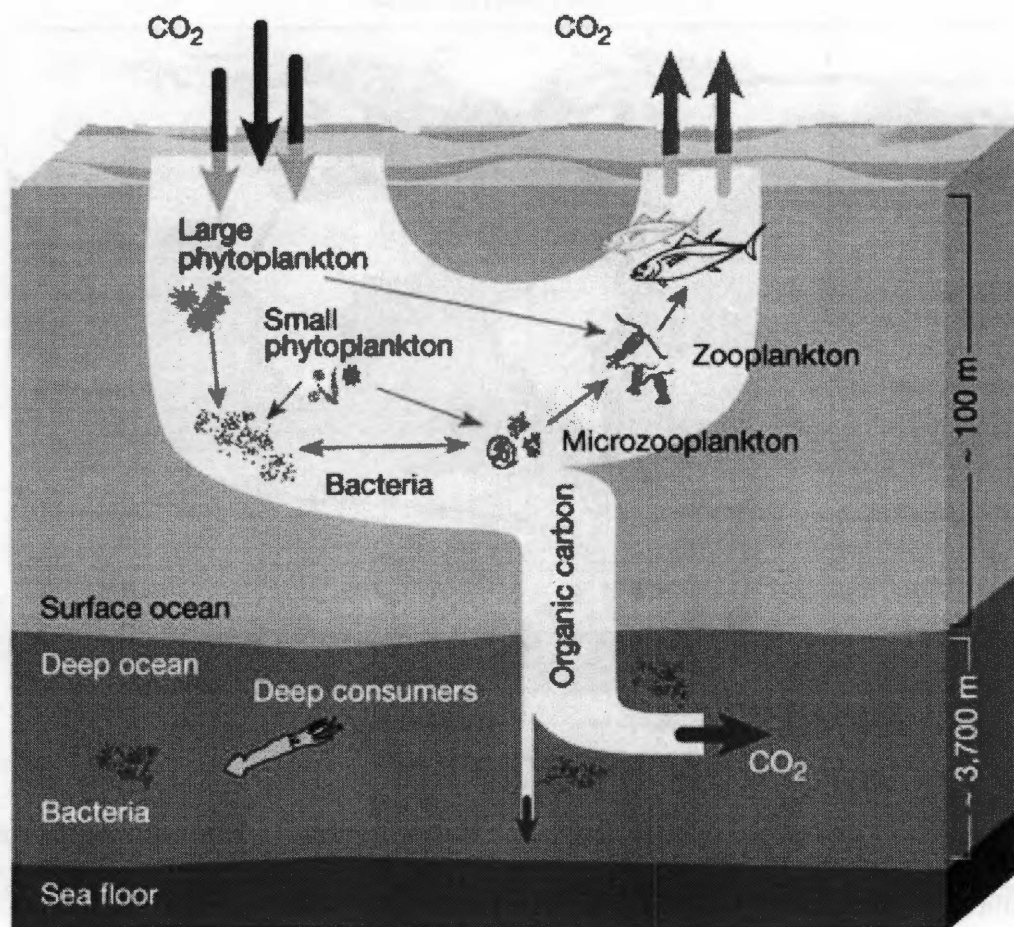


Figure I.2. Diagram of the marine food web. Phytoplankton convert CO₂ or HCO₃ to organic carbon, which then can be utilized by bacteria (the “microbial loop”), or transferred to higher trophic levels. Carbon not remineralised in the upper water column is vertically transported to the deep ocean (*aka* the “Biological Pump”). Adapted from Chisholm 2000.

The case for iron

The importance of iron (Fe) as a nutrient in marine systems has been acknowledged only relatively recently. In the early 1930's it was hypothesized that factors other than the conventional macronutrients, temperature and light could impact phytoplankton growth (Gran 1931). It was not long before Fe was put forth as a possible limiting nutrient (Harvey 1937; Hart 1941). However, the concentration of Fe in seawater was thought to be sufficient for the demands of the community. It was not until trace metal clean techniques were developed for seawater collection and analysis (Bruland et al. 1979) that the previously high concentrations estimates for Fe concentrations in marine surface waters were found to be the result of contamination.

In the mid-late 1980's, bottle incubation experiments by John Martin and co-workers (Martin and Fitzwater 1988; Martin and Gordon 1988; Martin et al. 1989) utilized trace metal clean techniques to examine the effects of Fe enrichment of seawater on the growth of phytoplankton. These studies indicated that the rate of biomass accumulation of some members of the phytoplankton community was Fe-limited. Rather heated debate questioning Martin's conclusions followed this work (Banse 1990; Martin et al. 1990). Martin and others (Martin et al. 1991) convincingly laid out "the case for iron" in a 1991 paper which analyzed shipboard Fe enrichment experiments in the Gulf of Alaska, the Ross Sea, and the equatorial Pacific. In these experiments, addition of subnanomolar concentrations of Fe resulted in phytoplankton growth rate increases of 2-3 fold. It was not until after Martin's death

that the first mesoscale Fe addition experiment was performed in the equatorial Pacific (Martin et al. 1994). This and subsequent open ocean Fe addition experiments in the equatorial Pacific (Coale and et al 1996), the north Pacific (Tsuda et al. 2003; Boyd et al. 2004) and the Southern Ocean (Boyd and Law 2001; Coale et al. 2004) have conclusively demonstrated phytoplankton to be Fe limited in high nutrient, low chlorophyll (HNLC) areas of the open ocean. In addition to these pelagic HNLC regions, recent work has shown coastal upwelling areas along the west coast of North (Hutchins et al. 1998; Fierman et al. 2003) and South (Hutchins et al. 2002; Eldridge et al. 2004) America to be Fe limited. Coastal upwelling areas, while only accounting for 0.5-1% of the ocean's surface, nonetheless are responsible for ~11% of the net global primary production (Hutchins et al. 2002).

Complexation of Fe

Almost all of the dissolved Fe in seawater is complexed to organic ligands (Rue and Bruland 1995; van den Berg 1995; Wu and Luther 1995; Rue and Bruland 1997; Powell and Donat 2001). The concentration of these ligands are generally 2 - 7 nM (Powell and Donat 2001) and have stability constants ($\log K_{\text{Fe}^{3+}\text{L}}$) of 18.6 - 22.9 (Witter et al. 2000). There is evidence that siderophores make up at least a portion of this ligand pool (Macrellis et al. 2001; Gledhill et al. 2004). Siderophores are low molecular weight Fe ligands (Table I.1.) which have exceptionally high Fe affinities. Siderophores specifically bind Fe (III) and are produced by many microorganisms to aid in Fe uptake (Figure I.3.) (Neilands 1995; Ratledge and Dover 2000).

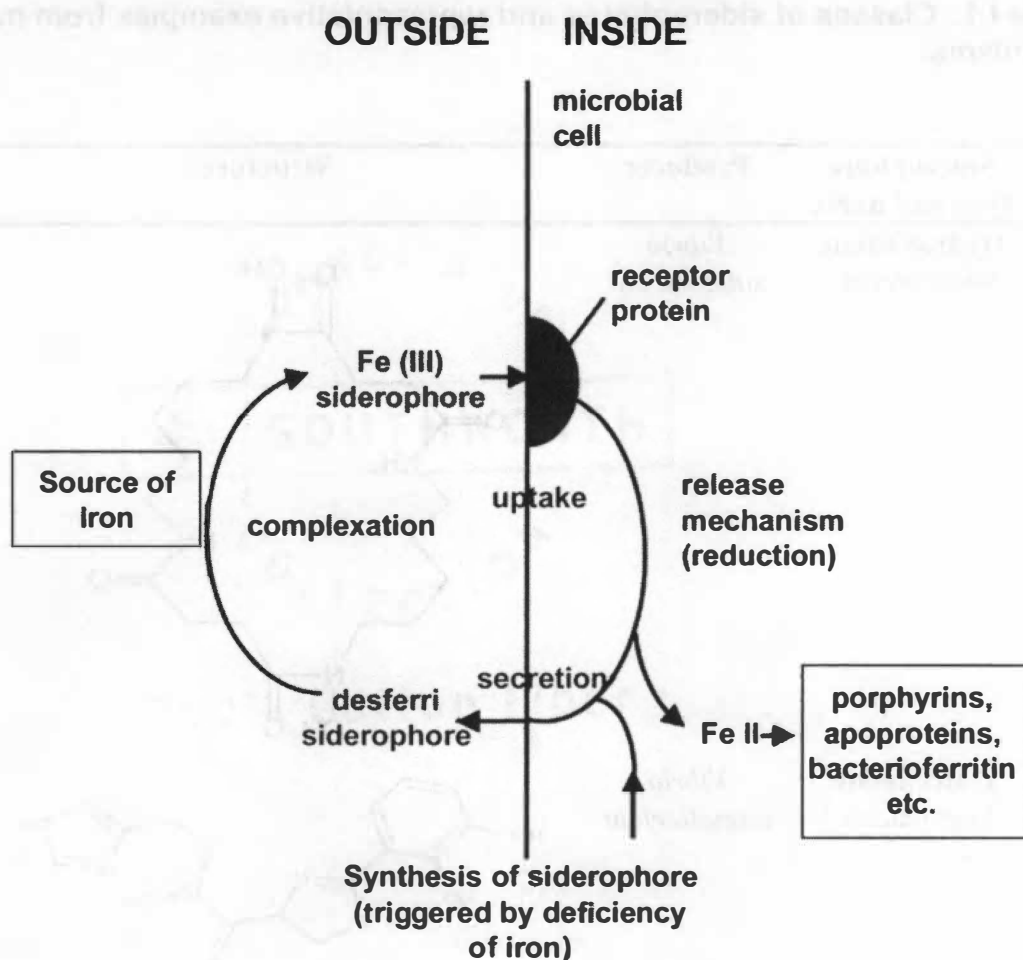
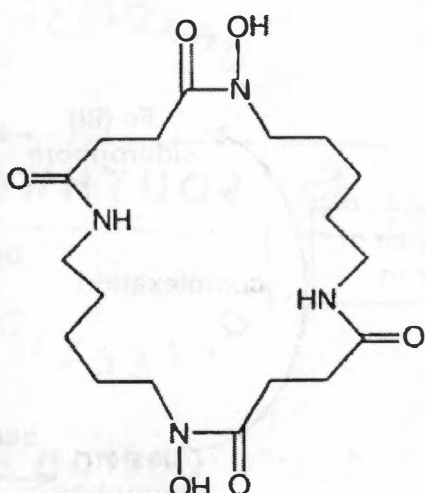
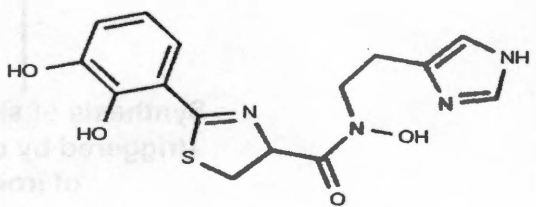
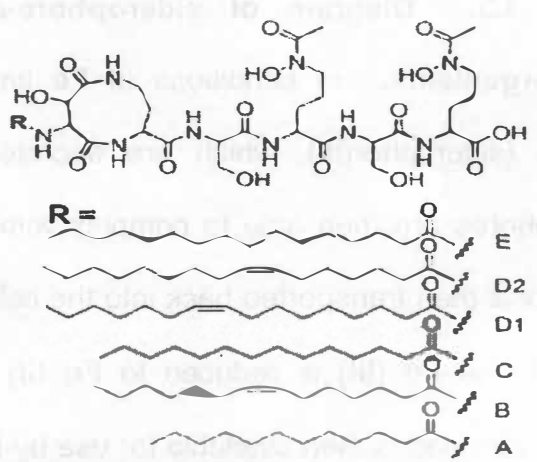


Figure I.3. Diagram of siderophore-mediated Fe transport in aerobic microorganisms. In conditions of Fe limitation, the cell produces Fe binding ligands (siderophores), which are secreted into the cell's environment. The siderophores are then able to complex with extracellular Fe. The Fe-siderophore complex is then transported back into the cell via an uptake carrier system. Once in the cell, the Fe (III) is reduced to Fe (II) which results in its release from the siderophore and is then available for use by the cell. The uncomplexed siderophore can then be re-released back into the environment. Adapted from Ratledge and Dover 2000.

Table I.1. Classes of siderophores and representative examples from marine organisms.

Siderophore type and name	Producer	Structure
Hydroxamate Bisucaberin ^a	<i>Vibrio salmonicida</i>	
Catecholate Anguibactin ^b	<i>Vibrio anguillarum</i>	
Amphiphilic Marinobactins ^c	<i>Marinobacter</i> sp.	

Marine bacteria and cyanobacteria have been shown to produce siderophores exclusively in response to Fe limitation (Reid et al. 1993; Wilhelm and Trick 1994; Wilhelm and Trick 1995). Interestingly, during the Iron-Ex II mesoscale Fe addition experiment in the equatorial Pacific, Fe ligands were found to increase 4 fold after fertilization (Rue and Bruland 1997). It has been suggested that this increase in ligand concentration may be the result of active siderophore production by plankton (Butler 1998). It seems counterintuitive however that an increase in siderophore production, which is associated with Fe limitation, would occur in response to an increase in available Fe.

The importance of viruses

After prokaryotes, viruses represent the second largest pool of organic carbon in the oceans (Wilhelm and Suttle 1999; Suttle 2005). It was only relatively recently that transmission electron microscopy (TEM) revealed the high abundance of viruses in marine waters (Bergh et al. 1989). Current estimates of $\sim 4 \times 10^{30}$ total viruses in the world's oceans mean they are the most abundant marine organism (Suttle 2005). In most marine systems examined, viruses outnumber bacteria 3-10 fold (Wommack and Colwell 2000). Several types of analysis have indicated that bacteriophage make up the majority of marine viruses. Marine viruses share morphological similarity with many known bacteriophages (Maranger et al. 1994; Wommack et al. 1992), changes in bacterial abundances correlate to changes in viral abundances (Cochlan et al. 1993; Hara et al. 1996) and most marine viruses

have genome sizes (20 to 60 kb) similar to bacteriophages (Wommack and Colwell 2000). The majority of isolated marine phages are DNA viruses and appear to have contractile tails, placing them in the *Myoviridae* family (Weinbauer 2004).

Attempts have been made to quantify the impact of virus-mediated mortality on marine microbes. Depending on the techniques employed and environments surveyed, viral mortality ranges from undetectable to 100% of total microbial mortality (Suttle 2005). A total of ~20-40% of marine bacteria killed daily by viruses has been suggested as a reasonable average mortality (Suttle 1994). The majority of viral production is believed to arise from lytic phages; a model developed by Jiang and Paul (1998) suggests that lysogenic production is responsible for < 1% of total production in normal, non-inductive environments.

Virus-mediated mortality may have significant biogeochemical effects. Viral lysis can effectively convert a large portion of the pool of particulate organic carbon (POC), namely marine prokaryotes, to dissolved organic carbon (DOC). This can have an important effect on the overall community as POC is more likely to be transferred to higher trophic levels via zooplankton grazing (Wilhelm and Suttle 1999) whereas more of the DOC stays in the microbial loop (Azam et al. 1983; Bratbak et al. 1994). This could have the effect of increasing total bacterial production, which is believed to often be limited by labile DOC (Wilhelm and Suttle 1999). This is supported by studies that demonstrate a significant correlation between viral abundance and bacterial production (Heldal and Bratbak 1991; Maranger and Bird 1995) and between frequency of visibly infected cells (FVIC) and bacterial production (Steward et al. 1996). Although correlations have been noted, it

is difficult to demonstrate a causal relationship. While higher rates of viral lysis could drive increased bacterial production, it is also possible that greater host abundance results in more host-virus contacts and greater rates of infection (Murray and Jackson 1992). One study (Middelboe et al. 2003) has examined the effects of viral lysis on organic carbon transfer between two marine bacterial cultures. In this study a *Photobacterium* sp. was able to take up and convert to biomass organic carbon released via lysis of a culture of *Cellulophaga* sp. while little or no uptake was observed from unlysed cells.

Although the potential for virus-mediated recycling of other nutrients has been theorized (Bratbak et al. 1994; Wilhelm and Suttle 1999; Fuhrman 1999), little work has actually been completed in this area. Gobler et al. (1997) examined the release and subsequent bioavailability of C, N, P, Se and Fe from lysis of the marine chrysophyte *Aureococcus anophagefferens*. Lysed cultures released ca. 1.5 fold more C and Se into the dissolved phase ($< 0.2 \mu\text{m}$) as compared to unlysed controls. Lysis initially released more Fe and P into the dissolved phase as well, but within 3 days decreased to the levels released by controls. This decrease may have been due to particle scavenging or by uptake by bacteria present in the non-axenic *A. anophagefferens* cultures. The diatom *Thalassiosira pseudonana* was able to take up some of the released C from the lysed cultures but were able to take up equivalent amounts from the unlysed controls. Exposure of the lysate to sunlight increased uptake of Se by *T. pseudonana* when compared to unexposed lysate, no comparison to unlysed controls was made. A small fraction (~25%) of Fe released in the lysate was available to the diatom regardless of exposure to sunlight.

Objectives

The goals of this study are to address a series of hypotheses associated with the idea that viruses play an important role in the regeneration of bioavailable Fe in marine surface waters.

H1: Virus-mediated cell lysis releases Fe-organic complexes into seawater that are bioavailable to marine prokaryotic and eukaryotic plankton.

H2: The rate of release of Fe-organic complexes into marine surface water by the activity of viruses is sufficient to satisfy a significant component of the Fe-demand of marine planktonic communities.

H3: Fe-organic complexes released by the lysis of marine plankton are not similar to Fe-siderophore complexes and may account for a significant portion of the naturally occurring Fe-ligand complexes found in seawater.

What follows is a series of three studies, examining the role of viruses in the regeneration of Fe-organic complexes in controlled laboratory studies, estimates of the rate that this process occurs in seawater, characterizations of the bioavailability of these mixed Fe-organic complexes to a series of model organisms, and a preliminary characterization of the functional groups and Fe-binding kinetics of the organics in virus-mediated cell lysates relative to siderophore produced by the same organism. These provide convincing evidence that the activity of viruses is important in the regeneration of Fe to marine microbial communities, and may result in changes in Fe-speciation that influence the pathways in the marine Fe cycle.

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This section is a version of a paper with the same title published in the journal *Limnology and Oceanography* in 2004 by Leo Poorvin, Johanna M. Rinta-Kanto, David A. Hutchins and Steven W. Wilhelm:

Poorvin, L., J. M. Rinta-Kanto, D. A. Hutchins, and S. W. Wilhelm. Viral release of iron and its bioavailability to marine plankton. *Limnol. Oceanogr.* **49**: 1734-1741.

My use of “we” in this section refers to my co-authors and myself. My primary contributions to this paper were: (1) all phases of experimentation and data analysis, with the exception of virus production experiments, (2) most of the writing of the manuscript.

Abstract

The biological availability of Fe has been demonstrated to strongly influence both primary and secondary production in pelagic as well as coastal upwelling high nutrient low chlorophyll (HNLC) regimes. Although nearly all of the dissolved Fe in marine surface waters is thought to be complexed by organic ligands, the character and origin of these Fe-organic complexes remains a mystery. Here we report that the activity of naturally occurring viral populations in an HNLC coastal upwelling system can regenerate sufficient concentrations of dissolved Fe to support the growth of the native phytoplankton community. When combined with studies demonstrating that Fe in virus-mediated lysates of heterotrophic bacteria and cyanobacteria is highly bioavailable to model marine plankton, our data demonstrate

that viral activity in this (and potentially other) marine systems is critical to the recycling of organically-complexed Fe that supports as much as 90% of primary production in HNLC surface waters.

Introduction

Mesoscale fertilization experiments over the past decade have conclusively shown iron (Fe) to be a limiting nutrient of primary production in high nutrient/low chlorophyll (HNLC) regions of the open ocean (e.g., Boyd et al. 2000). Additionally, shipboard experiments have shown that Fe limitation may also control primary productivity in coastal upwelling areas (Hutchins et al. 1998; Bruland et al. 2001). In total, recent models suggest that roughly half of the world's oceans may be Fe-limited (Moore et al. 2002).

Dissolved Fe in marine systems has been demonstrated to be almost completely bound to strong organic ligands (Rue and Bruland 1995) that influence the biological availability of this Fe to marine plankton (Hutchins et al. 1999a). While various Fe-chelates including siderophores and grazing byproducts have been shown to act as sources of Fe to marine plankton (Wilhelm and Trick 1994, Butler 1998, Hutchins et al. 1999b), there remains to date no field evidence of the source (and supply rates) of the Fe-organic compounds that marine plankton assimilate during in situ growth. In HNLC areas the recycling of Fe plays an important role, as new Fe inputs to the system can only support 4 - 20% of the total primary production observed (Hutchins 1995).

While their existence has been known for many years, marine viruses have recently been recognized as important factors that influence microbial communities. In marine surface waters viral abundance generally exceeds 10^9 L^{-1} , approximately one order of magnitude higher than the typical bacterial abundance (Fuhrman 1999). Estimates the virus-mediated lysis of planktonic bacteria range from 20-50 % of the population d^{-1} (Fuhrman 1999, Wilhelm and Suttle 1999). During viral lysis, the host cells are transformed from a series of uniform particles to a gradient of dissolved ($< 0.20 \text{ } \mu\text{m}$) through particulate ($> 0.20 \text{ } \mu\text{m}$) materials, and this can result in the potential release of significant quantities of organically complexed nutrients (Wilhelm and Suttle 2000). In their study using the pelagophyte *Aureococcus anophagefferens*, Gobler et al. (1997) demonstrated this idea through an examination of Fe released from this cell to bacteria as well as the model organism *Thalassiosira pseudonana*. While this work established the idea in principle, heterotrophic bacteria as well as cyanobacteria are thought to be the dominant targets of viruses in most marine surface waters (Fuhrman 1999). Given this high virus-induced mortality as well as the high abundance of bacteria and cyanobacteria in many HNLC environments, we set out to investigate the role the virus-mediated Fe recycling may play in these systems. Specifically, we hypothesized that viral activity may provide a significant proportion of the recycled Fe that is critical to community maintenance in HNLC systems, and that the character of this Fe may influence its bioavailability to marine plankton.

Material and methods

Production and fractionation of viral lysates - Fractionation of Fe in lysis products was determined for the cyanobacterium *Synechococcus* sp. WH7803 grown in A⁺ medium (Wilhelm and Trick 1995) and the heterotrophic *Vibrio natriegens* PWH3a grown in carbon supplemented ESAW (enriched seawater, artificial water) (Berges et al. 2001) with all media containing added Si (12.5 $\mu\text{mol L}^{-1}$ final concentration) and ⁵⁵Fe (4.7 $\mu\text{mol L}^{-1}$ final Fe concentration). All cultures employed in these studies were originally axenic, although minimal bacterial contamination was noted in some of the *Synechococcus* sp. WH7803 cultures. Cultures were grown at 25°C at an illumination of 65 - 85 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ in acid washed polycarbonate tubes. Cells were cultured for *ca* 12 days (for *Synechococcus* sp. WH7803) or *ca* 4 days (for *V. natriegens*), collected by filtration on 0.2- μm nominal pore-size polycarbonate filters (for *Synechococcus* sp. WH7803) or by centrifugation (for *V. natriegens*) and treated with Ti (III)-citrate-EDTA (Hudson and Morel 1989) to remove surface associated ⁵⁵Fe. The cells were resuspended in A⁺ medium with Si (12.5 $\mu\text{mol L}^{-1}$) and Fe (31 nmol L^{-1}). For each treatment, 100 μl of phage in Fe-free medium was added to lyse the cells: cyanophage SYN-M3 ($\sim 3.5 \times 10^7$ total particles) for *Synechococcus* sp. WH7803 and bacteriophage P1 ($\sim 2.0 \times 10^8$ total particles) for *V. natriegens*. Controls received no phage. The cells were incubated for *ca* 24 h (for *V. natriegens*) or *ca* 6 days (for *Synechococcus* sp. WH7803) to allow for infection and lysis.

Fe fractionation was initially examined in virus-mediated cellular lysates by filtration through 0.2- μm nominal pore-size polycarbonate filters. The ^{55}Fe in the filtrate was analyzed by liquid scintillation counting using a Wallac Tri-Lux scintillation counter with a ^{55}Fe quench correction program and Wallac SuperMix scintillation cocktail. Subsequent filtrate was further size-fractionated using Millipore Amicon Centriprep centrifugal filter devices (3 and 30 kDa nominal cut off, corresponding to pore sizes of ca 1.8 and 4.3 nm respectively, pore size values provided by manufacturer) using the manufacturer's recommended protocol.

Lysate uptake studies – All lysates used in uptake experiments were generated as in the fractionation experiments described above. Uptake of EDTA-Fe was determined using A+ medium with 80.5 $\mu\text{mol L}^{-1}$ EDTA and $^{55}\text{FeCl}_3$ (31 nmol L^{-1} total Fe concentration; 0.82 nmol L^{-1} $^{55}\text{FeCl}_3$). Uptake of inorganic Fe was measured from A+ medium supplemented with $^{55}\text{FeCl}_3$ but no added EDTA (31 nmol L^{-1} total Fe concentration; 0.82 nmol L^{-1} $^{55}\text{FeCl}_3$). Lysates of *Synechococcus* sp. WH7803 and *V. natriegens* for uptake studies were gently filtered through 0.2- μm nominal pore-size polycarbonate filters to remove any unlysed cells. Aliquots (30 ml) of the filtrates were then transferred to acid clean 40 ml polycarbonate Oak Ridge tubes. Cultures (1 ml) of model marine organisms (the cyanobacterium *Synechococcus* sp. PCC 7002, the heterotrophic bacterium *Vibrio harveyi* or the diatom *Thalassiosira pseudonana*) were added to the filtrates. The cultures were then incubated under appropriate conditions for each model organism. At $t = 0$ and 24 h, 2 ml samples of

each culture were filtered through 0.2- μm polycarbonate filters and the extracellular Fe was removed as described previously. The filters were placed into 4 ml scintillation vials and dissolved for 1 h with 500 μl of ethyl acetate. Scintillation fluid was then added and the samples analyzed for ^{55}Fe . Cell abundances (for normalization of uptake rates) were determined by Acridine Orange staining (Hobbie et al. 1977) or autofluorescence on a Leica DMRXA epifluorescent microscope. To allow for a comparison between our model organisms, Fe uptake rates were normalized to cellular carbon content using previously determined cellular quotas of 23.3 fg (femtograms) for heterotrophic bacteria, 210 fg for cyanobacteria and 5.94 pg (picograms) for diatoms (Lee and Fuhrman 1987; Waterbury et al. 1986; Montagnes et al. 1994).

Viral production estimates in the eastern subtropical Pacific Ocean- Viral production rates in the subtropical equatorial eastern Pacific Ocean off the coast of Peru were estimated as described by Wilhelm et al. (2002). This region has been well characterized in several recent studies (Hutchins et al. 2002, Eldridge et al. 2004). Surface seawater (~ 5 m depth) was cleanly collected with a PTFE Teflon diaphragm pump and PFA Teflon tubing connected to a PVC “fish” that was lowered off the ship outside of the wake (Bruland et al. 2001). Seawater (300 ml) was filtered under gentle vacuum through 0.22- μm nominal pore-size polycarbonate filters (Millipore). In order to prevent bacteria from settling of the filter, a retentate volume of > 50 ml was maintained throughout filtration by addition of virus-free ultrafiltered (< 30 kDa) seawater produced as ultrafiltrate from an Amicon M12 System equipped with an

acid cleaned S10Y30 cartridge. Additionally, bacteria were continuously resuspended with an acid clean transfer pipette during filtration. The final volume of the retained cell suspension was brought to 300 ml through the addition of ultrafiltered water. As such, the concentration of naturally occurring viruses was reduced to ca 4 – 40% of the initial concentration. Aliquots (100 ml) of the retained cell suspension were transferred into three acid-clean 250-ml polycarbonate flasks, which were incubated in the dark at in situ temperature. Subsamples (5 ml) were taken at $t = 0$ from whole water (before filtration) and from each bottle and then every 2-3 h for up to 12 h from each incubation bottle and preserved in 2.5% glutaraldehyde (final concentration) and stored in a refrigerator (4°C) until processed. The abundance of viruses and bacteria in samples were determined in the laboratory by staining samples with either SYBR Green I (Noble and Fuhrman 1998) or Acridine Orange staining (Hobbie et al. 1977) prior to direct enumeration by epifluorescence microscopy.

The production rate of viruses at each station was determined from regressions of samples in independent, replicate ($n = 3$) bottles for each station as previously described. To determine virus-induced mortality rates, a burst size of 25 viruses per lytic event was assumed (Wilhelm et al. 1998). This information was used to estimate the rate of virus-mediated mortality of bacteria ($\text{cells L}^{-1} \text{d}^{-1}$) as:

$$\text{Virus induced bacterial mortality} = \text{viral production} / \text{burst size}$$

To infer impacts on the regeneration of Fe, we assumed the cellular Fe quotas for marine bacterioplankton of 1.1 ag (attograms) per cell (Tortell et al. 1996) and calculated Fe remobilized by viral lysis from the following:

$$\text{Fe remobilization} = \text{virus-induced bacterial mortality} \times 1.1 \text{ ag}$$

Fe uptake by natural communities – Fe assimilation rates for the natural marine assemblages were estimated at two stations in the Pacific Ocean (90° 33.7'W, 8° 41.5N and 73° 24.9'W, 16° 27.3'S). Surface seawater was cleanly collected in acid washed 2.7-L polycarbonate bottles. Saturating amounts of ^{55}Fe (2 nmol L⁻¹, as $^{55}\text{FeCl}_3$ in 0.5 mol L⁻¹ HCl, ca 42 mCi mg⁻¹, from New England Nuclear, Boston MA) were added to replicate bottles, which were then incubated for 48 h at in situ temperatures and light levels. Following incubation, the water was filtered in parallel through 0.2- μm and 1.0- μm nominal pore-size polycarbonate filters that were then washed with Ti (III)-citrate-EDTA as above to remove surface associated ^{55}Fe . Assimilated ^{55}Fe was measured by scintillation counting as above with the Wallac Tri-Lux scintillation counter.

Results

Fe fractionation in virus-mediated lysates – Lysis of *V. natriegens* laboratory cultures released a significantly greater percentage ($24.9 \pm 2.9\%$) of intracellular Fe into the dissolved ($< 0.22 \mu\text{m}$) size class than was released from the unlysed control

($10.6 \pm 2.1\%$) ($p < 0.01$). Differences were observed between lysed and unlysed cultures in fractionation of the dissolved Fe (Figure II.1). Dissolved Fe released from virus-lysed cells was predominantly ($66.3 \pm 3.7\%$) in the < 3 kDa fraction with a lesser amount ($28.8 \pm 4.7\%$) in the ≥ 30 kDa class. A lesser percentage ($37.0 \pm 3.7\%$) of the Fe from the unlysed cells was found in the < 3 kDa fraction and a greater percentage ($48.9 \pm 2.2\%$) was in the ≥ 30 kDa class.

Following lysis of *Synechococcus* sp. WH7803, the majority ($81.6 \pm 5.1\%$) of the original intracellular Fe was released into the dissolved phase (Figure II.2). This is in contrast to the unlysed controls where $33.5 \pm 1.1\%$ was released into the dissolved phase in the absence of viruses. All of the dissolved Fe ($103.0 \pm 6.0\%$) released by the unlysed cells was found in the < 3 kDa fraction, whereas significantly less ($p < 0.05$) of the dissolved Fe released by virus lysis was present in this fraction ($82.1 \pm 7.7\%$). There were no significant differences seen in the other size fractions.

Assimilation rates of Fe from lysates – Fe present in the lysate of *V. natriegens* cultures was differentially bioavailable to the different model organisms over a period of 24 hours (Figure II.3). The bacterium *V. harveyi* assimilated ^{55}Fe from the lysates at a rate of $3.1 (\pm 1.0) \times 10^{-2} \text{ mol Fe (g C)}^{-1}$ (Figure II.4). This was significantly higher than the rate of ^{55}Fe assimilation from lysate for *Synechococcus* sp. PCC 7002 ($8.7 (\pm 0.7) \times 10^{-4} \text{ mol Fe (g C)}^{-1}$) ($p < 0.01$) and *T. pseudonana* ($9.8 (\pm 1.8) \times 10^{-4} \text{ mol Fe (g C)}^{-1}$) ($p < 0.01$).

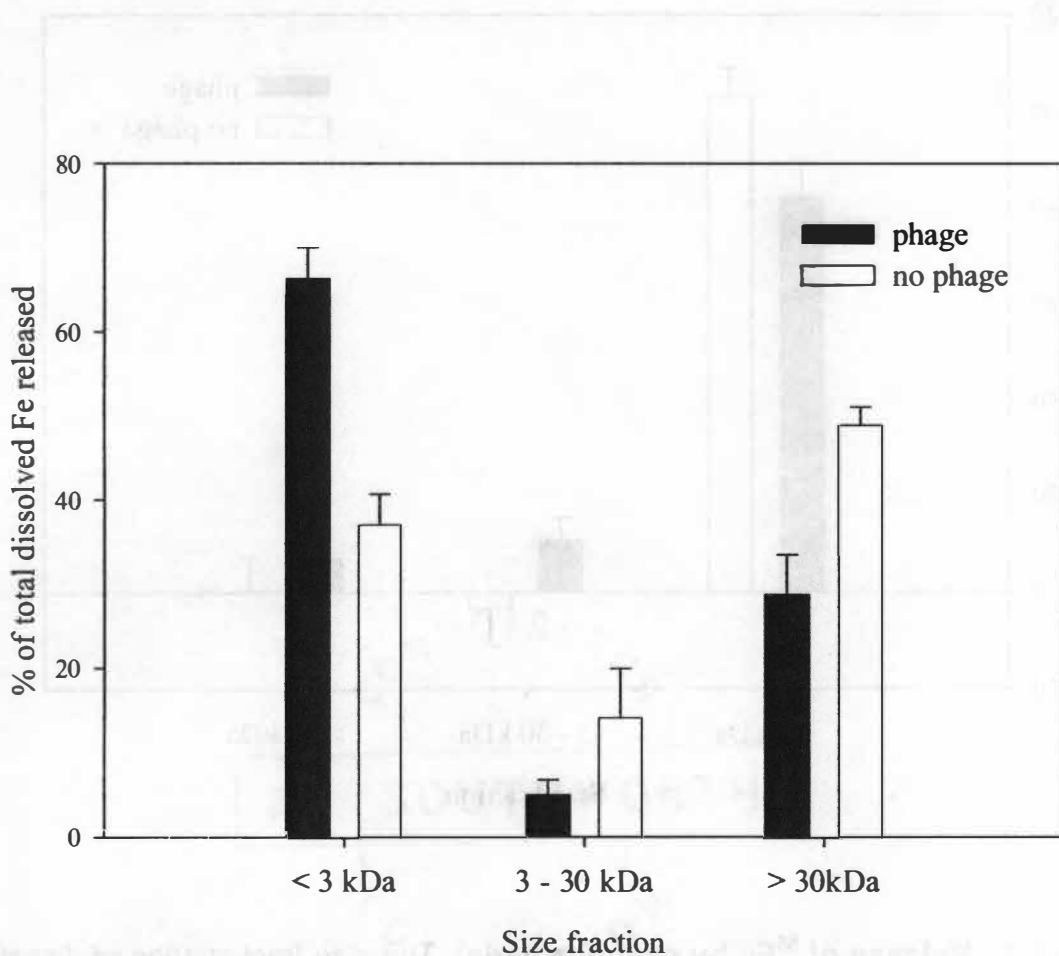


Figure II.1. Release of ^{55}Fe by heterotrophic bacteria. The size fractionation of dissolved released ^{55}Fe from *Vibrio natriegens* was measured ($n=3$, $\pm$ SD) both with and without added bacteriophage. In the cultures with phage, the greatest percentage of ^{55}Fe was present in the smallest (< 3 kDa) size class. The cultures that had no added phage released more ^{55}Fe into the larger size fractions.

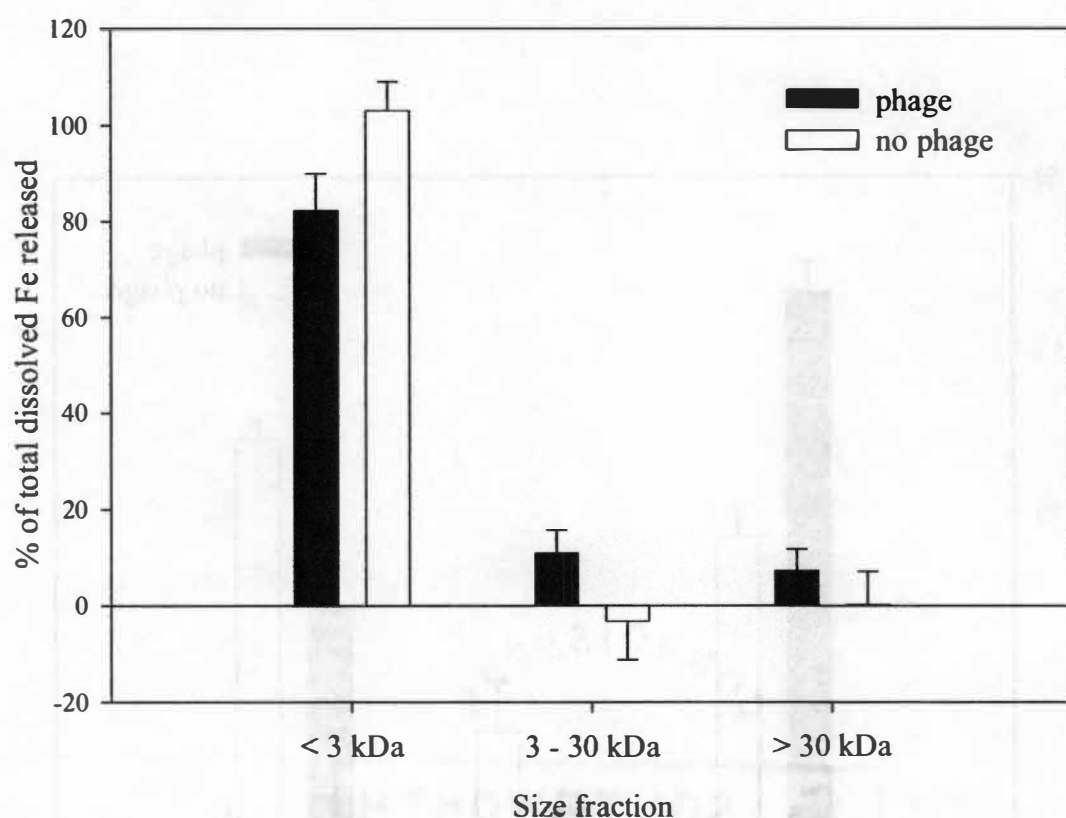


Figure II.2. Release of ^{55}Fe by cyanobacteria. The size fractionation of dissolved released ^{55}Fe from *Synechococcus* sp. WH7803 was measured ($n=3$, $\pm$ SD) both with and without added cyanophage. All of the ^{55}Fe released in absence of phage was found in the smallest (< 3 kDa) size fraction. Although most of the ^{55}Fe released with added phage was also found in the smallest fraction, a significant proportion was in the larger classes.

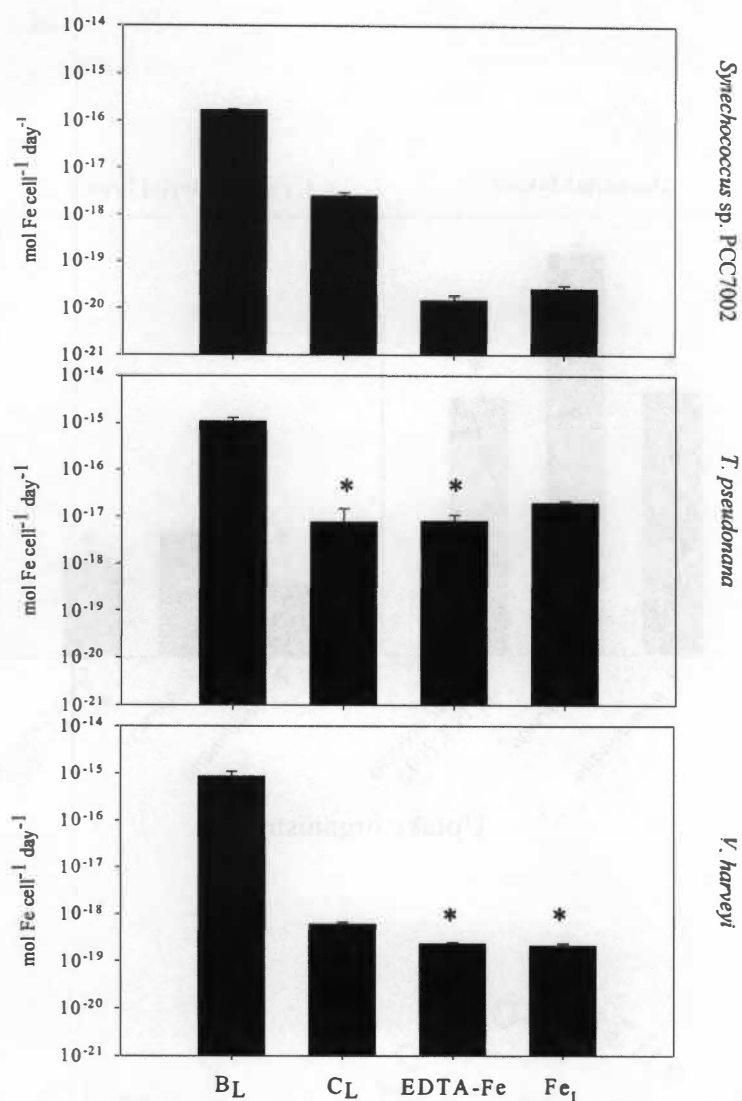


Figure II.3. Uptake of various Fe sources ($\text{mol Fe cell}^{-1} \text{d}^{-1}$). The uptake of Fe from bacterial lysate (B_L), cyanobacterial lysate (C_L), EDTA bound Fe (EDTA-Fe), and inorganic Fe (Fe_i) by laboratory cultures of *Thalassiosira pseudonana*, *Vibrio harveyi* and *Synechococcus* sp. PCC7002 was determined. Fe from the bacterial lysate had the greatest bioavailability for all three organisms tested. Values marked with an asterisk were not significantly different from one another. All other values showed significant differences ($p < 0.05$).

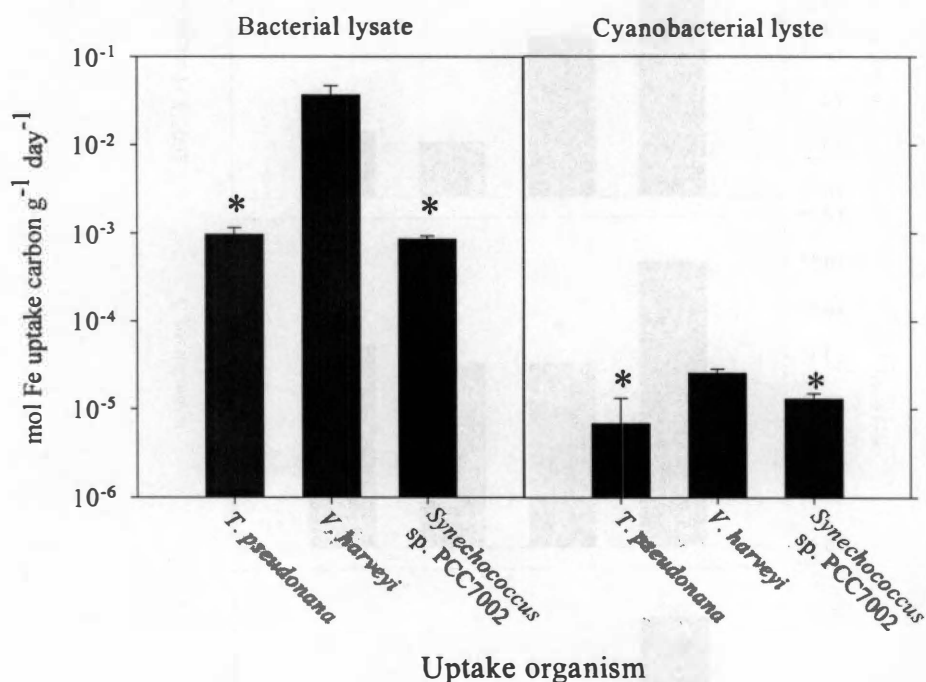


Figure II.4. Uptake of Fe released by viral lysis (mol Fe (g carbon)⁻¹ d⁻¹). The uptake of Fe by laboratory cultures of *Thalassiosira pseudonana*, *Vibrio harveyi* and *Synechococcus* sp. PCC7002 was measured over 24 hours to compare uptake rates between the three organisms. *V. harveyi* took up Fe from bacterial lysate at a rate more than an order of magnitude greater than either *T. pseudonana* or *Synechococcus* sp. PCC7002. Values marked with an asterisk were not significantly different from one another. All other values showed significant differences ($p < 0.05$).

In all cases, ^{55}Fe in the dissolved phase of the *V. natriegens* lysates assimilated at a statistically ($p < 0.05$) greater rate than either inorganic ^{55}Fe or ^{55}Fe -EDTA.

Fe released from lysis of *Synechococcus* sp. WH7803 was assimilated more rapidly by *V. harveyi* ($2.6 (\pm 0.3) \times 10^{-5}$ mol Fe (g C) $^{-1}$) than by *Synechococcus* sp. PCC7002 ($1.3 (\pm 0.2) \times 10^{-5}$ mol Fe (g C) $^{-1}$) ($p < 0.01$) and *T. pseudonana* ($6.9 (\pm 6.3) \times 10^{-6}$ mol Fe (g C) $^{-1}$) ($p < 0.01$) (Figure II.4). While this Fe was also more readily assimilated than Fe from the inorganic or EDTA-complexed treatments by the prokaryotes, there was no significant difference between the rates of assimilation by *T. pseudonana* of Fe from the EDTA-complex treatments.

Virus production and estimated Fe remobilization rates – The impact of viral activity on Fe recycling in situ was determined at a station in the Humboldt Current and at three stations in the Peruvian upwelling (Table II.1). Viral abundance at these stations ranged from ca $2 - 6 \times 10^7$ ml $^{-1}$, while bacterial abundance ranged from $5 - 18 \times 10^6$ ml $^{-1}$. Virus-induced mortality in these regions is consistent with results from previous studies (Fuhrman 1999; Wilhelm and Suttle 2000) ranging from ca 5.3 – 35.8% of the bacterial population lysed on a daily basis given a burst size of 100 viruses per lytic event. We employed an estimated burst size of 25 viruses per lytic event since this appears to be typical of those found in environments similar to that of the current study.

Table II.1. Virus-mediated recycling of dissolved organic nutrients.

Date	09 Sep 00	12 Sep 00	21 Sep 00	23 Sep 00
Location	84°40.3'W 4°10.7'S	80°13.1'W 7°7.6'S	75° 21. 1'W 15° 23.4'S	73° 24.9'W 16° 27.3'S
Bacterial abundance (x 10 ⁶ particles ml ⁻¹)	9.78	18.2	ND	5.39
Viral abundance (x 10 ⁶ particles ml ⁻¹)	27.8	60.7	ND	20.0
Viral production rate (x 10 ⁶ particles ml ⁻¹ h ⁻¹)	4.67	4.04	16.01	8.03
Total Fe remobilisation (ng L ⁻¹ d ⁻¹)	4.92	4.28	16.92	8.48
Dissolved Fe remobilisation (ng L ⁻¹ d ⁻¹)	1.23	1.07	4.23	2.12
Virally-generated dissolved Fe flux (pmol L ⁻¹ d ⁻¹)	22.0	19.2	75.7	38.0

ND: not determined

Fe release rates from virus-lysed cells were determined from the inferred destruction rates of bacterioplankton, a burst size of 25 viruses per lytic event and an estimated bacterial cellular quota for Fe (Tortell et al. 1996). Fe partitioning into the dissolved phase was estimated assuming 25% partitioning after viral lysis (see text for details).

In the Gulf of Mexico burst sizes were estimated to be 10 – 20 viruses per lytic event in offshore waters, and 30 – 60 viruses per event in nearshore, mesotrophic waters (Wilhelm et al. 1998). These estimates, in conjunction with our *ca* 25% partitioning into the dissolved phase, suggest that viral activity would result in a recycled dissolved Fe flux ranging from 19.2 – 75.7 pmol L⁻¹ d⁻¹ (Table II.1).

Fe uptake by natural communities – Saturated Fe uptake rates were measured at two stations in the Pacific Ocean. Fe assimilation rates over 48 h for phytoplankton (> 1.0 µm size class) ranged from 19.5 (± 7.8) to 30.6 (± 1.5) pmol L⁻¹ d⁻¹. Bacterioplankton (0.2 – 1.0 µm size class) uptake rates ranged from 160 (± 17) to 181 (± 28) pmol L⁻¹ d⁻¹.

Discussion

The results of this study demonstrate that the virus-mediated lysis of cyanobacteria and heterotrophic bacteria releases intracellular Fe into the dissolved size class at a greater rate than Fe is released from cells in the absence of phage. While this is not surprising, the size-fractionation experiments demonstrate that some of this virus-released Fe partitions into a different size class relative to Fe released from unlysed cells. Moreover, Fe assimilation experiments demonstrate that Fe released *via* lysis is highly bioavailable to model marine planktonic organisms: Fe in bacterial lysates and in most cases cyanobacterial lysates was more rapidly assimilated than either inorganic Fe or EDTA-Fe by our model organisms. When coupled with *in situ* measurements of viral production (and associated host lysis),

these experiments suggest that virus-mediated Fe release may provide an important fraction of the total bioavailable Fe in pelagic marine systems.

Fe release, fractionation and uptake – Viral lysis of laboratory plankton cultures demonstrated that the size class of Fe released by virus-mediated lysis was dependent on the host organism. In our experiments the lysis of cyanobacteria released a greater proportion of total intracellular Fe into the dissolved size class relative to the lysis of heterotrophic bacteria. In many ways this is not surprising, given that these prokaryotes are quite different both structurally and in terms of where the highest concentrations of Fe are located within the cells. Moreover, the high cell densities reached by heterotrophic bacteria in culture probably enhances aggregation. As such, the actual fate of Fe during the lysis of these prokaryotes in nature undoubtedly differs. In their studies with *A. anophagefferens* Gobler et al. (1997) found that only 5% of the Fe was released into the dissolved phase during viral lysis. In their cultures a marked increase in bacterial abundance concurrent with lysis may have combined with the release of high molecular weight materials to scavenge any free Fe.

Dissolved Fe ($< 0.2\text{-}\mu\text{m}$) released from lysis of *V. natriegens* was predominantly in the $< 3\text{ kDa}$ size-class, although ca one-third was found to be associated with larger components. For unlysed control cells (cultures not exposed to a virus) approximately one half of the dissolved Fe partitioned into the $> 30\text{kDa}$ size class with the majority of the remainder in the $< 3\text{kDa}$ class. Dissolved Fe released by the virus-mediated lysis of *Synechococcus* sp. WH7803 was also

predominantly in the < 3 kDa class, with a small but significant ($p < 0.05$) proportion found in the larger size classes. In contrast, Fe released from unlysed *Synechococcus* sp. WH7803 cells was found only in the < 3 kDa class.

Many studies make operational distinctions based on the ability to fractionate samples – in our own case we use 0.2- μm , 30-kDa, and 3-kDa cut offs. It remains difficult to draw comparisons between the size fractionation of Fe observed in these lysis experiments and previously observed in situ Fe fractionation as there are currently no universally recognized size standards for classifying “Fe-complexes” in the ocean. Estimates of “dissolved” Fe concentrations in near surface ocean waters range from 95% in the “soluble” fraction (< 200 kDa, Nishioka et al. 2001) to 90% in the “colloidal” size class (0.02 – 0.4 μm , Wu et al. 2001).

The data above provides direct evidence that different approaches to Fe fractionation in natural systems may result in samples containing Fe of different origins (and as we will show bioavailabilities) – as such comparisons using different size fractions (or methods) may yield inconsistent results. For example, a previous study (Wang and Dei 2003) demonstrated that the cyanobacteria *Synechococcus bacillarus* (CCMP 1333) and *Trichodesmium* sp. were able to take up low molecular weight Fe (< 1 kDa) at a faster rate than “colloidal” Fe (> 1 kDa – 0.2 μm). However, Mioni et al. (2003) recently demonstrated that removal of the > 0.2- μm size-class markedly reduced the bioavailability of Fe to a halotolerant Fe reporter strain (*Pseudomonas putida* FeLux) in a freshwater system (Lake Erie).

Fe released by the virus-mediated lysis of plankton showed varying levels of bioavailability to our model organisms. When assimilation rates were normalized to

estimates of cellular carbon (g), the Fe released from lysis of *V. natriegens* was significantly ($p < 0.05$) more bioavailable than Fe from autotrophic *Synechococcus* sp. WH 7803. This was consistent for the three model organisms we studied. Fe contained in the bacterial lysate was assimilated by *V. harveyi* at a rate almost three orders of magnitude greater than Fe from cyanobacterial lysis. Both lysates were significantly ($p < 0.05$) more bioavailable than either inorganic Fe or EDTA-Fe during our incubations (24 h).

The Fe from the lysed bacterium was also more readily assimilated by *T. pseudonana*, our model marine diatom. Uptake of Fe from the bacterial lysate by *T. pseudonana* occurred at a rate more than two orders of magnitude faster than the uptake rate of Fe from the cyanobacterial lysate. There was no significant difference in uptake rate of Fe from cyanobacterial lysates as compared to EDTA-⁵⁵Fe by *T. pseudonana*. Inorganic ⁵⁵Fe was slightly but significantly ($p < 0.05$) more bioavailable than either the cyanobacterial lysate or the EDTA-Fe during these short term experiments (although lab observations over longer period have demonstrated that EDTA is required for the cells to grow in this synthetic medium). These uptake experiments show that prokaryotic and eukaryotic organisms can assimilate Fe released from the lysis of both heterotrophic and autotrophic marine plankton, but may preferentially assimilate different forms.

Resolution of the marine Fe cycle remains complicated by our inability to determine how organic complexation with individual ligands affects Fe availability. Macrellis et al. (2001) have demonstrated that the marine Fe-ligand pool is a complicated mixture of many different organic complexes. Unfortunately the exact

bioavailability (and source) of these compounds have yet to be determined. In freshwaters, several groups have employed genetically-engineered strains of cyanobacteria (Porta et al. 2003) and heterotrophic bacteria (Mioni et al. 2003) that report (via bioluminescence) on the in situ Fe physiology of the cells to gain insight into how these cells perceive bioavailable Fe in a background of naturally occurring ligands; unfortunately similar strains have yet to be employed in marine systems. As such, other than the use of model Fe-binding ligands (e.g., Hutchins et al. 1999b), estimates of variation in the bioavailability amongst Fe-ligand complexes in marine systems remain a mystery. The results of the current study highlight the fact that, while model ligands such as siderophores may provide useful insights into high-affinity transport systems used by prokaryotes (e.g., Butler 1998), that these systems must interact and compete with a complex combination of different Fe-bound complexes.

Estimating Fe turnover by viruses in the eastern subtropical Pacific - To determine whether the estimated virus-mediated release rates of Fe were enough to satisfy phytoplankton demands, we measured ^{55}Fe -uptake rates at two stations in the Pacific Ocean. It should be noted here that, as mentioned in the results, the estimated release rates that we compare to the uptake rates are potentially confounded (both positively and negatively): Wilhelm et al. (1998) found that burst sizes of only 10-20 were common in oligotrophic waters of the Gulf of Mexico with near shore infected cells producing burst sizes of 30 – 60. As such, our estimates of virus-mediated Fe regeneration may be under- or over estimates by as much as 2-

fold. A further caveat to this is that we assume all the viral production is the result of bacteriophage lysis of heterotrophic bacteria: although it has been shown that both cyanobacteria and eukaryotic phytoplankton can be infected by viruses, it is believed that the majority of viruses in marine systems infect heterotrophic bacteria (Fuhrman 1999, Wilhelm and Suttle 1999). Finally, we used the conservative estimate of 25% partitioning into the dissolved phase for lysates. We hypothesize that in field samples this value is most likely higher, as lower cell densities in the field no doubt lead to less aggregation than in higher density lab cultures.

In total the results imply that viruses could supply a major fraction of the daily algal demand for recycled Fe. Although our lab experiments show that heterotrophic bacteria are better able to take up Fe from lysis products than are algal cultures, in HNLC upwelling regimes heterotrophic bacteria have been shown to be limited by carbon, not Fe (Kirchman et al. 2000). Incubation studies in the South American eastern boundary current upwelling regime have demonstrated that augmentation of ambient dissolved Fe concentrations by $0.1 - 2.5 \text{ nmol L}^{-1}$ can increase in phytoplankton biomass by up to 250 % within 48-72 h (Hutchins et al. 2002, Eldridge et al. 2004). Given the sensitive nature of these natural populations to dissolved Fe concentrations, it becomes obvious that the Fe released by viral lysis may greatly affect the Fe-bioavailability in this system. As well, given this work and previous studies using model ligands (Hutchins et al. 1999b, Eldridge et al. 2004) it appears that a key to understanding this cycle depends on the differentiation between the Fe (II) and Fe (III) pools as well as the mechanism by which Fe is recycled.

The chemical speciation of Fe in viral lysis products is not known, but Fe released from cells is most probably in a variety of different Fe (II) and Fe (III) organic complexes. Uncomplexed intracellular Fe is virtually nonexistent due to its toxic nature and Fe (III) in cells is generally concentrated in storage components (e.g., bacterioferritins), which would presumably not be found in cells from Fe-limited environments. In contrast, regenerated Fe released as a byproduct of grazing is mixture of both organic and (primarily) inorganic forms: the inorganic Fe being generated as Fe-organic complexes pass through the acidic digestive system of the grazer (Fok et al. 1982), followed by rapid oxidation of Fe (II) to Fe (III) in the oxic marine environment. In combination with photolysis in surface waters (Barbeau et al. 2001), these processes provide for distinct pools of the two valences of Fe that marine plankton can draw from.

To better visualize this, we have generated a schematic for the biological cycling of Fe in marine systems (Figure II.5) based on the assimilation efficiencies of Fe-ligand complexes described here as well as by Hutchins et al. (1999b). As discussed above the current dogma suggests that “new” Fe entering marine surface waters (as inorganic dust or from vertical advection) must be complexed to metal-free ligands to keep it in solution and make it bioavailable. The presence of siderophores in a system allows for siderophore-producing members of the community (which are mostly prokaryotes) (Wilhelm and Trick 1994, Butler 1998) to acquire this Fe. The Fe is then recycled back to either the inorganic pool (via grazing) or to the pool of organically complexed compounds (primarily via viral lysis). The organic Fe from the lysis products then becomes available to marine eukaryotes

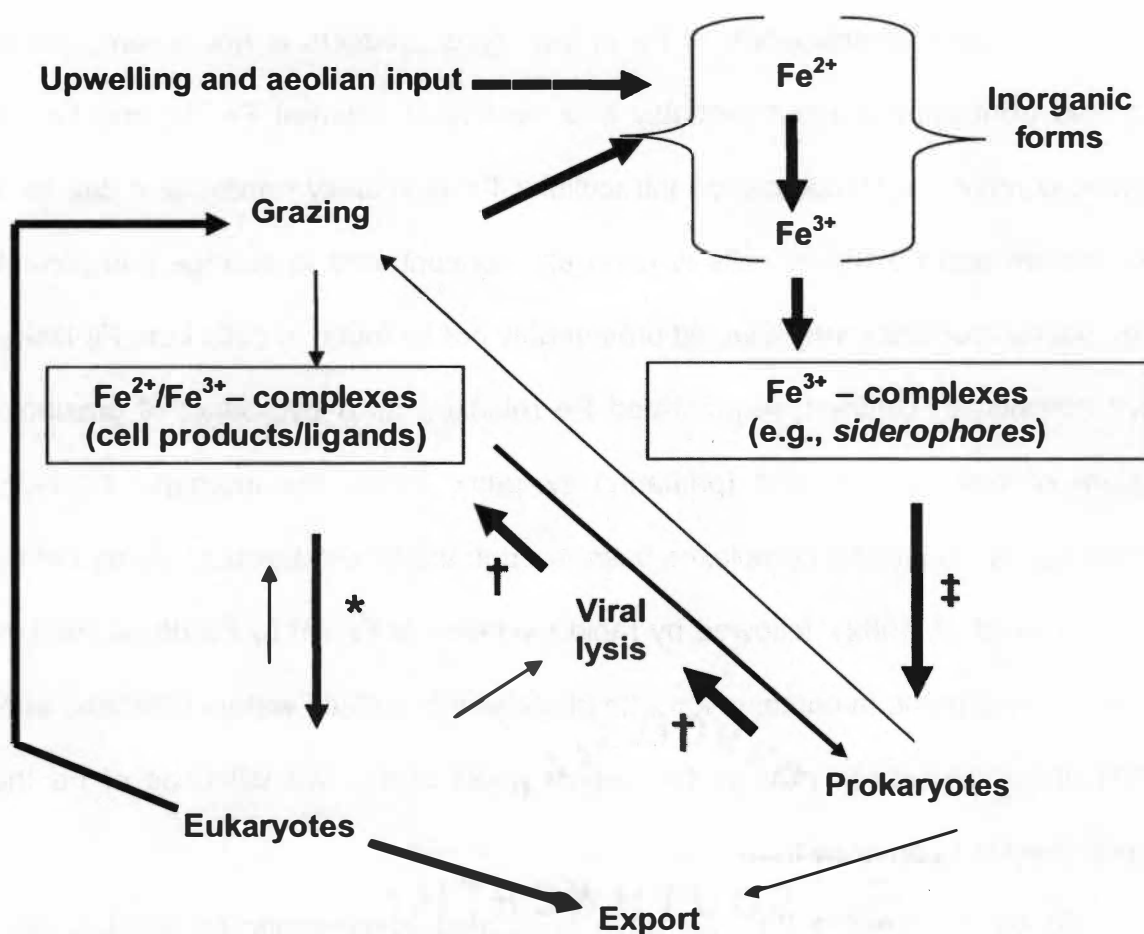


Figure II.5. Flow diagram of the major pathways for the steady-state biological cycling of iron in marine systems. Arrow weight reflects hypothetical importance of pathways in HNLC environments. The current study demonstrates that the phytoplankton Fe-demand (*, $19.5 - 30.6 \text{ pmol L}^{-1} \text{ d}^{-1}$) can be completely offset by dissolved, organically-complexed Fe generated from the virus-mediated lysis of bacterioplankton (†, $19.2 - 75.7 \text{ pmol L}^{-1} \text{ d}^{-1}$). The bacterial Fe uptake (‡, $160 - 181 \text{ pmol L}^{-1} \text{ d}^{-1}$) is sufficient to satiate this, and is itself supported by regenerated Fe from grazing activity, virus-mediated lysis and new Fe (aeolian and upwelling) inputs.

(potentially through surface reductases activity, Maldonado and Price 2001). This study demonstrates that the release of Fe by viral activity may be a major pathway in this cycle.

Support for these ideas comes from previous mesoscale Fe addition experiments which have demonstrated that the addition of Fe results in an increase in Fe-binding organic ligands (Croot et al. 2001). Previous researchers have suggested that ligand production may be an active response to added Fe by the native microbial population. While siderophore production may account for at least some of these compounds (Butler 1998) it is important to remember that siderophore production is a response to growth limiting levels of Fe (Wilhelm and Trick 1994). In lab studies, siderophore production by marine bacteria and cyanobacteria is an exclusive response to decreases in available Fe (Butler 1998, Wilhelm and Trick 1994, 1995). So, while siderophores may play a significant role under normal conditions in Fe-limited environments, when Fe is added (as in mesoscale addition experiments), siderophore production should be repressed. We propose that the increased production of Fe-binding ligands is largely the result of microbial mortality. As pelagic assemblages respond to Fe inputs from fertilization, the increase in cell density results in a direct increase in microzooplankton grazing as well as viral infection. The latter point is due to the fact that the frequency of virus-host contact is directly proportional to both the densities of the host cell and virus (Wilhelm et al. 1998). It is therefore evident that viral lysis rates will increase with Fe-induced biomass increases, and suggests that Fe-ligand concentrations could increase in

marine systems after Fe fertilization because more cellular debris from viral activity will be produced.

This hypothesized viral control of the cell abundance suggests that prokaryotic abundance should stay low relative to increases in carbon production during fertilizations: observations made during the IronEx fertilization experiments demonstrated only a *ca* 1.7-fold increase in bacterial abundance relative to a *ca* 4-fold increase in bacterial carbon production (Cochlan 2001), with similar observations arising from bottle incubations in the Californian upwelling (Hutchins et al. 1998). Although usually attributed to a grazer-mediated control of bacterial biomass, it is evident that viruses should be major players in this process. The role of viral activity in the release of cellular Fe-ligand complexes is further supported by the fact that significant Fe (II) was detected during the SOIREE experiments, presumably bound to Fe (II) ligands (Croot et al. 2001). This Fe (II) could possibly have been released as a result of cell mortality.

Resolving the complex web of factors that influence marine Fe-cycles is critical to our understanding of the biological role that marine plankton play in global carbon budgets, and to an eventual resolution of the “ocean Fe fertilization” debate. Over the past two decades we have gained an appreciation for the importance of the microbial components in marine systems and their influence on biogeochemical cycles: this report highlights this role and demonstrates that even in their death microbes exert this considerable influence. Viral lysis produces a suite of Fe-binding compounds of varying bioavailability to marine plankton. While there is little doubt that other “top-down” mechanisms and processes (e.g., heterotrophic consumption

of Fe-ligand complexes leading to Fe release) are involved, our results demonstrate that viral activity is of vital importance and along with grazing regenerates supplies of organically-complexed Fe that are critical to biological communities growing in Fe-limited HNLC conditions.

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Part III

Virus and siderophore-mediated transfer of available Fe between heterotrophic bacteria: characterization using a Fe-specific bioreporter

This section is a version of a paper with the same title currently in press in the journal Aquatic Microbial Ecology in 2005 by Cécile E. Mioni*, Leo Poorvin*, and Steven W. Wilhelm.

*These authors contributed equally to this study.

My use of “we” in this section refers to my co-authors and myself. My primary contributions to this paper were: (1) production of virus-mediated lysates, (2) extraction and characterization of *Pseudomonas putida* FeLux siderophores (collective work with Cécile E. Mioni), (3) determination of Fe bioavailability (collective work with Cécile E. Mioni), (4) assisting in writing, editing and responding to reviewers comments.

Abstract

Although marine chemists can accurately quantify both the concentration of dissolved iron (Fe) and the high-affinity organic ligands which complex Fe in surface waters, tools to characterize the relative bioavailability of such organically-bound Fe complexes remain unavailable. In this study, we have compared the bioavailability of Fe released from the lysis of the heterotrophic bacterium *Vibrio natriegens* PWH3a to that of Fe complexed to synthetic chelators (EDTA) and siderophores (including the trihydroxamate desferrioxamine B and two catecholates isolated from Fe-limited heterotrophic bacterial cultures) using a heterotrophic bioluminescent reporter of Fe

availability (*Pseudomonas putida* FeLux). Using the bioluminescent response of *P. putida* FeLux, we were able to rank the Fe sources tested here in a decreasing order of bioavailability: lysates > Fe- homologous catecholate (from a *P. putida* FeLux culture) ~ Fe- exogenous catecholate (from *V. natriegens* culture) > inorganic Fe (FeCl_3 , 15 nM) ~ $\text{Fe(III)}'$ from EDTA-buffered treatment (pFe 18.12) > Fe:DFB. Combined with estimates of Fe assimilation of ^{55}Fe -labeled lysates, our data further demonstrate that organic Fe complexes released during virus-mediated cell lysis are ca. 1,000 times more bioavailable and efficiently assimilated by bacterial cells than $\text{Fe(III)}'$. Our results validate the utilization of *P. putida* FeLux as a bioreporter of Fe-bioavailability. Our results also support the assumption that virus activity plays a crucial role in the regeneration of biologically available Fe complexes in surface seawater.

Introduction

A number of independent studies have clearly demonstrated that approximately half of the World's oceans are chronically or seasonally Fe-deplete (Moore et al. 2004). This growth-limiting concentration of Fe has been suggested to control phytoplankton production (Boyd 2004). Most (> 99%) of the dissolved Fe in these regions is complexed by strong organic ligands, leaving less than 0.1 pmol kg^{-1} as free ferric species (Rue & Bruland 1995, Wu & Luther 1995). During the past decade, several studies have demonstrated that organic complexation can control Fe concentration and Fe speciation and thus its bioavailability to marine plankton (Boye et al. 2001, Wu & Luther 1995, Gledhill & van den Berg 1994). Although

uncertainties remain regarding the source(s) and chemical structure of these organic ligands, it appears that they fall into two distinct ligand classes: a strong (L1) and a weak (L2) ligand class. It is currently assumed that L1 is actively produced by plankton resident in the water column while the weaker ligand class L2 results from both active production as well as the degradation of the L1 group, (Tortell et al. 1999, Butler 1998, Rue & Bruland 1995). Recently we have proposed that a significant component of these Fe-ligand complexes are the products of cellular mortality (Poorvin et al. 2004). While little is known about the relative bioavailability and the fate of these organic ligands in natural systems, recent experiments suggest that the chemical nature of these Fe complexes may influence competition between prokaryotes and eukaryotes (Hutchins et al. 1999a, Poorvin et al. 2004).

The similarities between the stability constants (Rue & Bruland 1995, Lewis et al. 1995) and structural groups (Gledhill et al. 2004, Martinez et al. 2003) of the natural organic Fe ligands and those for naturally produced siderophores has led to a surge of interest in the microbial component of the marine plankton assemblage. It has been demonstrated that marine heterotrophic bacteria account for as much as 50% of the biomass (and thus biogenic Fe) in oceanic systems (Tortell et al. 1999). Furthermore, bacteria contain significantly more Fe per biomass unit than phytoplankton and are responsible for significant Fe uptake in Fe-depleted seawaters (Tortell et al. 1996). Although several studies have shown that bacterial productivity was co-limited by carbon and Fe (Church et al. 2000, Kirchman et al. 2000), other studies suggest that heterotrophic bacterial productivity may be directly Fe-limited at low, *in situ* concentrations (Pakulski et al. 1996, Tortell et al. 1996).

Finally, conflicting reports suggest that Fe may (Eldridge 2004) or may not (Arrieta et al. 2004, Hutchins et al. 2001) influence bacterial diversity. And although it is well established that Fe is efficiently recycled in marine surface waters, the question of how resources (bottom-up) and predation (top-down) control this process is not fully resolved.

In marine ecosystems, viruses are typically 5 to 10 times more abundant than bacteria (Weinbauer 2004) and persist at densities ranging from $< 10^4$ to $> 10^8$ mL⁻¹ (Wommack & Colwell 2000), resulting in a global population of ca. 3.5×10^{29} viruses in the world's oceans (Wilhelm & Suttle 2000). Moreover, findings suggest that phages dominate the virus population in aquatic systems (Breitbart et al. 2002, Fuhrman 1999). As such, viruses may play an important role in the regeneration of organically-complexed nutrients in aquatic systems (Fuhrman 1999, Wilhelm & Suttle 1999, Gobler et al. 1997). Very little is known about the chemical nature, molecular size and bioavailability of nutrients such as Fe that are released during the phage-mediated lysis of prokaryotes. Recent laboratory experiments, using model planktonic organisms, have shown that viral lysis resulted in the release of a range of dissolved to particulate Fe-containing components and that this Fe can be rapidly assimilated by other plankton (Poorvin et al. 2004). Consequently, virus-mediated Fe regeneration may provide a substantial fraction of total bioavailable Fe in oceanic systems and may support as much as 90% of the primary production in recycling-based HNLC systems (Poorvin et al. 2004). In parallel, virus activity may also control prokaryotic abundance in marine systems, especially when growth conditions are favorable (Weinbauer 2004). As such, the control exerted by viruses on prokaryotic

proliferation, and the subsequent destruction of cells, may explain the increase in organic ligand concentrations observed during mesoscale Fe fertilization – an event which should suppress prokaryotic siderophore production (Poorvin et al. 2004).

In this study, we hypothesized that lysates may increase Fe bioavailability by adding to the concentration and diversity of bioavailable Fe species that are, at least in part, readily available to the oceanic prokaryotes. We compared the bioavailability of Fe released from lysates of the heterotrophic bacterium *Vibrio natriegens* PWH3a to that of Fe complexed to a model synthetic chelator (EDTA) using a heterotrophic bioluminescent reporter for Fe availability, *Pseudomonas putida* FeLux (Mioni et al. 2003). This bioreporter produces a luminescent signal which is inversely correlated to Fe bioavailability. Combined with estimates of Fe assimilation from ^{55}Fe -labeled lysates by the bioluminescent bacterial reporter, our data suggest that organic Fe complexes released during lysis are both highly available and efficiently assimilated by bacterial cells. The bioavailability of Fe complexed to siderophores produced by the two heterotrophic bacteria (*V. natriegens* and *P. putida*) was also examined. Our data suggest that Fe complexes released from virus-mediated lysis are more bioavailable than Fe-siderophore complexes, and validate the use of the *P. putida* FeLux bioreporter for these studies.

Material and Methods

Production of virus-mediated lysates - To reduce Fe contamination, all nutrient stocks and water used in this study were treated with Chelex-100 resin (Price et al. 1988/89). All culture materials were soaked in dilute HCl and rinsed with Chelex-100

treated Milli-Q water prior to use. All manipulations were performed under a class-100 conditions to maintain aseptic and trace-metal clean conditions.

Virus-mediated lysates of the heterotrophic bacterium *Vibrio natriegens* PWH3a were prepared as previously described (Poorvin et al. 2004). In brief, *V. natriegens* PWH3a cultures were grown in modified ESAW medium (Berges et al. 2001) supplemented with glycerol and ^{55}Fe (3.33 nM total Fe concentration) at 25°C for 4 days. Cells were collected by centrifugation and washed with Ti(III)-citrate-EDTA (Hudson & Morel 1989) to remove surface associated ^{55}Fe . Cells were then resuspended in carbon-free ESAW medium supplemented with non-radioactive Fe (1 nM) and the lytic bacteriophage PWH3a-P1. Bacteria cells and virus particles were incubated for 24 h to allow for virus infection and subsequent cell lysis. The dissolved fraction of the lysate was collected after filtration through 0.22- μm , 47-mm diameter polycarbonate filters. The final Fe concentration in the lysate was estimated at 4.33 nmol L⁻¹ (3.33 nM ^{55}Fe and 1 nM Fe).

Estimation of iron bioavailability to P. putida FeLux - *P. putida* FeLux (Mioni et al. 2003) stock cultures were maintained at 30°C on Pseudomonas Isolation Agar (Remel) supplemented with 50 $\mu\text{g mL}^{-1}$ of tetracycline (Tc). For each experiment an individual colony was selected and amplified to provide a stock culture for use with all treatments. Cultures from the selected colony were maintained at 25°C (with shaking) in microwave-sterilized (Keller et al. 1988) marine broth 2216 (Difco) supplemented with 50 $\mu\text{g mL}^{-1}$ Tc. Bioreporter cells were acclimated in Fe-deficient BESAW medium (pFe 21.15, Mioni 2004) and incubated overnight at 25° C with

shaking. The experiment was started by transferring 2 mL of the overnight-acclimated cell culture to each replicate tube. All treatments were performed in triplicate. The optical density at 600 nm (OD_{600}) and light production were measured every 2 h over an experimental period of 12 h using a spectrophotometer (Biomate 5, Thermospectronic corp.) and luminometer (FB-15, Zylux corp.). At each time point, light production was normalized to estimated cell abundance (OD_{600}).

Treatments supplemented with Fe-EDTA were prepared as described elsewhere (Mioni 2004). Sterile aliquots of Fe-free BESAW (18 mL) were dispensed into acid washed and microwave sterilized Oakridge tubes. Fe species within the BESAW medium were determined using Mineql+ software (version 4.5, Environmental Research software). Both pFe ($-\log [Fe^{3+}]$) and $Fe(III)'$ (sum of the major inorganic Fe species) was determined for a series of total Fe concentrations so as to chemically describe the medium (Sunda and Huntsman 2003, Table III.1). The concentration of Fe was altered to create an increasing range of modeled free Fe^{3+} spanning from pFe 21.15 ($Fe(III)' = 0.93 \text{ pM}$) to pFe 16.84 ($Fe(III)' = 18.6 \text{ nM}$). The final concentration of EDTA was maintained constant in all treatments (100 μM). As previous studies have shown that Fe-complexed to EDTA is not available to *P. putida* (Meyer & Hohnabel 1992), the bioluminescent signal is assumed to be correlated to the free inorganic Fe species present in the EDTA-buffered treatments. Light production per OD_{600} values were converted to light production per cell using the empirically determined linear function: $y = 3.459 \times 10^8 - 3.611 \times 10^6 x$ (where y

Table III.1. Chemical speciation of Fe in EDTA buffered BESAW medium.

Total Fe pFe	5.0×10^{-9} 21.15	5.0×10^{-8} 20.15	2.0×10^{-7} 19.54	5.0×10^{-7} 19.14	7.5×10^{-7} 18.97	5.0×10^{-6} 18.12	5.0×10^{-5} 16.84*
Fe^{3+}	7.15×10^{-22}	7.16×10^{-21}	2.87×10^{-20}	7.19×10^{-20}	1.08×10^{-19}	7.53×10^{-19}	1.43×10^{-17}
Fe(OH)_2^+	6.94×10^{-12}	6.94×10^{-11}	2.78×10^{-10}	6.97×10^{-10}	1.05×10^{-9}	7.31×10^{-9}	1.39×10^{-7}
$\text{Fe(OH)}_{3\text{aq}}$	3.53×10^{-12}	3.53×10^{-11}	1.41×10^{-10}	3.55×10^{-10}	5.34×10^{-10}	3.72×10^{-9}	7.07×10^{-8}
Fe(OH)_4	2.80×10^{-13}	2.80×10^{-12}	1.12×10^{-11}	2.81×10^{-11}	4.23×10^{-11}	2.95×10^{-10}	5.61×10^{-9}
Fe(OH)^{2+}	6.77×10^{-17}	6.77×10^{-16}	2.71×10^{-15}	6.80×10^{-15}	1.02×10^{-14}	7.13×10^{-14}	1.36×10^{-12}
$\text{Fe}_2(\text{OH})_2^+$	1.59×10^{-30}	1.59×10^{-28}	2.55×10^{-27}	1.60×10^{-28}	3.62×10^{-28}	1.76×10^{-24}	6.36×10^{-22}
$\text{Fe}_3(\text{OH})_4^+$	1.66×10^{-39}	1.67×10^{-36}	1.07×10^{-34}	1.69×10^{-33}	5.74×10^{-33}	1.94×10^{-30}	1.34×10^{-26}
FeHPO_4^+	1.21×10^{-18}	1.21×10^{-17}	4.87×10^{-17}	1.22×10^{-16}	1.83×10^{-16}	1.28×10^{-15}	2.43×10^{-14}
$\text{FeH}_2\text{PO}_4^{2+}$	1.68×10^{-24}	1.69×10^{-23}	6.75×10^{-23}	1.69×10^{-22}	2.55×10^{-22}	1.77×10^{-21}	3.37×10^{-20}
FeBr_2^+	3.55×10^{-25}	3.55×10^{-24}	1.42×10^{-23}	3.57×10^{-23}	5.37×10^{-23}	3.74×10^{-22}	7.11×10^{-21}
FeCl_2^+	3.83×10^{-22}	3.83×10^{-21}	1.53×10^{-20}	3.84×10^{-20}	5.78×10^{-20}	4.03×10^{-19}	7.66×10^{-18}
$\text{FeCl}_3(\text{aq})$	5.81×10^{-24}	5.81×10^{-23}	2.33×10^{-22}	5.84×10^{-22}	8.78×10^{-22}	6.12×10^{-21}	1.16×10^{-19}
FeCl_2^+	1.01×10^{-21}	1.01×10^{-20}	4.07×10^{-20}	1.02×10^{-19}	1.53×10^{-19}	1.07×10^{-18}	2.03×10^{-17}
FeNO_3^{2+}	6.70×10^{-25}	6.70×10^{-24}	2.68×10^{-23}	6.73×10^{-23}	1.01×10^{-22}	7.05×10^{-22}	1.34×10^{-20}
FeHSeO_3^{2+}	2.02×10^{-28}	2.03×10^{-27}	8.12×10^{-27}	2.04×10^{-26}	3.06×10^{-26}	2.13×10^{-25}	4.05×10^{-24}
FeSO_4^+	5.78×10^{-21}	5.78×10^{-20}	2.32×10^{-19}	5.81×10^{-19}	8.73×10^{-19}	6.09×10^{-18}	1.16×10^{-16}
$\text{Fe}(\text{SO}_4)_2^-$	9.30×10^{-22}	9.31×10^{-21}	3.73×10^{-20}	9.35×10^{-20}	1.41×10^{-19}	9.79×10^{-19}	1.86×10^{-17}
Fe(III)' pM	0.93	9.3	37	93	140	980	18,600*

Values for Fe species were calculated using Mineql+ software (version 4.5, Environmental Research software) with EDTA = 100 μM , pH 7.8 @ 25 °C and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. Fe(III)' and pFe estimates are giving for comparison to other lab studies only, as it is anticipated that Fe concentrations in the highest treatments* may result in precipitation in seawater.

is the number of bioreporter cell per mL and $\times$ the corresponding OD₆₀₀ value). Values are the means of triplicate cultures from the 12 h time-point.

Treatments amended with ⁵⁵Fe-labeled lysate were prepared similarly to Fe-EDTA treatments. In these treatments, EDTA was omitted so that the lysate mixture was the only source of Fe and Fe ligands. Increasing volumes of radiolabeled lysate were added to sterile Fe-free BESAW medium (total volume = 18 mL) to obtain the following final total Fe concentrations: 43.3 pmol L⁻¹, 0.11 nmol L⁻¹, 0.16 nmol L⁻¹, 0.21 nmol L⁻¹, 0.54 nmol L⁻¹, and 4.33 nmol L⁻¹. The last treatment was composed completely of lysate (100% v/v). Chelex-100 treated glycerol was supplied directly to replicate tubes to obtain a similar carbon in all treatments. For all lysate-amended treatments, in parallel to bioluminescence and OD₆₀₀ monitoring, 1 mL of culture was fixed with glutaraldehyde (2.50% v/v) at $t = 12$ hours and enumerated by epifluorescence microscopy after treatment with acridine orange (Hobbie et al. 1977) on a Leica DMRXA epifluorescence microscope.

Lysate assimilation studies - Fe-assimilate in the lysate-amended treatments was assessed as previously described (Poorvin et al. 2004). At time $t = 12$ hours, 2 mL of each lysate-amended treatment were collected by filtration through 47-mm diameter, 0.2- μ m nominal pore-size polycarbonate filters. Extracellular Fe was removed by washing the filters with Ti(III)-citrate-EDTA (Hudson & Morel 1989). Filters were placed into 4-mL scintillation vials and dissolved for 1 hour with 500 μ L of ethyl acetate. Scintillation fluid was added prior to the analyses of ⁵⁵Fe.

Preliminary tests demonstrated that residual bioluminescence from the bioreporters does not interfere with the results from the Wallac scintillation counter (data not shown).

Isolation of siderophores - Batch cultures (ca. 10 L) of *P. putida* FeLux and *V. natriegens* were grown in a modified BESAW culture medium to optimize siderophore production. The medium was supplemented with 0.5 mL of glycerol (100 %) for *V. natriegens* PWH3a and 1 mL of glycerol (100 %) for *P. putida* FeLux. All trace metals except Fe were identical to that of ESAW medium (Berges et al. 2001). Inorganic Fe was supplemented to the medium to reach a final concentration of 1 nM. EDTA was omitted as it has been it reacts with siderophore assays (Granger & Price 1999). No effort was made to limit trace-metals contamination: the culture media were not treated with Chelex-100 and were autoclave sterilized. However, all manipulations were performed with aseptic techniques. Batch cultures were grown to stationary phase at room temperature in 10-L polycarbonate carboys containing a Teflon stir bar (ca. 5 days).

Deferrated siderophores were extracted from the 0.22- μ m filtrate of the bacterial cultures as described previously (Wilhelm & Trick 1994, 1995). After acidification to pH 3.0, organics in the filtrate were collected using Amberlite XAD-16 resin (BioRad). The column was allowed to dry overnight and was washed subsequently with Chelex-100 treated Milli-Q water. The organic fraction

containing the siderophores was subsequently eluted with methanol. Extracts were concentrated by rotary evaporation and stored at 4°C.

Characterization of siderophores - Extracts were assayed for Fe-binding compounds by thin layer chromatography (TLC) on cellulose plates (Merck) using methanol:H₂O (70:30, v/v) as a solvent. Fe-binding compounds were resolved by spraying the dried TLC plate with 1% FeCl₃ in ethanol (Wilhelm & Trick 1994). To estimate the total Fe-binding capacity of these compounds, we performed the Chrome Azurol S (CAS) assay (Schwyn & Neilands 1987). Standard curves for the CAS assay were generated with the fungal siderophore desferrioxamine B (DFB, Sigma). To determine the chemical nature of the Fe-binding compounds, we performed two more assays. Catecholate moieties were detected using the Rioux assay (Rioux et al. 1983) standardized with 2,3-dihydroxybenzoic acid (2,3-DHBA). The modified Csaky test was used to detect hydroxamate functional groups with DFB as a standard (Csaky 1948, Gillam et al. 1981).

Determination of the bioavailability of Fe-siderophore complexes - To assess the bioavailability of the siderophore extracts, *P. putida* FeLux cultures were maintained and acclimated as described above. At time zero, acclimated cells were inoculated into 18 mL of BESAW medium supplemented with 15 nM FeCl₃ and 5 nM or 15 nM (Fe-binding equivalents as determined by the CAS assay) of the siderophore extract. Comparisons to two other treatments were also made:

inorganic Fe (15 nM FeCl₃) and Fe:DFB (15 nM:5 nM). The Fe-DFB treatment was used here as a positive control as we demonstrated previously that Fe complexed to DFB is not bioavailable to the *P. putida* FeLux bioreporter (Mioni et al. 2003). Bioluminescence and bacterial density were monitored as described above. All treatments were repeated in triplicate.

To determine whether Fe bound to the fungal siderophore DFB was bioavailable to the bioreporter cells, we evaluated the impact of equimolar Fe:DFB complex additions on the bioreporter in Fe-replete medium (FeCl₃ = 15 nM). Cells were acclimated and prepared following the same protocol described above. To insure chemical equilibrium between the added DFB and Fe(III), DFB was added using 1000-times concentrated Fe(III):DFB premixed (1:1) stock solutions. At time zero, acclimated cells were inoculated to 18 mL of BESAW medium supplemented with 15 nM of FeCl₃ plus the Fe:DFB premix to the final concentrations of 0 nM (control), 5 nM, 10 nM, 15 nM, and 20 nM. EDTA was omitted from the recipe. All treatments were triplicated. Assuming that the added DFB bound to Fe(III) in 1:1 ligand stoichiometry (Liu & Hider 2002, Rue & Bruland 1995) the total inorganic Fe concentration was 15 nM in all treatments.

Statistical analyses - Statistical analyses were performed using SPSS (ver. 12) software. Independent *t*-tests (two-tailed), analyses of variance (one-way ANOVA) and multiple comparison tests were performed assuming equal variance of mean values. The homogeneity of variance was tested in each analysis using the Levene test. Analysis of variance was used to establish the statistical

significances of variation among different treatments. In parallel, multiple comparison tests were performed to ascertain differences. Dunnett's test was used to analyze the significance of the variations in the means of Fe:DFB amended treatments relative to the control treatment (Corston & Colman 2003). The Tukey's honestly significant difference test (*Tukey-HSD* test) was used to establish the statistical significance of variations among treatment means (Corston & Colman 2003). Unless stated, results were considered significant at $p < 0.05$.

Results

Bioavailability of Fe released from virus-mediated lysates - Light production by *P. putida* FeLux bioreporter cells grown in the trace-metal buffered BESAW was characterized by a sigmoidal function with a linear dose-response portion extending from pFe 19.54 to pFe 16.84 (Figure. III.1A). Linear regression analysis performed over the linear region using Sigma-Plot software (ver. 9, SPSS Inc.) yielded the equation $y = 0.0271x - 0.1023$ ($r^2 = 0.994$; where y is the bioluminescent signal per cell and x the pFe). The luminescent signal was saturated for pFe values higher than 18.6 as no significant differences were observed between the treatments pFe 21.15, pFe 20.15 and pFe 19.54 (ANOVA, $p > 0.05$). The results suggest that all high-affinity transport systems were derepressed in this medium at free Fe³⁺ concentrations lower than $10^{-19.54}$ M (ca. Fe' = 37 pM; total Fe = 200 nM in EDTA-buffered synthetic medium).

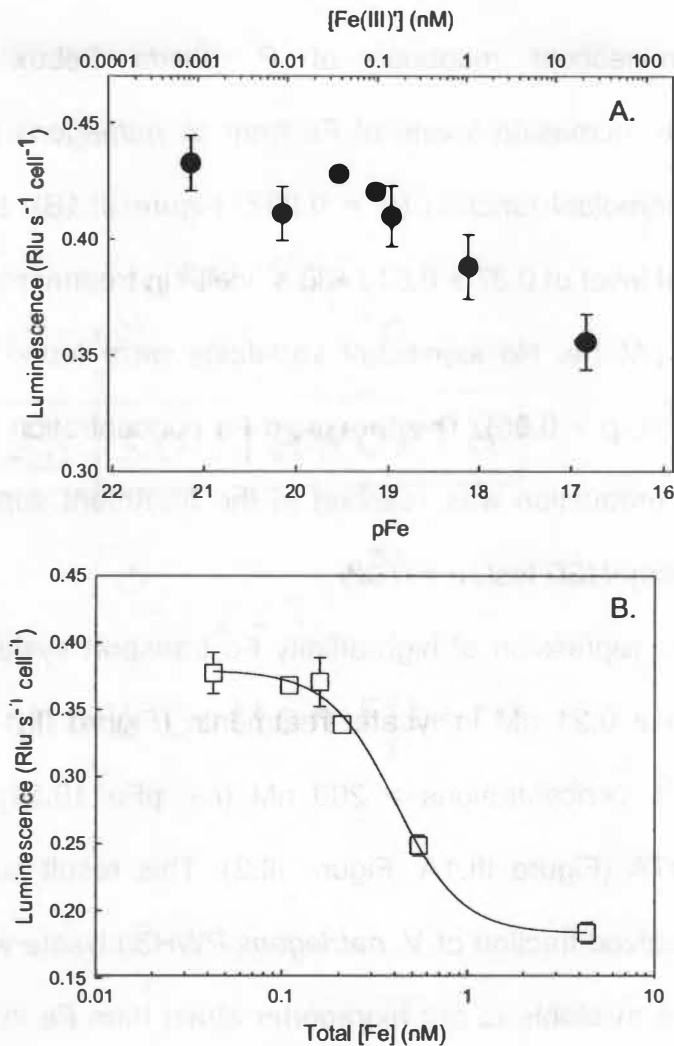


Figure III.1. Dose-response characterization of the Fe bioreporter *P. putida* FeLux in BESAW medium. A: Dose-response in the trace-metal buffered BESAW medium. Regression analysis extending from pFe 16.84 ($\text{Fe(III)}' = 215$ nM) and pFe 19.54 ($\text{Fe(III)}' = 43$ pM) is represented ($r^2 = 0.994$; $\text{slope} = 0.0271$ and $y\text{-intercept} = -0.1023$). B: Bioavailable Fe level (relative to total Fe concentrations) estimated with the *P. putida* bioreporter in BESAW medium supplemented with *V. natriegens* PWH3a lysate. Cells preconditioned in Fe-deficient BESAW medium were used to inoculate Fe-free BESAW medium amended with increasing amounts of bacterial lysate.

The bioluminescent response of *P. putida* FeLux in treatments supplemented with increasing levels of Fe from *V. natriegens* PWH3a lysates also followed a sigmoidal function ($r^2 = 0.997$; Figure III.1B). Light production reached a maximal level of $0.37 \pm 0.013 \text{ Rlu s}^{-1} \text{ cell}^{-1}$ in treatments supplemented with 43.3 to 160 pM Fe. No significant variations were found between these treatments (ANOVA, $p > 0.05$). The threshold Fe concentration for a significant decrease of light production was reached in the treatment supplemented with 0.21 nM of Fe (Tukey-HSD test, $p = 0.02$).

As such the repression of high-affinity Fe transport systems occurred at Fe concentrations $\geq 0.21 \text{ nM}$ in lysate treatments (Figure III.1B, Figure III.2), relative to total Fe concentrations $> 200 \text{ nM}$ (i.e. pFe 19.54) when Fe was complexed to EDTA (Figure III.1A, Figure III.2). This result suggests that Fe present in the dissolved fraction of *V. natriegens* PWH3a lysate was ca. 3 orders of magnitude more available to our bioreporter strain than Fe in the trace-metal buffered BESAW medium. The dose-response elicited by the addition of Fe in form of bacterial lysate was dramatically greater than when Fe source was provided as Fe-EDTA complex (Figure III.2). Light production decreased by a factor of 2 between the treatment amended with 0.16 nM Fe-lysate mixture and the full lysate treatment (ca. 4.33 nM of Fe) while it decreased only 20% between pFe 19.54 and pFe 16.84 treatments.

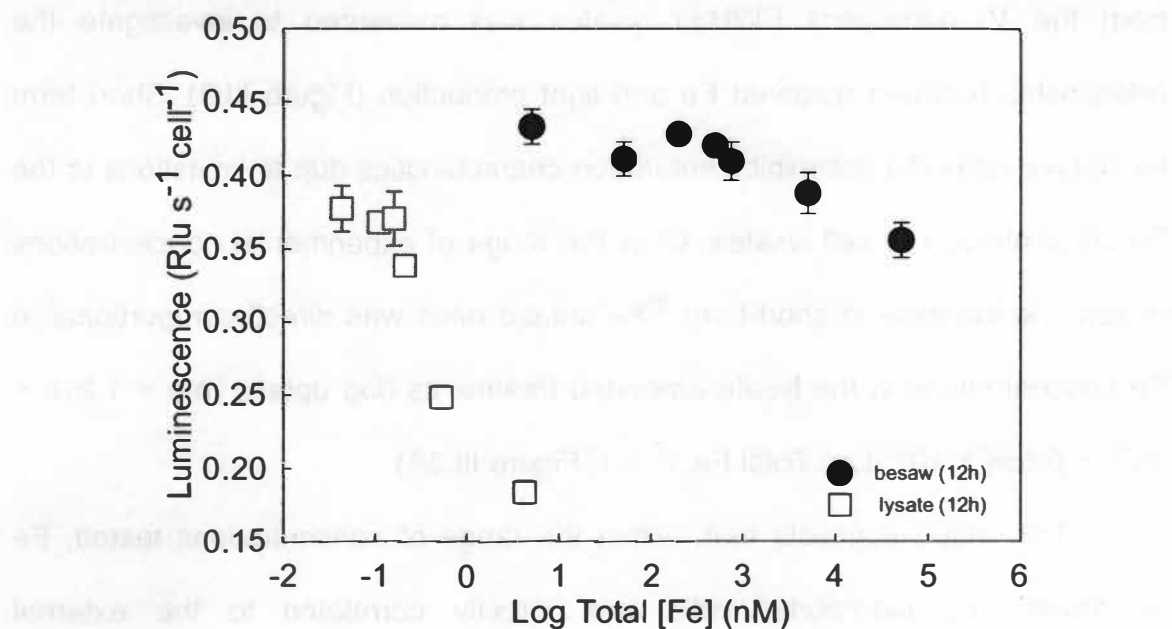


Figure III.2. Dose response of *P. putida* FeLux in both Fe-EDTA- and lysate-supplemented BESAW medium. Fe concentrations are reported as total concentrations of Fe to allow comparison between treatments. Bioluminescence production was measured following 12 h of incubation and normalized to the number of bioreporter cells per milliliter. Error bars represent the standard deviations between replicates ($n = 3$).

Fe assimilation from lysates - The rate of Fe assimilation by the bioreporter cells from the *V. natriegens* PWH3a lysates was measured to investigate the relationship between acquired Fe and light production (Figure III.3). Short term Fe uptake rates did not exhibit saturation characteristics due to limitations in the Fe concentration of cell lysates. Over the range of experimental concentrations tested, the increase in short-term ^{55}Fe uptake rates was directly proportional to Fe concentrations in the lysate amended treatments (log uptake rate = $1.218 \times 10^{-23} + 6.534 \times 10^{-21} \text{ Log Total Fe}$; $r^2 = 1$; Figure III.3A).

This result suggests that, within the range of concentrations tested, Fe assimilated by bioreporter cells was directly correlated to the external concentrations of Fe and thus that maximal velocity of transport was not reached. A comparison between uptake kinetics and bioreporter luminescent response (*i.e.* high-affinity transport systems expression) is presented in Figure III.3B. Over the range of concentrations tested, the bioluminescent signal produced by *P. putida* FeLux decreased as ^{55}Fe uptake rates increased. This suggests a strong correlation (inverse) between bioluminescent signal and Fe assimilation, and as such validates the use of *P. putida* FeLux to estimate the available fraction of Fe in the extracellular environment. The luminescent signal was saturated for uptake rates lower than $1.02 \times 10^{-21} (\pm 2.41 \times 10^{-23}) \text{ mol Fe cell}^{-1} \text{ day}^{-1}$ as no significant differences were observed between these three treatments (ANOVA, $p > 0.05$). However, for these treatments, ^{55}Fe uptake rates decreased linearly although Fe-transport systems were fully derepressed. Luminescence production also appeared quenched by our highest Fe treatment.

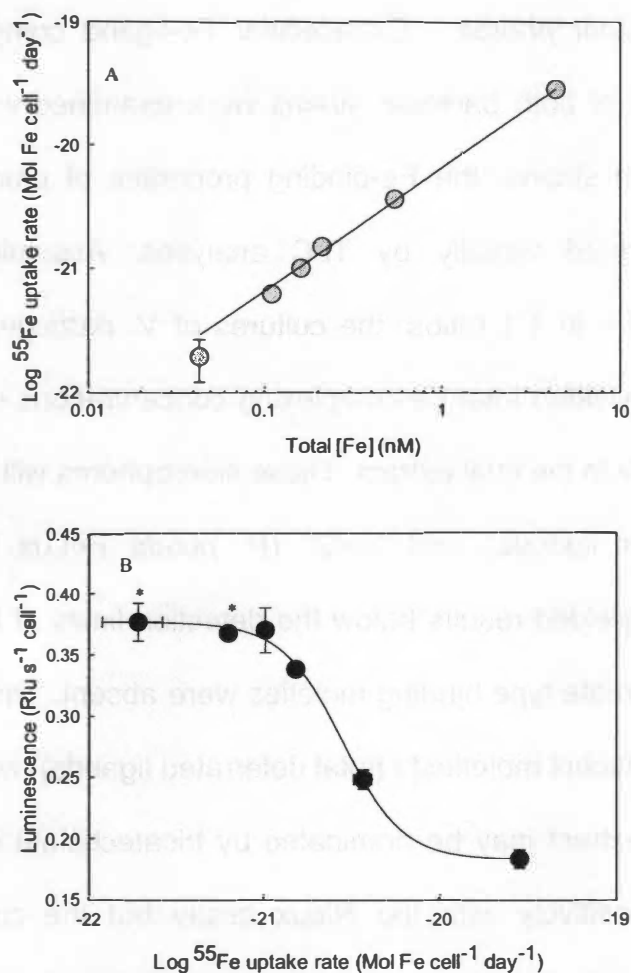


Figure III.3. Uptake of Fe from *V. natriegens* PWH3a lysate by *P. putida* FeLux. A: Fe uptake rate as a function of total Fe concentrations in lysate-supplemented BESAW ($r^2 = 1$, Log uptake rate = $1.218 \times 10^{-23} + 6.534 \times 10^{-21}$ Log Total Fe). Error bars represent the standard deviations between replicates ($n = 3$), and are not shown where they are smaller than the symbol size. B: Comparison of Fe bioavailability with Fe uptake. The four-parameter logistic function (linear; $r^2 = 0.9999$) characterizing the relationship between these two parameters as determined by using Sigma-plot software (ver. 9, SPSS Inc.) is represented. Values denoted with an asterisk were not significantly different from one another. All other values showed significant differences ($p < 0.05$).

Characterization of siderophores - Extracellular Fe-ligand complexes isolated from the supernatant of both bacterial strains were examined with CAS assay (Table III.2). For both strains, the Fe-binding properties of crude siderophore extracts were confirmed visually by TLC analyses. Assuming that these siderophores bound Fe in 1:1 ratios, the cultures of *V. natriegens* sp. PWH3a and *P. putida* FeLux yielded total Fe-complexing concentrations of 9.25 μ M and 12.00 μ M, respectively in the final extract. These siderophores will be referred as “cat1” (*V. natriegens* ligands) and “cat2” (*P. putida* FeLux ligands). Both siderophore extracts yielded results below the detection limits of the Csaky test, implying that hydroxamate-type binding moieties were absent. The ratio of Rioux to CAS reactants ([catechol moieties] / [total deferrated ligands]) was ~ 3 for cat2, suggesting that this extract may be dominated by tricatecholate siderophore(s). Cat1 also reacted positively with the Rioux assay but the concentration of catechols as in excess of the 3:1 (Rioux:CAS) predicted for tricatecholates, suggesting some other compounds (e.g. phenolics) that reduce Fe at low pH may have been present in the supernatant of *V. natriegens* cultures (Granger & Price 1999).

Assessment of siderophore bioavailability - The bioavailability of the siderophores to the *P. putida* FeLux bioreporter was examined in a separate set of experiments and compared to the bioavailability of inorganic Fe and Fe-DFB

Table III.2. Concentrations of the Fe ligands isolated from the supernatants of *V. natriegens* and *P. putida* FeLux.

Strain	CAS assay (μM ligands)	Csaky Test (μM hydroxamate)	Rioux Assay (μM catechols)
<i>V. natriegens</i> PWH3a	9.25	nd	> 50.00
<i>P. putida</i> FeLux	12.00	nd	40.90

nd - the concentration was below the detection limit of the assay (*i.e.* 10 nM in concentrated extracts analyzed by the Csaky test).

complex (Figure III.4). The concentration of bioavailable Fe in BESAW medium amended with inorganic Fe (FeCl_3) was not significantly different from BESAW medium supplemented with Fe-EDTA complex corresponding to a pFe level of 18.12 (*t*-test, $p > 0.05$).

DFB addition resulted in a significantly higher light production relative to the inorganic Fe treatment (*t*-test, $p = 0.003$). Light production from bioreporter cells grown in the medium amended with DFB corresponded to the saturation range of the calibration curve (*i.e.* pFe > 19.54) suggesting that high-affinity systems were completely derepressed. This result confirms that DFB complexed Fe is not available to the bioreporter.

The impact of DFB on Fe availability was further confirmed in a set of experiments in which increasing DFB concentrations were added as DFB:Fe complexes (1:1 molar ratio) to a BESAW medium supplemented with 15 nM of FeCl_3 (Figure III.5). No significant variation in the bioluminescent response (ANOVA, $p > 0.05$; *Dunnett's* test, $p > 0.05$) was observed between Fe(III):DFB amended treatments compared to the unamended control.

For all treatments, the bioluminescent signals fell into the linear range of the calibration curve, enabling us to determine the concentrations of bioavailable Fe (expressed as pFe) for these treatments (Table III.3). In all treatments, inferred bioavailable concentrations were not significantly different than that of the pFe 18.12 treatment (ANOVA, $p > 0.05$). These data demonstrate that DFB effects on Fe assimilation and bioreporter light production are a direct response to perceived bioavailable Fe in the extracellular environment (*i.e.* Fe

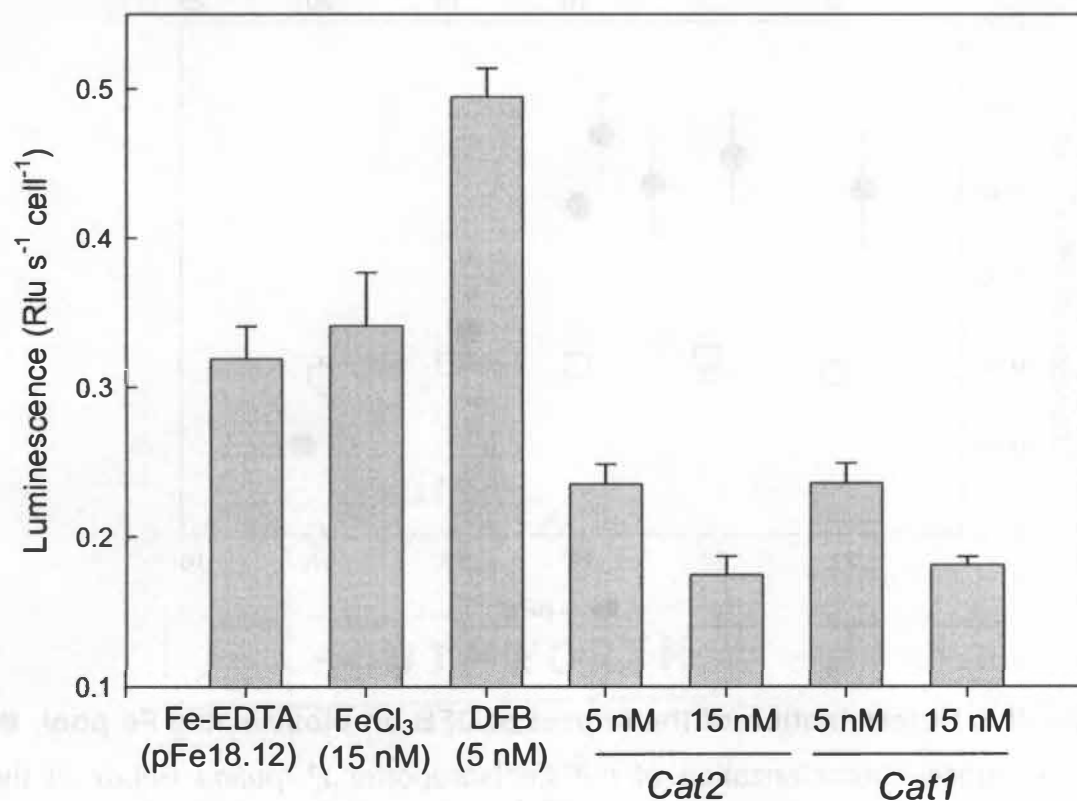


Figure III.4. Comparison of the bioavailability of Fe in presence of various ligands, including the siderophores produced by *V. natriegens* and *P. putida* FeLux. Error bars represent the standard deviations between replicates ($n = 3$).

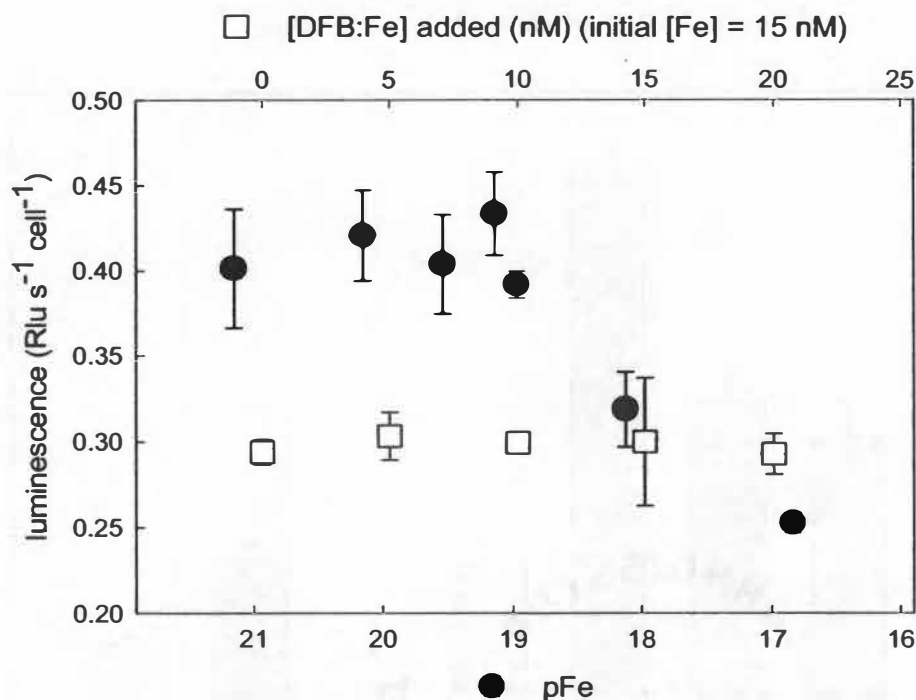


Figure III.5. Determination of the impact of DFB on bioavailable Fe pool. ●: Dose-response characterization of the Fe bioreporter *P. putida* FeLux in the trace-metal buffered BESAW medium.. Cells were prepared as described in Figure III.2. □: Bioavailable Fe level predicted from *P. putida* FeLux bioreporter cells luminescence analyses in BESAW medium supplemented with equimolar Fe:DFB complex. Cells preconditioned in Fe-deficient BESAW medium (pFe 21.15) were used to inoculate BESAW medium ([FeCl₃] = 15 nM, EDTA omitted) supplemented with increasing amounts of Fe:DFB complex (Fe = :DFB = 5, 10, 15 and 20 nM). Fe concentrations are reported as total concentration of Fe and DFB added to allow comparison between treatments. Control treatment was not amended with Fe:DFB complex (0 nM) and represent the bioavailable level of Fe in presence of 15 nM of inorganic Fe. Bioluminescence production was measured following 12 h of incubation and normalized to the number of bioreporter cells per milliliter. Error bars represent the standard deviations between replicates ($n = 3$). pFe values computed for each DFB:Fe amended-treatments are reported in Table III.1.

Table III.3 Evaluation of the impact of DFB addition on the bioluminescent signal of *P. putida* FeLux.

Treatments	Total Fe (nM)	DFB (nM)	Inferred pFe
0	15	0	17.95 ($\pm$ 0.05)
5	20	5	18.01 ($\pm$ 0.09)
10	25	10	17.99 ($\pm$ 0.03)
15	30	15	17.89 ($\pm$ 0.42)
20	35	20	17.94 ($\pm$ 0.10)

pFe values reported here represent the mean value ($\pm$ S.D.) of the pFe determined with Sigma-Plot (ver. 9.0, SPSS Inc.) "Plot equation" function for each replicate ($n = 3$ for each treatment) using the regression analysis performed in the linear range of the calibration curve in the trace-metal buffered BESAW medium. For comparison with Figure III.5, treatments are designated as the total concentration of FeCl_3 and DFB added.

complexation by DFB when DFB is in excess). Moreover, the consistency of the results (*i.e.* 15 nM FeCl₃ ~ 10^{-18.12} M Fe³⁺ ~ Fe(III)' = 1.63 nM) highlights the reproducibility and sensitivity of the bioreporter tool. In contrast to DFB amended treatments, addition of either bacterial siderophore extract resulted in a significant decrease in bioluminescent signal relative to treatments amended with inorganic Fe (*Tukey-HSD* test, $p < 0.001$). The results suggest that siderophore addition significantly increased Fe availability as compared to the inorganic Fe treatment. All siderophore-supplemented treatments were at the lower limit of the linear range of the calibration curve (*ca.* pFe ~ 16.84). No significant difference was observed between treatments amended with similar level of cat1 and cat2 (*Tukey-HSD* test, $p > 0.05$ for both 5 nM and 15 nM additions). This suggests that *V. natriegens* siderophores were available to *P. putida* FeLux. Slight but significant decreases in light production per cell were observed upon addition of 15 nM siderophore as compared to the treatments amended with 5 nM of siderophore (*t*-test, $p = 0.003$ [cat1 5nM – cat1 15 nM], $p = 0.004$ [cat2 5nM – cat2 15 nM]). However, light production was never completely “switched off”, suggesting that a basal level of activity of the high-affinity transport systems was not repressed during the 12h assay.

Discussion

Two main conclusions can be drawn from this study. First, independent lines of evidence are presented which validate the use of the *P. putida* FeLux bioreporter as a quantitative tool to estimate the biological availability of Fe in

marine medium. The two parameters used in this study to estimate Fe bioavailability, bioluminescent signal and Fe uptake, are strongly correlated. Light production (*i.e.* the expression of high-affinity transport systems) from *P. putida* FeLux decreased as assimilation rates increased, suggesting a tight relationship between Fe acquisition and the luminescent response from this bioreporter strain. Furthermore, the results demonstrate the suitability of our bioreporter strain to discriminate across ranges of bioavailability of various Fe-organic complexes. Using the bioluminescent response of *P. putida* FeLux, we were able to rank the Fe sources tested here in a decreasing order of bioavailability: Fe-cat1 = Fe-cat2 > inorganic Fe (FeCl_3 , 15 nM) = inorganic Fe species from EDTA-buffered treatment ($\text{pFe } 18.12$, $\text{Fe(III)}' = 980 \text{ pM}$) > Fe:DFB. Lastly, results from Fe(III):DFB amended treatments demonstrate that the bioluminescent response is impacted by the bioavailable Fe concentration and not the total dissolved Fe concentration in the extracellular environment. Taken together, our results support therefore the conclusion that the bioreporter *P. putida* FeLux could be a valuable tool to estimate variations in the bioavailability of naturally occurring Fe-ligand complexes in marine systems.

As suggested in Poorvin et al. (2004), this study also confirms that viruses infecting prokaryotes may play a crucial role in planktonic Fe regeneration. Both the bioluminescent reporter signal and ^{55}Fe assimilation rates of the bioreporter cells provided a dose-dependent response to increasing Fe from the dissolved (< $0.2 \text{ }\mu\text{M}$) fraction of *V. natriegens* lysate. As well, these results suggest that Fe from the lysis products enters the bioreporter cells *via* multiple transporters, as

ferric uptake regulator (FUR)-controlled high-affinity systems can be repressed (as demonstrated by reduced light production) while transport velocities continue to increase. Given the energetic cost of high-affinity Fe transport system production, multiple acquisition systems may be advantageous in Fe-limited environments. Finally, Fe released from *V. natriegens* lysate and presented to the bioreporter resulted in a more significant decrease in the bioluminescent signal than when the bioreporter was fed with its own siderophores. As discussed below, our results support the hypothesis that Fe released from lysates is highly bioavailable. Given that these lysates contain a diverse collection of organically-bound Fe sources, Fe released from virus-mediated lysis may be acquired through specific (e.g. outer membrane receptors) and non-specific (e.g. porin channels) transport pathways (Winkelmann 1990).

Determination of Fe bioavailability from virus lysates - Results from the bioreporter and ^{55}Fe assimilation assays demonstrated that Fe present in the dissolved phase of *V. natriegens* lysates was significantly (ANOVA, $p > 0.05$) more available to *P. putida* FeLux than Fe present in Fe-EDTA buffered medium. These results are consistent with recent reports (Poorvin et al. 2004). Using ^{55}Fe uptake assays and the marine bacterium *Vibrio harveyi* as a model organism, Poorvin et al. (2004) also observed that Fe released from the dissolved fraction of virus-mediated bacterial lysis was more readily assimilated than Fe complexed to EDTA.

Our inability to saturate Fe assimilation rates for *P. putida* FeLux in the lysate-amended treatments while repressing light production indicates that Fe complexes released from virus-mediated lysis entered the cells through more than one transport systems, *i.e.* both non-specific (low-affinity system) and specific routes (high-affinity systems) (Winkelmann 1990). It has been reported that high concentrations of Fe-ligands may contribute to a diffusion-like behavior while transport systems start operating inefficiently or are bypassed (Winkelmann 1990). This hypothesis is supported by the comparison of uptake rates to bioreporter bioluminescent response (*i.e.* high-affinity transport systems expression). Through the range of concentrations tested, the bioluminescent response was not linear, while the ^{55}Fe uptake rate increased linearly. An indirect linear relationship between light production and Fe concentrations was only observed for the three intermediate treatments (*ca.* 0.16 nM, 0.21 nM and 0.54 nM). High-affinity transport systems reached their maximal level of expression at total Fe ($< 0.2 \mu\text{M}$) concentrations lower than 0.21 nM, and were fully repressed (characteristic of Fe-replete conditions) in the “full lysates” treatment (*ca.* 4.33 nM). These data highlight one limitation of bioreporters as a tool: the relatively limited dynamic range through which they can be used as a quantitative tool (Durham et al. 2002). However, they also demonstrate that the linear portion of the dose-response occurs within the range of dissolved organic Fe concentrations commonly found in aquatic environments (Rue & Bruland 1995, Wu & Luther 1995), confirming that *P. putida* FeLux bioreporter is suitable to assess Fe bioavailability in HNLC environments.

The ^{55}Fe assimilation rates also provide important data that suggest Fe-uptake rates were directly proportional to extracellular Fe concentrations over the range of concentrations tested. Therefore, although the high-affinity Fe transport systems were fully derepressed, Fe assimilation rates were lower in the Fe-limited treatments. Furthermore, although high-affinity transport systems were reduced to minimal expression level in the higher range of concentrations tested, Fe assimilation increased linearly with higher ^{55}Fe concentrations. If transport rates were only influenced by the density of FUR-regulated membrane receptors (and thus light production), uptake rates should have followed Michaelis-Menten saturation kinetics within the range of Fe concentrations tested. Such a saturation curve was not observed even though we know FUR-regulated transport was repressed. This highlights the difficulty of characterizing Fe transport in systems where multiple transporters (and multiple Fe-complexes) persist. Taken together, these results suggest that Fe permeated the cells through pathways that were not FUR-regulated.

Previously size fractionation studies have shown that the virus-mediated lysis of *V. natriegens* cells releases Fe predominantly in the < 3 kD size-fraction (Poorvin et al. 2004). Previous studies with cyanobacteria (Wang & Dei 2003) have also demonstrated that Fe present in the < 1 kDa size fraction of seawater was assimilated ca. 1.7 times faster than Fe-complexes of higher molecular weight (1 kDa – 0.2 μm). It is therefore plausible that a fraction of the organic Fe-complexes released from virus-mediated lysis are small enough to pass through membrane porins (size limit ~ 600 Da, Andrews et al. 2003) and constitute a non-

negligible source of bioavailable Fe. The available literature data documenting “low-affinity” Fe transport systems are scarce, and mainly focuses on the pathogenic bacteria *Escherichia coli* and *Helicobacter pylori* (Andrews et al. 2003, Velayudhan et al. 2000). Several potential transport pathways, including outer-membrane receptors specific to other metals or cations, and nonbiologically-driven Fe binding to the cell surface, have been suggested; however, a low-affinity transport system has not been identified to date (Andrews et al. 2003). Recent experiments on the bacterium *H. pylori* suggest that low-affinity Fe uptake systems are not sufficient to support optimal growth even in presence of high concentration of Fe (Velayudhan et al. 2000).

Previous reports also suggest that Fe-uptake systems involved in the acquisition of Fe from heterologous ferric-binding ligand complexes are differentially regulated relative to those involved in the acquisition of Fe from cognate siderophores (Ratledge & Dover 2000, Venturi et al. 1995). It has been suggested that an Fe-acquisition system involving an outer-membrane ferric reductase associated to an Fe(II)-salicylate shuttle could be a common feature in bacteria (Ratledge & Dover 2000). Such a pathway of Fe acquisition would be less specific than the acquisition system involved in the uptake of cognate siderophores, and may allow bacteria to acquire Fe bound to a wide range of organic ligands.

These results suggest that Fe sources present in virus-mediated lysates are highly available to heterotrophic bacteria and may be internalized into microbial cells through several routes. Such a transport strategy is plausible

owing to the diversity of the potential Fe sources in natural systems. If *P. putida* FeLux (or other) bacterial cells relied only on highly specific high-affinity transport systems, cells would have to produce a large variety of specific outer membrane receptors. As such bacterial cells would be at a competitive disadvantage given the energetic costs required to produce and maintain these systems (Griffin et al. 2004). As organic Fe-ligands are often found in excess in surface seawater (Rue & Bruland 1995, Wu & Luther 1995), we speculate that microorganisms may employ multiple Fe acquisition strategies in oceanic environments.

Specificity of Fe-siderophore acquisition and cross-utilization - Under our experimental conditions, the bioluminescent signal was higher when Fe was provided as inorganic Fe than when Fe supplied as a Fe(III)-catechol complex (Figure III.4). These results suggest that cells require the activation of the high-affinity transport systems to internalize inorganic Fe. Although no inorganic Fe(III)-transporter has been found to date in the outer membrane of heterotrophic bacteria (Granger and Price 1999), inorganic Fe may be internalized into cells as Fe(III)-siderophore complexes through outer-membrane receptors (Winkelmann 1990) or through a broader Fe(III) exchange mechanism also requiring production of siderophores (Stintzi et al. 2000). Our results agree with previous reports which suggest that inorganic Fe(III)-acquisition by heterotrophic bacterial cells requires activation of high-affinity transport systems and the production of siderophores (Guan et al. 2001, Granger & Price 1999). Indeed our results confirm that the bioluminescence signal is indirectly correlated to the estimated

concentration of bioavailable Fe in the environment of the bioreporter and not to the total Fe concentration.

At comparable concentrations, Fe released from *V. natriegens* lysates appeared more efficient at satiating bioreporter cells requirements than Fe from Fe(III)-siderophore complexes. This result supports our hypothesis that Fe released from virus-mediated lysis enters cells through transport systems other than the specific outer-membrane receptors. In the case of siderophores, high-affinity systems need to remain active to maintain production of outer-membrane receptors (Andrews et al. 2003). Fe(III)-siderophores complexes are too large to be internalized by passive diffusion or non-specific transport in Gram negative bacteria (Andrews et al. 2003). Two main mechanisms for Fe(III)-siderophore transport have been previously described in Gram-negative bacteria: one specific, involving ligand exchange at the level of the outer-membrane receptor, and a second non-specific with a siderophore shuttle mechanism in which ligands pass serially through a channel with an Fe(III) exchange between siderophores (Wilhelm and Trick 1994, Stintzi et al. 2000). Although Fe acquisition through the ligand exchange mechanism has been shown to mediate cross-utilization of exogenous siderophores (Stintzi et al. 2000), it has also been shown that some outer membrane receptors recognize Fe-siderophore complexes produced by other species (Winkelmann 1990). The lack of significant difference observed in the bioluminescent signal between the cat1 and cat2 amended treatments suggests that in both cases high-affinity transport systems were probably involved in Fe-acquisition. And although both siderophores were

available to *P. putida* FeLux, the luminescent signal remained higher than observed in the lysate-amended treatments. These results suggest that a basal expression of the high affinity-transport systems was required to maintain the production of transport components and may reflect a homeostatic balance between the cost of the production of such high-affinity systems and the energy required to sustain cell metabolic functions (Andrews et al. 2003).

Interestingly, the bioluminescent signal was never completely repressed by the addition of the most available form of Fe, the lysates. This observation suggests that high-affinity transport systems, and probably other FUR-regulated genes, are not a “fully on/off” system. McHugh et al. (2003) observed differences in the level of derepression between genes involved in Fe-acquisition at identical Fe concentrations. Ferric-enterobactin uptake genes (such as the *fepA* acquisition system used in our bioreporter) were among the most weakly derepressed, indicating that such a system is controlled more by cellular Fe status than by the acquisition apparatus itself. Thus, we speculate that the background bioluminescent signal may reflect the basal level of high-affinity transport system expression required to sustain the increased Fe demand resulting from the induction of Fe-containing proteins synthesis (e.g. respiratory proteins). Such maintenance of high-affinity system expression, even under suboptimal Fe conditions, may also reflect a compromise between maintaining assimilation rates and reducing transporter proteins which also act as targets for bacteriophage and antibiotics (Andrews et al. 2003).

In contrast to cat2 and the exogenous cat1, the fungal siderophore DFB sequestered Fe away from the bioreporter cells. Although it has been shown that DFB has toxic effect on eukaryotic cells (Leardi et al. 1998, Fukuchi et al. 1994), we have demonstrated that this it is not the case for our heterotrophic bioreporter (Figure III.5). Our results do demonstrate that Fe:DFB complex were not available to *P. putida* FeLux, probably because DFB was not recognized by the outer membrane receptors (or Fe(III)-ligand exchange mechanism). Although results based on a single model organism must be extrapolated with caution, most studies in aquatic systems also suggest that the fungal siderophore DFB reduces the bioavailability of Fe to the planktonic community (Eldridge et al. 2004, Mioni et al. 2003, Weaver et al. 2003) and that the transport kinetics of Fe:DFB complexes are not sufficient to sustain growth (Hutchins et al. 1999b). However, Fe acquisition from Fe:DFB complexes is still a source of debate as it has been also suggested that phytoplankton communities can access to at least a fraction of this chelated Fe (Maldonado & Price 1999) and that the diatom *Phaeodactylum tricornutum* could reduce Fe:DFB complexes by reductases located on the cell surface and subsequently internalize the reduced Fe (Soria-Dengg & Horstmann 1995). The utilization of the siderophore DFB as a carbon source has been recently reported for one isolated soil bacterium, a *Rhizobium loti*-like organism (Pierwola et al. 2004), but could represent an adaptation to its ecological niche since actinomycetes (those that produce DFB) are relatively abundant in soil environments. To date, only one marine siderophore (DFG) closely related to DFB has been fully characterized, and it is produced by a

symbiotic *Vibrio* strain not commonly found as free living cells in the water column (Martinez et al. 2001). Indeed, one may presume that producing receptors specific to such a rarely occurring trihydroxamate siderophore would be needlessly expensive for natural bacterioplankton.

Although the cross-availability of various siderophores to diverse aquatic organisms has been documented for laboratory culture or enclosed-seawater samples (Weaver et al. 2003, Guan et al. 2001, Granger & Price 1999, Hutchins et al. 1999a, Trick 1989), the artificial conditions of these incubation experiments might be a source of bias. Indeed, it has been argued that relying on siderophores as a sole Fe source may be inefficient and energetically expensive in an aquatic environment due to the strong probability of a diffusion of the siderophores away from the cell (Völker & Wolf-Gladrow 1999), especially if other microbes could exploit these newly formed complexes (Griffin et al., 2004). The cost of siderophore excretion by an isolated heterotrophic cell is also metabolically expensive, especially where organic carbon may also be limiting (Kirchman et al., 2000). Models predict that Fe acquisition through freely diffusible siderophores may be an efficient strategy in oceanic environment only when the concentration of organic ligands in the surrounding environment is high enough to allow the bacteria cell to use siderophores (cognate and xenosiderophores) excreted by other cells (Völker & Wolf-Gladrow, 1999).

One way to counter this problem is to produce compounds with reduced diffusive potential in aquatic systems. Wilhelm & Trick (1994) proposed such a model, where production of relatively hydrophobic catechol-type siderophores

(like those discussed in this study) allows for cells to maintain a surface concentration of active chelates. More recently Martinez et al. (2003) identified cell-associated amphiphilic chelators in marine bacterial samples and suggested that this cell association could be a strategy to counter siderophore diffusion in the oceanic environment.

Conclusions

Results from this study reinforce that Fe released from virus-mediated lysis should be an important source of bioavailable Fe to bacterioplankton (Poorvin et al. 2004). Increasing evidence suggests that viruses play a pivotal role in Fe transfer in aquatic environments (Poorvin et al. 2004, Wilhelm & Suttle 2000, Gobler et al. 1997). In the case of heterotrophic bacteria, the bioavailability of Fe in the lysates may be related to the high diversity of organic Fe species released through virus-mediated lysis. Considering the importance of viral lysis in nutrient regeneration, we speculate that Fe acquisition through molecular recognition pathways (*i.e.* siderophore-specific outer membrane receptor) might not be the main system used by microbial cells under ambient conditions. The relatively small size of bacterial cells and the miniaturization of microbial cells observed under Fe limitation (Eldridge et al. 2004) might enable them to rely on non-specific Fe pathways such as diffusion through porins or oxydo-reduction through outer-membrane ferric reductases. Such processes may explain why marine prokaryotes are able to sustain higher Fe assimilation rates and cells Fe:C quotas than eukaryotic phytoplankton (Tortell et al. 1999).

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Part IV

Comparative bioavailability of a catecholate siderophore and virus-mediated lysates from the marine bacterium *Vibrio natriegens* PWH3a

This section is a version of a paper with the same title to be submitted to the journal Limnology and Oceanography by Leo Poorvin, Sylvia G. Sander, Keith A. Hunter, Steven W. Wilhelm.

My use of “we” in this section refers to my co-authors and myself. My primary contributions to this paper were: (1) all phases of experimentation and data analysis, with the exception of electrochemical analysis, (2) most of the writing of the manuscript.

Abstract

We compared the bioavailability of Fe complexed to siderophores produced by the heterotrophic marine bacterium *Vibrio natriegens* PWH3a and Fe from ligand-complexes present in virus-mediated lysates of this same bacterium. Fe-binding functional groups, stability constants and the bioavailability of Fe associated with these two separate ligand pools were determined. Under low-Fe growth conditions, axenic cultures of *Vibrio natriegens* PWH3a were shown to produce a catecholate siderophores, while neither catecholate nor hydroxamate-type Fe-binding moieties were detected in virus-generated cell lysates. Analysis of stability constants using electrochemical techniques revealed that the catecholate siderophore extract and the organics in the virus-mediated lysates had Fe-binding constants comparable to the organic ligands found in marine surface waters. In Fe assimilation studies, the lysate was found to be more bioavailable to our model heterotrophic bacterium, autotrophic

cyanobacterium and eukaryotic diatom cultures than the catecholate siderophore. The high bioavailability of Fe in the virus-mediated cell lysates coupled with the rapid turnover of prokaryotes by viruses observed in other studies suggests that virus driven recycling may provide a significant fraction of the Fe demand in marine systems.

Introduction

It has been well understood for many years that primary production in many aquatic environments is often rate-limited by the supply or speciation of essential nutrients, including iron (Fe) for phytoplankton growth (e.g. Gran 1931; Harvey 1937). During the past decade both mesoscale fertilization experiments (Martin et al. 1994; Boyd et al. 2000) and controlled mesocosm / bottle incubation studies (Hutchins et al. 1998; Bruland et al. 2001; Eldridge et al. 2004) have shown that the availability of iron (Fe) to primary producers in high-nutrient, low-chlorophyll (HNLC) areas of the world's oceans influences ecosystem level production. Current models suggest that approximately half of the world's oceans may be limited by Fe availability (Moore et al. 2002), and that as much as 90 % of the production in these regions could be fueled by virally regenerated Fe (Poore et al. 2004).

In the last decade, chemical analyses of the dissolved Fe in marine waters has suggested that the majority (> 95%) is complexed to organic ligands (Rue and Bruland 1995), implying that these ligands may influence the biological availability of the Fe to marine communities. In mesoscale Fe addition

experiments, the concentration of these ligands has been observed to increase following Fe addition (Croot et al. 2001). Indeed, one hypothesis for the origin of these ligands is that a significant fraction are siderophores produced by marine planktonic organisms (Rue and Bruland 1997; Powell et al. 1995). Siderophores are low molecular weight, high-affinity Fe(III)-binding ligands produced by many diverse organisms, that have in recent years been shown to be produced by numerous marine bacterial and cyanobacteria (Trick 1989; Wilhelm and Trick 1994; Butler 1998). In Fe-limited HNLC surface waters, siderophores may play a potentially important role in Fe assimilation by marine prokaryotes. Granger and Price (1999) have demonstrated that various heterotrophic marine bacteria can assimilate Fe complexed to siderophores. Interestingly, in their study all the bacteria examined were able to assimilate Fe from these complexes, regardless of whether the bacterium was able to produce the specific siderophores. In a study by Weaver et al. (2003), Fe complexed to siderophores and other organic ligands had differing bioavailabilities to alpha and gamma proteobacteria. Yet another series of studies have shown that Fe bound to a model siderophore from a terrestrial actinomycete, desferrioxamine B, is differentially available to microbial communities from various environments (Maldonado and Price 1999; Hutchins et al. 1999; Wells and Trick 2004; Eldridge et al. 2004). Given the ubiquitous distribution of prokaryotes in the ocean, the importance of siderophores in marine Fe cycles seems obvious. However, in laboratory experiments, marine heterotrophic bacteria and cyanobacteria produce siderophores only in response to Fe-limitation (Butler 1998; Wilhelm and Trick

1994), and the case for siderophores as the major Fe-binding ligands associated with Fe-fertilization experiments seems tenuous.

During the last 15 years research has also illuminated the important role that virus can play in marine microbial communities (e.g., see Wommack and Colwell 2000; Arrieta et al. 2004). These observations include, but are not limited to, a potentially important role in biogeochemical cycles (Wilhelm and Suttle 1999; Gobler et al. 1997). Combining lab and field studies, recent work has extended observations on viruses to their role in marine Fe cycles and suggested that a significant fraction of the Fe-binding ligands in seawater may be released during from the lysis of marine plankton (Poorvin et al. 2004). To this end we have labored under the hypothesis that the regeneration of Fe-organic complexes in seawater could be of some significance, as new Fe inputs in HNLC regions can only support 4-20% of the primary production that occurs (Hutchins 1995). In the current study we have continued to characterize this potential source of Fe-ligand complexes by contrasting Fe ligands released from the virus-mediated lysis of a model marine bacterioplankton with siderophores produced by this same cell line. The results of this work clearly demonstrate that the Fe-binding ligands released by cell lysis are distinctly different from siderophores the cell produces under Fe-limited growth conditions.

Material and Methods

Production and isolation of siderophores – Throughout all experiments, acid-cleaned polycarbonate containers, Chelex-100 treated water (Price et al. 1989),

and trace metal clean protocols (Bruland et al. 1979) were employed to reduce Fe contamination. Siderophores produced by the marine bacterium *Vibrio natriegens* PWH3a, were isolated following previous protocols used with marine prokaryotes (Wilhelm and Trick 1994). To induce siderophore production, a low Fe adapted culture of the heterotrophic bacterium *V. natriegens* PWH3a was inoculated into 10 L of ESAW (enriched seawater, artificial water) (Berges et al. 2001) containing a final concentration of 1.0 nM. total Fe, no EDTA, 1/10th the standard vitamin stock and glycerol at a final concentration of 6.85 mM. The *V. natriegens* PWH3a culture was incubated on the benchtop with gentle agitation at ca 24 °C. After 24 hrs, cells were removed from the culture by centrifugation (~10,000 g for 10 min) and the supernatant filtered through a 0.22 µm Millex-GV PVDF membrane to remove residual cells and debris. After the pH was reduced to 3.0 (via the addition of trace metal clean HCl) to disassociate Fe-siderophore complexes, it was gravity fed through a Amberlite XAD-16 resin column (approximately 100 mL total bed volume) to bind organic molecules. The column was then rinsed with 1 L of Chelex-100 treated Milli-Q water to remove any salts or other unbound materials (Wilhelm and Trick 1994). Organic material, containing the Fe-free siderophores bound to the column was eluted with 300 mL of methanol (Fisher Optima trace metal grade). The siderophores-enriched eluent was concentrated by rotary evaporation to a final volume of ca 15 mL, and stored at 4°C in the dark until use.

Production of bacterial lysates—Virus-mediated lysates of the marine bacterium *V. natriegens* PWH3a were generated for comparison to isolated siderophores using previously established protocols (Poorvin et al. 2004). Briefly, cultures of *V. natriegens* PWH3a were inoculated into Fe-replete ESAW medium. After 24 hrs incubation, the cells were removed and resuspended in low Fe (1 nM) ESAW and a lytic phage (PWH3a-P1) was introduced to lyse the cells. After ~24 hrs unlysed cells and debris were removed *via* filtration through a 0.22 μ m Millex-GV PVDF membrane. Following filtration, organic material was isolated and concentrated using Amberlite XAD-16 resin and rotary evaporation using the same protocol as in isolation of siderophores above.

Analysis of Fe-complexing properties—Thin layer chromatography (TLC) was undertaken on the isolated organic material to detect the presence of Fe-binding compounds. Concentrated eluent (5 μ L) was spotted on silica gel TLC (Whatman PE SIL G) plates and run with a solvent of methanol:water (70:30 v/v). After the sample was run, the plates were developed with I_2 to identify any organic material present. Separate plates were developed with $FeCl_3$ (1% w/v in ethanol) to identify Fe binding compounds (Wilhelm and Trick 1994).

In parallel the Fe binding capacity of the samples was determined using the liquid Chrome Azurol S (CAS) assay (Schwyn and Neilands 1987). The CAS assay measures overall binding capacity of a sample without differentiating functional binding moieties. The model trihydroxamate siderophore desferrioxamine B (DFB, Sigma Chemicals) was used to generate a standard

curve (and as such all results are reported in DFB equivalents). To further characterize the Fe binding species present in the samples, two additional assays, commonly used to study siderophores, were employed. A modified Csaky assay (Csaky 1948; Gillam et al. 1981) was performed to detect hydroxamate functional groups, again using DFB as a standard. To identify catecholate moieties, the Rioux assay (Rioux et al. 1983) was completed using 2,3-dihydroxybenzoic acid (2,3-DHBA) as a standard. Results reported represent concentrations of ligands present in initial cultures prior to extraction and concentration.

Fe association kinetics were also determined for the samples. Electrochemical binding stability analysis of all samples were performed with TAC as a competing ligand using the method of Croot and Johansson (2000). Samples, prepared as above, were stored frozen (-20° C) prior to analysis.

Assimilation of Fe from organic complexes- The catecholate siderophores and DFB were used in Fe assimilation studies. Organics were complexed to ⁵⁵Fe at a 2:1 ratio of chelator:⁵⁵Fe (determined using the CAS assay results) and allowed to equilibrate overnight. The labeled siderophore was added to ESAW media (as above but with no other Fe source) to a final concentration of 3.26×10^{-8} M. Three model uptake organisms were used in uptake studies; the cyanobacterium *Synechococcus* sp. PCC 7002, the heterotrophic bacterium *Vibrio harveyi* and the diatom *Thalassiosira pseudonana*. Low Fe adapted inocula of the model organisms were added to the labeled media and the cultures incubated at

appropriate conditions for each organism. After 24 hrs the cells were filtered, extracellular Fe was removed and cellular uptake of Fe was determined (Poorvin et al. 2004). Fe uptake rates were normalized to cellular carbon content using literature cellular quotas of 210 fg (femtograms) for cyanobacteria, 23.2 fg for heterotrophic bacteria, and 5.94 pg (picograms) for diatoms (Waterbury et al. 1986; Lee and Fuhrman 1987; Montagnes et al. 1994). The potential for Fe uptake up to meet cellular growth demands was estimated as well. This was accomplished by monitoring growth rates of cells and applying literature cellular Fe quotas of 2.62×10^{-18} mol for cyanobacteria, 1.97×10^{-20} mol for heterotrophic bacteria and 9.89×10^{-19} mol for diatoms (Tortell et al. 1996; Wilhelm 1995) to determine the minimum Fe required for production of new cells.

Results

TLC analysis of extracted siderophore - Exposure of the TLC plates containing extract from the low Fe *V. natriegens* culture to I_2 indicated only one band of organic material. Additionally, development with $FeCl_3$ of plates run concurrently showed an Fe binding band with identical mobility. This suggests that the extract consisted of one species of Fe-complexing ligand.

Fe binding assays - CAS assays of both the low Fe *V. natriegens* (which presumably contains siderophores) extract (9.25 nM) and the viral lysis extract (40.9 nM) demonstrated Fe binding. Positive results from the Rioux assay of the low-Fe *V. natriegens* extract indicated the presence of catecholate type species

(> 75.0 nM) (Table IV.1). No catecholates were detected in the viral lysates. Csaky assays failed to detect hydroxamate type moieties in either the low Fe or viral extracts.

Electrochemical binding assay - The isolated siderophore and the viral lysate showed similar binding constants, with each sample appearing to have a single ligand species. The bacterial siderophore had a binding stability of $\log K'_{\text{Fe}'} = 10.87 (\pm 0.25)$ while the viral lysate showed a $\log K'_{\text{Fe}'} = 10.35 (\pm 0.41)$ (Table IV.1). Conversion to $\log K'_{\text{Fe}^{3+}}$ notation using the inorganic side reaction coefficient $\alpha_{\text{Fein}} = 10^{10}$ (Hudson et al. 1992) produces values of $20.87 (\pm 0.25)$ and $20.35 (\pm 0.41)$ for the siderophore and lysate respectively.

Uptake of Fe from siderophore - The isolated catecholate siderophore was significantly ($p < 0.05$) less bioavailable than DFB to the diatom *T. pseudonana* (Table IV.2). However, it was significantly ($p < 0.01$) more bioavailable than DFB to the cyanobacterium *Synechococcus* sp. 7002. No significant difference was noted in bioavailability to the heterotrophic bacterium *V. harveyi*.

Discussion

The goals of this study were to contrast the Fe-binding ligands released by a heterotrophic bacterium during Fe-limited growth (siderophores) and the Fe-organic compounds that result from the lysis of the heterotrophic bacterium by a bacteriophage. The results of this study indicate that the Fe ligands released by

Table IV.1. Comparison of biochemical analyses of siderophores and virus released organics produced by *Vibrio natriegens* PWH3a.

Fe ligand source	CAS (nM ligands)	Csaky (nM hydroxamate)	Rioux (nM catechols)	FeL stability constant logK' $_{Fe^{3+}}$
Low-Fe cell filtrate	13.9	N.D.	>75.0	20.87 ($\pm$ 0.25)
Lysate	61.3	N.D.	N.D.	20.35 ($\pm$ 0.41)

N.D.=none detected.

Table IV.2. Fe assimilation rates by model organisms. (mol Fe g C⁻¹ day⁻¹)

Organism	Catecholate siderophore	DFB
<i>T. pseudonana</i> ^a	7.87 (± 1.86) x 10 ⁻⁷	1.07 (± 0.10) x 10 ⁻⁶
<i>V. harveyi</i>	2.74 (± 0.17) x 10 ⁻⁷	2.68 (± 0.34) x 10 ⁻⁷
<i>Synechococcus</i> sp. 7002 ^b	7.79 (± 0.32) x 10 ⁻⁷	1.55 (± 0.39) x 10 ⁻⁷

^a significant difference (p<0.05)

^b significant difference (p<0.01)

lysis of *V. natriegens* PWH3a are not siderophores. Only one Fe ligand was observed to be produced by *V. natriegens* PWH3a under low-Fe conditions, which was identified as a catecholate siderophore whereas lysis of the organism produced neither catecholate nor hydroxamate functional groups at detectable concentrations. The two ligands did however have similar Fe binding constants. Uptake experiments, when coupled with previous studies (Poorvin et al. 2004), indicate that Fe bound to both the isolated catecholate siderophore and a model trihydroxamate siderophore were relatively less bioavailable to a selection of model unicellular plankton than was Fe released by viral lysis of either heterotrophic bacterial or cyanobacterial organisms. This suggests that lysis products, should they make up a significant fraction of organically complexed Fe in marine systems, may provide a readily available Fe source for plankton.

Analysis of Fe ligands—Both the extract from low-Fe cultured cells and the viral lysate contained a single Fe binding ligand. Electrochemical analysis of both samples indicated ligands with similar $\log K'_{\text{Fe}^{3+}}$ values. These values are comparable to those found in surface marine waters by various researchers (Witter et al. 2000). Interestingly, the binding constants were also similar to constants observed during a dinoflagellate bloom in the eastern Atlantic (Croot and Johansson 2000). While these data do not indicate that the ligands present in surface marine waters necessarily are lysis products, it does not rule it out.

Our analysis of the extract of low-Fe grown cells indicated only the presence of catecholate type moieties. This was somewhat unexpected as

others (Granger and Price 1999) had previously detected hydroxamate type ligands from *V. natriegens* PWH3a via a Csaky assay. However that study utilized growth medium with complex organic enrichments (bactopeptone and casein hydrolysate) whereas our medium was enriched only with glucose. It is possible that *V. natriegens* PWH3a is unable to synthesize hydroxamate siderophores in our more minimal medium. Previous studies have shown that differing mediums (Gram 1996) and particularly carbon sources (Mackie and Birkbeck 1992) can influence amount and types of siderophores produced by bacteria, so it is not unreasonable to suggest that lack of observed hydroxamate type ligands in the current study can be attributed to the medium used.

Uptake of Fe ligands- The current study demonstrated that the catecholate siderophore isolated had varying levels of bioavailability to the three model organisms as compared to the fungal trihydroxamate siderophore DFB (Table IV.2). However, when compared to the uptake rates determined for viral lysates in a previous study (Poorvin et al. 2004), both siderophores were relatively unavailable (Figure IV.1). Viral lysates (both from the heterotrophic bacterium *V. natriegens* PWH3a and the autotrophic cyanobacterium *Synechococcus* sp. WH7803) showed a much greater bioavailability to every uptake organism studied. This is particularly striking when a comparison is made between uptake of Fe contained in the viral lysate of *V. natriegens* PWH3a versus Fe bound to siderophore isolated from the same organism. The heterotrophic bacterium *V. harveyi* was able to take up Fe from the bacterial lysate at a rate more than 5

Uptake comparison

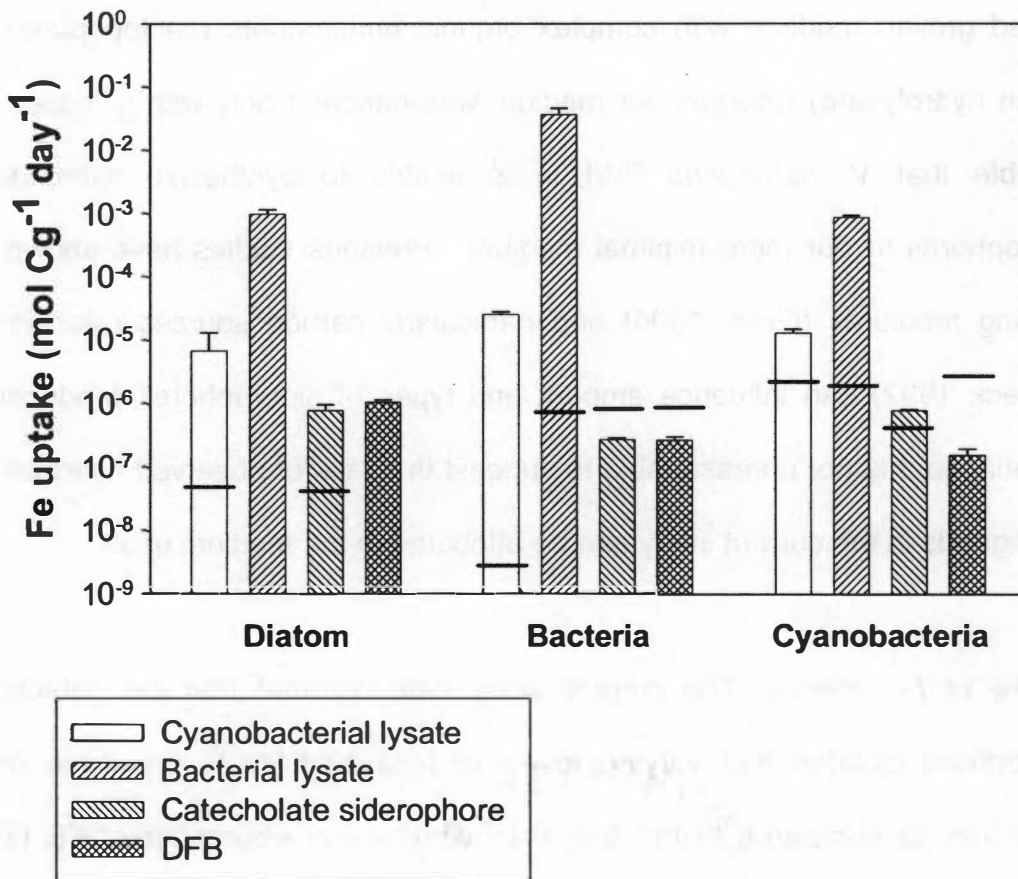


Figure IV.1. Comparison of the bioavailability of lysates and siderophores to three model organisms. With the exception of the bacterial uptake catecholate and DFB siderophores, all values within an uptake organism were significantly different ($p < 0.05$). Data on Fe uptake rates from lysates taken from Poorvin et al., (2004). Horizontal black bars represent calculated Fe uptake required to support growth in cultures where growth was observed. Treatments without black bars had no increase in cell abundance.

orders of magnitude greater than Fe bound to the catecholate siderophore. Comparisons to the cyanobacterial lysate and to the other uptake organisms, while not as dramatic, clearly show the Fe contained in the lysates to be more bioavailable than either siderophore.

Although each organism was able to uptake Fe bound to siderophores to some degree, rates of uptake were not enough to support the cellular growth observed in some cases (Figure IV.1.). Uptake by *V. harveyi* of Fe complexed to either the catecholate siderophore or DFB and uptake of DFB by *Synechococcus* sp. PCC 7002 was less than the rate needed to support the growth observed in the experiment. As the uptake experiment was relatively short (24 hrs) and the cultures were not Fe starved prior in inoculation into the labeled siderophore media, stored Fe may have allowed the increase in cell abundances observed. Had cell growth been monitored over a longer span, Fe limitation may have been noted. Uptake rates of Fe bound to the catecholate siderophore by *Synechococcus* sp. PCC 7002 and *T. pseudonana*, while significantly lower than uptake of either lysate, appeared to meet the organisms' growth requirements. No overall increase in cell abundance was observed for *T. pseudonana* in either the DFB supplemented medium or bacterial lysate, therefore no Fe requirements were calculated. All three organisms assimilated Fe from the cyanobacterial lysate at a rate in excess of what was required for growth. The rates of Fe uptake by *Synechococcus* sp. PCC 7002 and *V. harveyi* from the bacterial lysate were also in excess of that required simply for growth. This excess uptake of Fe may be "luxury" uptake, cellular Fe quotas of both marine cyanobacteria (Wilhelm

et al. 1996) and diatoms (Sunda et al. 1991) have been shown to increase with greater environmental Fe concentrations. Alternatively, it may be possible that the model organisms were able to take up the Fe in the lysates but were unable to utilize it. We believe this possibility to be unlikely however. Previous work (Mioni et al. 2004) has shown *V. natriegens* PWH3a lysate to very effectively repress light production in the *Pseudomonas putida* FeLux heterotrophic bacterium Fe bioreporter, which indicates that it is available.

In recent years it has been established that the vast majority of Fe in ocean waters is organically complexed. However, the nature of the organic ligands is still relatively unknown. The majority of in-situ examinations have focused primarily on the Fe affinities of the ligands. Both siderophores and viral lysis products have been suggested as possible components of this organic ligand pool. In this study we show that while these two types of ligands may be indistinguishable in their affinity for Fe as determined by electrochemical methods, important biological differences exist between them. The Fe released by viral lysis was significantly more bioavailable to a range of model plankton than was Fe bound to siderophores. This is of critical importance as efforts are made to resolve the cycling of Fe in marine systems.

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Part V

Summary and Conclusions

In these studies we examined the potential for viral lysis of plankton to recycle Fe in marine systems. Prior to our work, it was known that the virus-mediated lysis of bacteria could release biologically available organic carbon (Middelboe et al. 2003). However, only one study had experimentally examined the potential for viral lysis to recycle Fe (Gobler et al. 1997). As up to half of the world's marine systems are thought to be Fe-limited (Moore et al. 2002), determining the factors influencing cycling and availability of Fe is necessary to understanding the components of primary production in the oceans. We have extensively examined the role that viral lysis of plankton has on both the release of Fe and its subsequent bioavailability.

Through our investigations we have demonstrated the following:

i.) Viral lysis of plankton can release Fe into the dissolved phase. Laboratory experiments with two model planktonic microorganisms demonstrated the potential for viruses to play a major role in repartitioning Fe from particulate (intracellular) to dissolved phases. Virally mediated lysis of the heterotrophic bacterium *V. natriegens* resulted in a ca. 2.5-fold increase in release of intracellular Fe into the dissolved size class as was released from unlysed cells. Similarly, lysis of the autotrophic cyanobacterium *Synechococcus* sp. WH7803 released approximately 2.5 times as much dissolved Fe as was released from unlysed controls. Coupled with viral production experiments from stations in the Peruvian upwelling and Humboldt Current, we have estimated that virally

mediated lysis could result in dissolved Fe remobilization in the range of 19.2 – 75.7 pmol L⁻¹ d⁻¹.

ii.) Viral lysis releases Fe in a form physically and chemically different than is released from unlysed cells. Fe released in lysates was found in different size partitions when compared to unlysed cells. When *V. natriegens* was lysed a greater proportion of released dissolved Fe was found in the < 3 kDa size fraction than Fe released from unlysed cells. In contrast, less of the Fe released following lysis of *Synechococcus* sp. WH7803 was found in the < 3 kDa size class as compared to unlysed cells. There is evidence that at least some of the Fe-ligand complexes found in seawater may be siderophores (Macrellis et al. 2001; Gledhill et al. 2004). However, our analysis of *V. natriegens* lysate indicated the presence of neither hydroxamate nor catecholate type moieties in cell lysates. In contrast we have shown that under low-Fe conditions without added lysogenic phage, *V. natriegens* produces a catecholate-type siderophore.

iii.) Fe released via virus-mediated cell lysis is highly bioavailable to model marine plankton. We have demonstrated that Fe present in lysates of *Synechococcus* sp. WH7803 is more bioavailable to both the heterotrophic bacterium *Vibrio harveyi* and the autotrophic cyanobacterium *Synechococcus* sp. PCC 7002 than is inorganic Fe, EDTA complexed Fe, or Fe bound to either a catecholate siderophore or a model trihydroxamate siderophore. Uptake studies have also shown that Fe in this cyanobacterial lysate is more bioavailable to the diatom *Thalassiosira pseudonana* than is Fe complexed to either siderophore. Fe released via lysis of *V. natriegens* is more bioavailable than Fe in

cyanobacterial lysate, inorganic Fe or Fe bound to EDTA or either siderophore to our model heterotrophic bacterium, cyanobacterium and diatom. We have also utilized the heterotrophic bioluminescent Fe bioreporter *Pseudomonas putida* FeLux to examine bioavailability of Fe released by lysis *V. natriegens*. As demonstrated by both reporter response and uptake measurements, the Fe in the viral lysate was highly bioavailable to the bioreporter. Of particular note is the observation that Fe present in the *V. natriegens* lysate showed a greater bioavailability than Fe complexed to a catecholate siderophore produced by *P. putida* FeLux.

These conclusions support the overarching hypothesis that viral lysis, which may result in the destruction of up to 40% of bacterioplankton daily (Suttle 1994), could play a major role in marine Fe cycles. Our work shows that virus-mediated lysis has the potential to provide a significant source of bioavailable Fe to the planktonic community. As much of the primary production in the World's oceans is at least sporadically limited by Fe availability, the effect of viruses should be considered in any analysis of global carbon cycles. This study also has implications for the other major nutrient elements (i.e., P, N) as it suggests their co-regeneration in organic form may also influence community structure. Today, in light of mounting evidence of global climate change, much effort is being put forth to determine both the oceans' role in carbon sequestration and predict how this role will be affected as changes occur. Our studies have indicated that virus-mediated Fe recycling, which previously has attracted little

attention, must be addressed in any meaningful examination into the importance of marine systems to global climate regulation.

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