12-2004

Using a Bioluminescent Bacterial Bioreporter to Assess Iron Bioavailability in the Oceans

Cecile Elise Mioni
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Cecile Elise Mioni entitled "Using a Bioluminescent Bacterial Bioreporter to Assess Iron Bioavailability in the Oceans." I have examined the final electronic 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.

Stephen W. Wilhelm, Major Professor

We have read this dissertation and recommend its acceptance:

Lee W. Cooper, Gary S. Sayler, Robern N. Moore

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Vice Chancellor and
Dean of Graduate Studies

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USING A BIOLUMINESCENT BACTERIAL BIOREPORTER TO ASSESS IRON BIOAVAILABILITY IN THE OCEANS

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Cécile Élise Mioni
December 2004

DEDICATION

This dissertation is dedicated to my parents, Charles Mioni and Dolorès Mioni; my sister, Amandine Mioni; my fiancé, Lawson Worrell IV, and the rest of my family, for their love and continuous support, for their encouragements and infinite patience, for sharing my sacrifices, and because they have never left the boat when the fire drill was going off.

"The sea is a mirror which confronts us with our own ignorance."

Anita Conti

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Steven W. Wilhelm, for his guidance and patience, as well as for giving me the opportunity to work on such a beautiful, although challenging, project. His enthusiasm about Sciences and his contagious motivation were inspiring throughout this work.

I would like to thank also Dr. Gary S. Sayler, for his generosity and his advice all along my Ph.D. research. I would like to thank as well the other members of my committee, Dr. L. Cooper, Dr. D.A. Hutchins, and Dr. R.N. Moore, for their advice and feedback on this research project.

I am also very grateful to my friends Dr. M. Allen and Amy Allen, for their help, for their practical advices and for sharing their tips throughout this work. I would like to thank as well all my friends and colleagues at the Wilhelm's lab and at the Center for Environmental Biotechnology, who helped me in many ways. The list is not exhaustive but I would like to thank especially, Matt Carberry, Amanda Dean, Melanie Eldridge, Shannon Efteland, Julie Higgins, Lynn Neal, Andy Ouellette, Leo Poorvin, Renhui Li, Casey Rentz, Johanna Rinta-Kanto, Janet Rowe and Matt Smith. I have learned a lot from you guys and you really made me feel home since the very beginning. I would like to thank also Dr. H. Dionisi, Dr. S. Ripp, Dr. J. Sanseverino, A. Fraley, A. Ozen, L. Mitchell, V. Garrett, L. Saidak, D. Williams and his wife Cathy, who were all very supportive and always ready to help. I have never felt bad to ask them questions, they have always taken the time to reply to my questions with kindness, I am really indebted to all of them.

I would like to thank as well everybody at the Microbiology Department. I would like to thank especially my friends Steve Minkin, Shannon Efteland, Judson Hervey and Heather Connelly for their priceless friendship and continuous support. I would like to thank also to my friends Johanna Rinta-Kanto, Melanie Eldridge and Chris Pack, they have always been here, even during the down times. I would not have made it through without their help; they always anticipated my needs without waiting for me to ask. They

were all very caring and supportive when I became sick and I will always be indebted to them. I would like to thank the Center for Environmental Biotechnology, the International Women's Fishing Association 2003, and Daniel Jouvance to provide fundings during this PhD project.

I would like to thank as well some exceptional instructors and professors who has the truly rare gift to transmit their passion for atmospheric and marine sciences and to inspire their students. I thank Dr. P.-J. Labourg and Dr. X. de Montaudoüin (Marine Station of Arcachon) for initiating me to marine ecology and encouraging me to pursue in oceanography. I thank Dr. P. Castaing (University of Bordeaux I) for being such a wonderful and supportive professor; through his encouragements and advice, I eventually found my way to graduate school. I would like to thank especially Pr. P. Tréguer and Dr. S. Blain who initiated me and guided my first steps in the research on iron limitation in oceans while I was at the European Institute of Marine Studies. They transmitted me their enthusiasm for this field and are still a great source of inspiration for me. I would like to thank Dr. A. Milligan and Pr. F.M. Morel for helping me in pursuing my research work in the iron field. I would have not been here without the encouragements and the help of my friend Dr. A. Milligan, I am really grateful for his support and advices. Lastly, I would like also to thank Dr. V. Garçon, D. Thouron and J. Sudre (LEGOS) for introducing me to other aspects of oceanography.

I would like to thank my dear friend Lucile Storti who, despite the distance, was always here for me. She helped me in many ways and has shared so much with me, I am so glad to have her as a friend for more than a decade now.

I would like to thank my parents, Charles and Dolorès Mioni, my sister, Amandine Mioni, my grand-parents, Santiago and Josepha Martinez, and Antonio and Anna Mioni, as well as the rest of my family. Since the very beginning, their encouragements, their moral and material support, greatly help me through it all. They are my roots that keep my feet on Earth. I would like to thank as well my fiancé, Lawson Worrell IV, I could not imagine life without him; he just made it so much more beautiful. His love and support helped me a lot to go through this both at sea and on land.

ABSTRACT

Recent improvements in modern analytical methods have considerably increased our understanding of Fe biogeochemistry in the Ocean. Compiled data have shown that Fe concentrations are low in most open ocean surface waters and that the bioavailability of this Fe is influenced by organic complexation. Of presumed biotic origin, the importance of this organic complexation to Fe availability remains to be elucidated. Unfortunately, current analytical tools do not allow for a linkage to be established between Fe speciation to Fe bioavailability.

To supplement chemical analyses, we have developed a bioanalytical tool: a heterotrophic bacterial bioluminescent reporter system that responds quantitatively to bioavailable Fe concentrations. Data collected during one field study in a freshwater system as well as three independent field studies in marine systems demonstrate that these whole-cell biosensors are a powerful tool for environmental monitoring, providing both qualitative and quantitative insights on Fe biogeochemistry.

To complement this work, laboratory studies have been carried out to characterize the influence of various sources of Fe-complexing organic ligands, including synthetic chelators, bacterial and fungal siderophores and virus-mediated bacterial lysis products. The results of this study will be presented in the context of attempting to clarify the role that the organic complexation of Fe may play in regulating Fe bioavailability to the diverse planktonic components of the aquatic microbial community.

To improve our abilities to standardize this tool in the field, a green fluorescent protein gene expressed under control of a constitutive promoter has been introduced in the genomic DNA of *P. putida* FeLux. The resulting dual bioreporter will allow for the enumeration of bioreporter cells in environmental samples in parallel to the detection the bioluminescent signal in response to changes in Fe bioavailability.

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LIST OF ABBREVIATIONS

Amy	α-amylase
Amp	Ampicilin (antibiotic)
ATP	Adenosine triphosphate
BL	Bioluminescence
BSi	Biogenic Silica
Chl *a*	Chlorophyll a
CL	Chemiluminescence
CLE-ACSV	Competitive ligand equilibration – adsorptive cathodic stripping voltammetry
CO_2	Carbon dioxide
CR	Colorimetric
CSV	Cathodic stripping voltammetry
CTD	Conductivity-temperature-depth probe/profiler
DFB	Desferrioxamine B (actinomycete siderophore, tri-hydroxamate, binds preferentially to Fe(III))
DNA	Deoxyribonucleic acid
rDNA	Ribosomal Deoxyribonucleic acid
DOC	Dissolved organic carbon
DmdR	Divalent metal dependent repressor
DP	2,2'-dipyridyl (a synthetic Fe chelator with soft donor atoms, binds preferentially to Fe(II))
EC	Electrochemical
EDTA	Ethylenediaminetetraacetate
ESAW	Enriched seawater, artificial seawater medium
ETP	Eastern tropical Pacific Ocean
ETS	Electron Transport System
FC	Flow cytometry
Fe	Iron
dissolved Fe	Fe that passes through 0.2 or 0.4 μm pore filter (depending on authors)
particulate Fe	Fe retained by 0.2 or 0.4 μm pore filter (depending on authors)
Fe'	Combined soluble inorganic Fe(II) and Fe(III) species
Fe^{2+}	Ferrous iron (Fe(II))
Fe^{3+}	Ferric iron (Fe(III))
Fe-L	Iron-ligand complex
FL	Fluorescence
FMN	Flavin Mononucleotide
$FMNH_2$	Flavin Mononucleotide reduced
FUR	Ferric Uptake Regulator
HC	Histochemical
Ina	ice nucleation protein

IRONEX	Iron enrichment experiments
JGOFS	Joint Global Ocean Flux Study
HNHC	High Nutrient, High Chlorophyll
HNLC	High Nutrient, Low Chlorophyll
HNLSiLC	High Nutrient, Low Silicic acid, Low Chlorophyll
Km	Kanamycin (antibiotic)
LNLC	Low Nutrient, Low Chlorophyl
lux	bacterial luciferase
NaH_2PO_4	Sodium Phosphate
NH_4	Ammonium
NH_4NO_3	Ammonium Nitrate
NAD(H)	Nicotinamide Adenine Dinucleotide
NADP(H)	Nicotinamide Adenine Dinucleotide Phosphate
NO_3	Nitrate
PCR	Polymerase Chain Reaction
pFe	- Log $[Fe^{3+}]$
POC	Particulate organic carbon
RA	Rhodotorulic acid (a yeast siderophore, dihydroxamate coprogen, binds preferentially to Fe(III))
RI	Radioisotope
Rlu s^{-1}	Relative light unit per second
Si	Silica
$Si(OH)_4$	Silic acid
SOIREE	Southern Ocean iron enrichment experiment
Tc	Tetracyclin (antibiotic)

Part I

LITERATURE REVIEW

Part I

LITERATURE REVIEW

1. Background

1.1. Marine carbon cycle and climate

In his satirical *Devil's dictionary*, Ambrose Bierce (1911) defines the ocean as follows:

Ocean, *n*. *A body of water occupying about two-thirds of a world made for man – who has no gills*.

Indeed, nearly 75% of the surface of Earth is covered by oceans, in which *ca.* 3.45 Ga ago appeared the first unicellular photoautotrophic organisms (cyanobacteria). Since this time these organisms have profoundly influenced the geochemistry of our planet and subsequently the course of the evolution of life (Falkowski, 1997; Falkowski et al., 1998). Indeed, the oxygen-rich atmosphere (21%) which has made Earth hospitable to complex eukaryotes, including humans, is derived from the byproducts of oxygenic photosynthesis (*i.e.* the production of carbohydrate and oxygen from the reduction carbon dioxide and the oxidation of water molecules using light energy) by these marine unicellular algae (Falkowski, 1997).

During oxygenic photosynthesis, microscopic oceanic plants (*e.g*. phytoplankton) also trap carbon dioxide (CO_2) from the atmosphere to generate massive amounts of organic carbon, which are subsequently transferred to higher trophic levels of the marine food web and ultimately to the deep sea, either through the vertical transport of sinking dead cells and fecal pellets or through advection in the course of global circulation patterns (Fig. I.1). Although marine phytoplankton represent less than 1% of the total photosynthetic biomass (Bryant, 2003), they are responsible for nearly the half of the total annual photosynthetic (*i.e.*, "primary") production due to a rapid turn over time on the order of days to weeks (Field et al., 1998). The global primary productivity has been

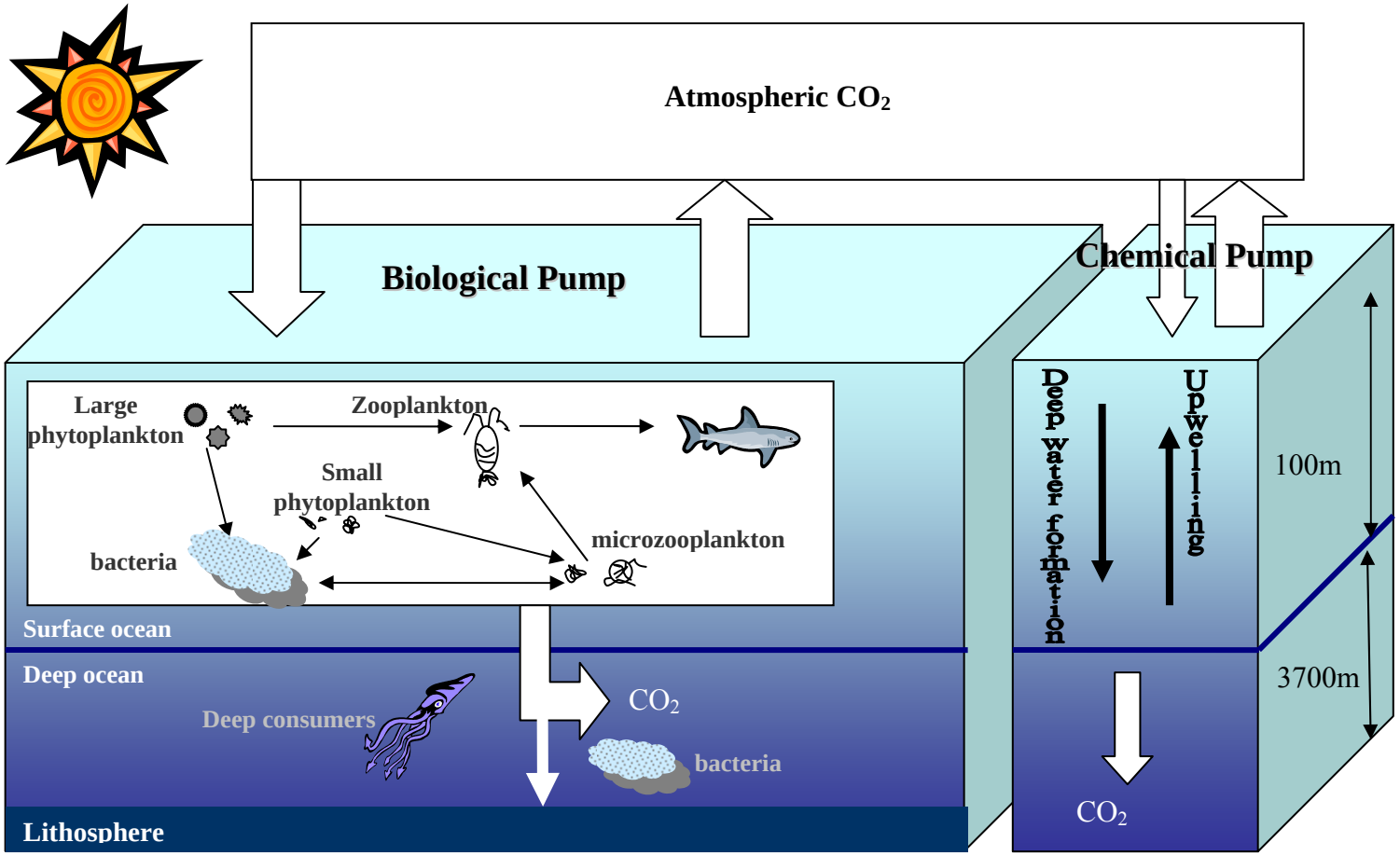

Fig. I.1. Marine carbon cycle (adapted from Chisholm, 2000)

estimated of 0.8×10^{15} mol C y^{-1} in the coastal areas, which represent 10% of the World's oceans, and 18×10^{15} mol C y^{-1} in the open ocean (deBaar and deJong, 2001). Most of the organic particles that reach the deep sea are remineralized by marine heterotrophic bacteria, which regenerate it into inorganic form; however, a small fraction eventually reaches the lithosphere where it fossilizes (reviewed by Falkowski et al., 1998). As such, this "hidden forest" contributes significantly to the production of resources that have been intensively exploited by Man over the course of the last century: phytoplankton are at the base of the marine food-chain, thereby they greatly influence fishery yields, and a small fraction of organic carbon derived from fossil marine phytoplankton preserved in the lithosphere has contributed to fuel the industrial world. Marine phytoplankton, their predators (grazers) and marine bacteria play an important role in the global biogeochemical cycle of carbon by acting as a "biological pump", which transfers atmospheric carbon to the deep sea, where it is concentrated and sequestered for roughly 1000 years (thus on time scales relevant to anthropogenic-mediated changes) (Chisholm, 2000).

Marine unicellular algae also influence other major biogeochemical cycles, including those of phosphorus and nitrogen. Indeed, the bulk chemical composition of marine phytoplankton is similar to the proportion of major elements (*i.e.* nitrogen and phosphorus) in seawater (Redfield, 1934). Although variations exist between species, the average elemental composition of marine phytoplankton is generally characterized by the Redfield ratio: 106C:16N:1P (by atoms) (Falkowski, 1997; Falkowski and Davis, 2004). These elements, which are structural components of the algal biomass and are generally present at micromolar concentrations in seawater, are commonly defined as macronutrients (as opposed to essential trace elements, such as iron, which are generally used as enzyme cofactors and are present at subnanomolar levels, and are referred as micronutrients). In the deep ocean, where photosynthesis does not occur, the remineralization of sinking organic matter derived from phytoplankton material regenerates these nutrient elements in similar C:N:P proportions following approximately the stoichiometric reaction (Redfield, 1934; Falkowski, 1997):

$$(CH_2O)_{106}(NH_3)16H_3PO_4 + 138O_2 \rightarrow 106CO_2 + 122H_2O + 16NO_3^- + PO_4^{3-} + 19H^+$$

In contrast to CO_2, phosphorus and nitrogen do not have atmospheric sources (with the exception of nitrogen-fixing cyanobacteria, which can use molecular nitrogen, but represent less than 1% of the marine phytoplankton; Falkowski, 1997). Therefore, the utilization of CO_2, and as such the strength of the biological pump, is tightly coupled to the rate of renewal of these two macronutrient in the euphotic layer (deBaar, 1994; Falkowski, 1997). There are several routes of resupply of these macronutrients including river inputs, upwelling of the nutrient-enriched deep waters, and deep convective mixing. As a consequence, phytoplankton biomass and production is generally concentrated along coastal boundaries where riverine nutrient fluxes, upwelling circulation and deep convective mixing occur (Falkowski, 1997). However, in three major oceanic areas (subarctic Pacific, equatorial Pacific and Southern Ocean) where nitrogen and phosphorus are present in excess, the rates of supply and assimilation are not coupled (Gran, 1931; Thomas, 1969, 1979). These areas are more than an exception to the rule since they represent roughly the half of the world's ocean (Moore et al., 2002). In these high-nutrient regions, phytoplankton biomass remains paradoxically low and the biological pump is not efficient. Since the 1980's, extensive research has tried to determined which factors are responsible for the inefficiency of the biological pump in these regions. Indeed, these three regions, and especially the Southern Ocean, are of special interest as it is thought that they may play a crucial role in climate changes (Martin, 1990b).

CO_2 is the most important anthropogenic greenhouse gas in terms of a contribution to climate change, *i.e*. global warming. Since 1850 and as a consequence of burning of fossil fuel, deforestation and land changes, human activities have contributed to introduce about 340 Pg of carbon to the atmosphere (Falkowski, 1997). As a result, climate models predict that the oceanic carbon cycle will be affected by the rising atmospheric CO_2 concentration (Joos et al., 1999; Sarmiento et al., 2004). Because the mechanisms driving carbon sequestration by the ocean are not fully understood, the biological response to the predicted changes in ocean dynamics is unknown. Simulations predict increase stratification and an alteration of the mixed layer depth, with consecutive decrease in nutrient availability (Sarmiento et al., 2004). Field data also suggest that

alteration of mixed-layer depth would cause a shift in community structure, toward species that are less efficient in carbon removal (Arrigo et al., 1999). Other recent studies suggest that species favored by warming changes would be as efficient (Coale et al 2004; Schneider et al., 2004; Riebesell, 2004). To elucidate how the anthropogenically induced changes will impact the oceanic biota and how these organisms will impact the global warming, it is therefore crucial to gain insight on the factors controlling the marine biota, and thereby the biological carbon pump (Falkowski et al, 1998).

1.2. The case for iron: from hypothesis to paradigm

In July 1988, while the scientific community wondered about the climatic consequences of the 7 billions of tones of anthropogenically generated atmospheric carbon dioxide (CO_2) emitted yearly, "*putting on his best Dr. Strangelove accent*", the late John Holland Martin uncovered his theory of iron (Fe) limitation to an audience of academics at the Woods Hole Oceanographic Institute by using these inflammatory words that sparked a tremendous debate over the role of iron in ocean productivity and community structure (Martin, 1990a; Chisholm and Morel, 1991; Fig. I.2):

"I suggested that with half a ship load of Fe … I could give you the ice age."

He went on to estimate that adding 300,000 tons of the trace metal Fe into the Southern (*aka* Austral) Ocean would be enough to biologically "pump" two billions tons of CO_2 (Martin, 1990a). However, the hypothesis of the Fe limitation in some oceanic habitats was not a new concept. More than a half-century before Martin revitalized this hypothesis, Gran (1931), Harvey (1933) and Hart (1934) were the first to postulate that Fe could limit phytoplankton growth in oceanic systems (deBaar, 1994). Nevertheless, until the late eighties this hypothesis was not testable owing to the biases introduced by the ubiquitous contamination of seawater samples and by the low sensitivity of contemporaneous analytical tools (deBaar, 1994). Therefore, the concept of Fe limitation was ignored while macronutrients (such as silicic acid, nitrogen and phosphorus) and physical factors (*e*.g. sunlight, mixing) were considered the main factors influencing regulate primary productivity in aquatic systems (see deBaar, 1994; for review).

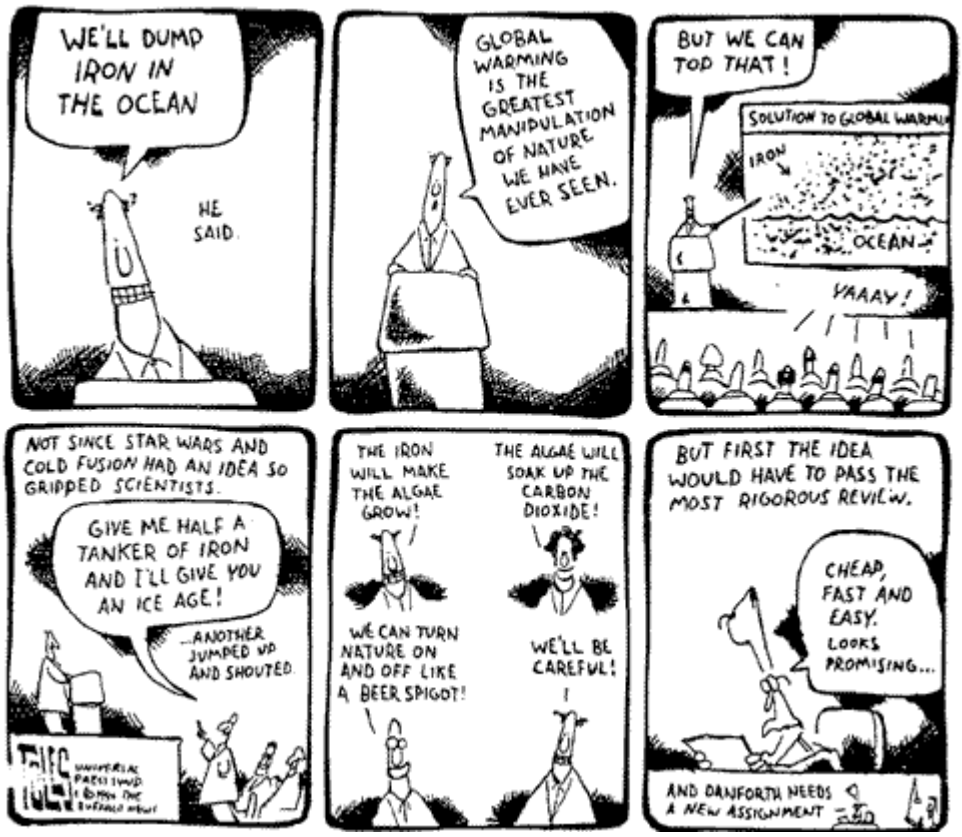

Fig. I.2. The "Geritol solution". First presented by Martin in July 1988 to an audience of academics at the Woods Hole Oceanographic Institute, the idea was soon picked up by some "scientists", the press and eventually was considered as a policy option to remedy to Global Change. TOLES ©1990 The Buffalo News. Reprinted with permission of UNIVERSAL PRESS SYNDICATE. All rights reserved.

Throughout this period conceptual models failed to explain the paradoxically low standing stocks of primary producers (phytoplankton) observed in large areas of the world's ocean (specifically the subarctic gyre of the Northwestern Pacific, the Equatorial Pacific, and the Southern Ocean) characterized by a persistent excess of macronutrients (Fig. I.3; Gran, 1931; Thomas, 1969, 1979). To illustrate this paradox, these areas have been termed "High-Nutrient, Low-Chlorophyll" (HNLC) regions (Hart, 1934).

It was not until 1980's that "*the ferruginous feline* [*was*] *out of the bag*" (Chisholm and Morel, 1991). With the application of trace-metal clean sampling equipments (Bruland et al., 1979), incubation procedures (Fitzwater et al., 1982) and new analytical methods (Bruland et al., 1979) pioneered by Californian research teams, reliable dissolved and particulate Fe concentrations became available enabling the establishment of a link between this micronutrient and biological processes (deBaar, 1994). Concomitantly, accepted estimates of total Fe concentrations in the euphotic zone decreased by several orders of magnitudes, from micromolar (Tranter and Newell, 1963) to sub-nanomolar (Gordon et al., 1982; Landing, 1983; Landing and Bruland, 1987). There was then some "*hope to retest this old hypothesis using modern techniques in which spurious Fe contamination is avoided*" (Martin and Gordon, 1988). The increased accuracy and sensitivity in measurements of dissolved Fe from seawater showed that the vertical profile of this trace metal was strikingly similar to those of other major nutrients such as nitrate (Fig. I.4; Martin and Gordon, 1988; Martin et al., 1989), suggesting that Fe played a key role in biological processes (Martin and Fitzwater, 1988). Indeed, it was shown that dissolved Fe concentrations in various HNLC regions (Subarctic Pacific and Austral Ocean) were characterized by a strong vertical gradient typical of bioactive nutrients, with a depletion in surface waters (20-50 pM; Martin et al., 1989, 1990, 1991), due to biological uptake as well as particulate adsorption of Fe (Martin and Gordon, 1988; Sunda, 1991). The results also demonstrated a significant increase with depth, resulting from the oxidation of sinking organic material driven by marine heterotrophic bacteria (Cho and Azam, 1988; Martin and Gordon, 1988; Sunda, 1991).

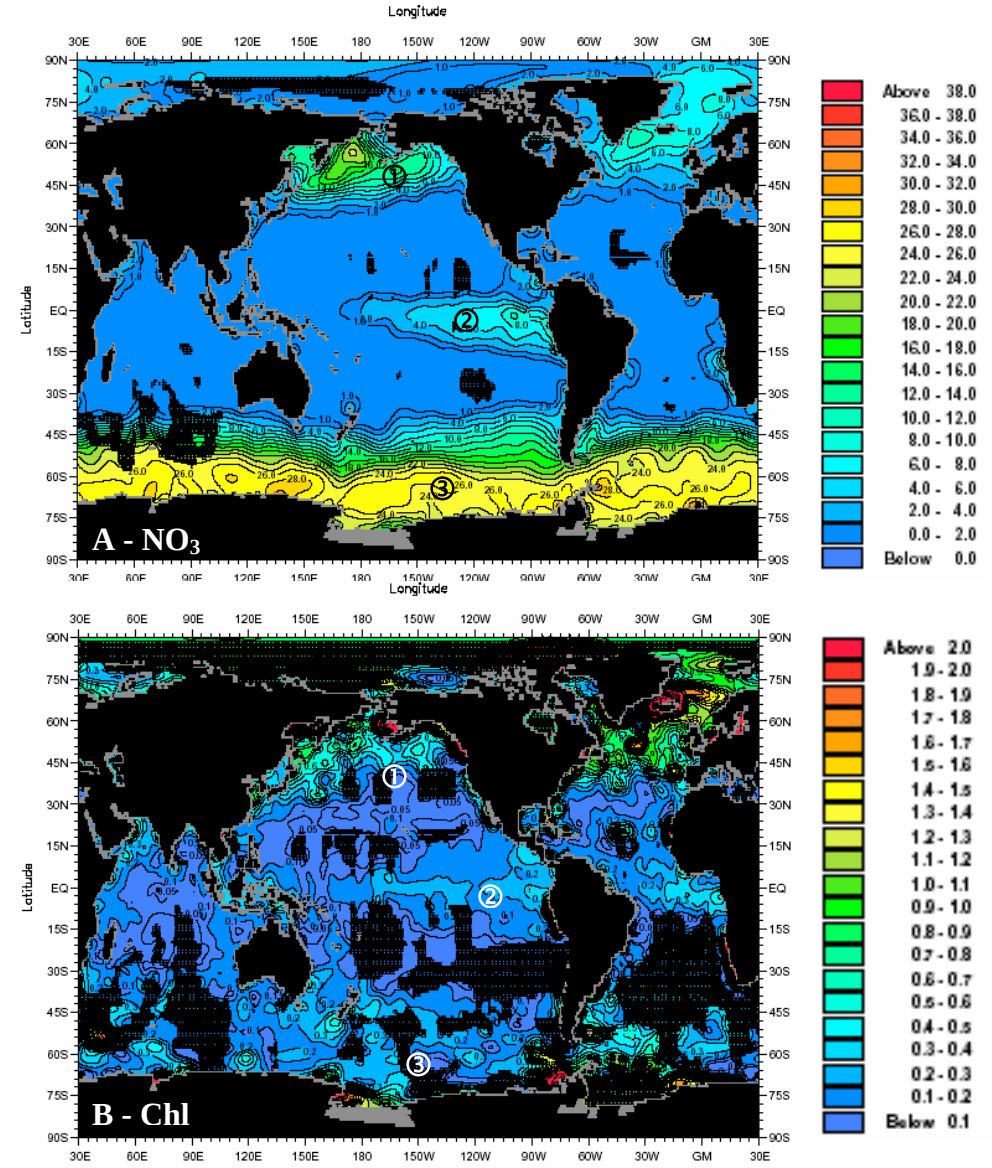

Fig. I.3. Annual means of Nitrate and Chlorophyll in surface waters (10 m depth). A: Nitrate (NO_3, μM), Contour interval: 2.00 μM increment (Minimum value = 0.00, Maximum value = 32.95); B: Chlorophyll (Chl, μg L^{-1}), Contour Interval: 0.10 μg L^{-1} increment (Minimum Value= 0.00 Maximum Value= 8.41)**.**These images clearly shows the high levels of nitrate and low level of Chlorophyll in the subarctic Pacific (①), the equatorial Pacific (②) and the Southern Ocean(③). Taken from World Ocean Atlas 2001 Ocean Climate Laboratory/NODC (http://www.nodc.noaa.gov/).

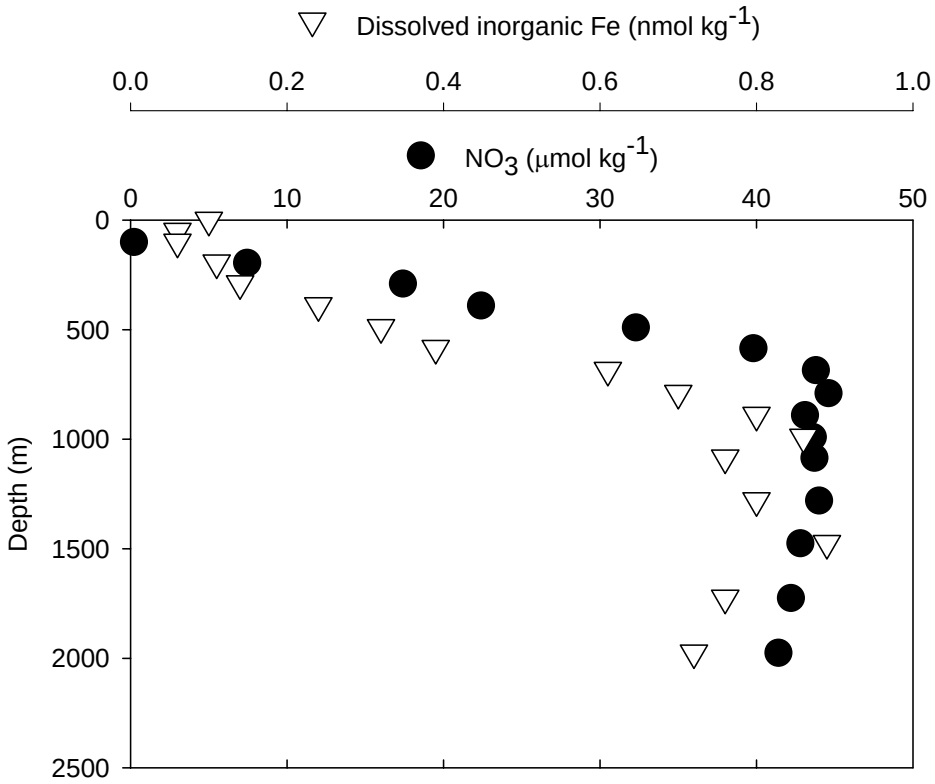

Fig. I.4. Typical vertical distribution of nitrate (NO_3) and dissolved inorganic Fe in offshore surface waters. Taken from Martin and Gordon (1988; their Table 3).

Parallel to the determination of Fe distributions, the Moss Landing team performed a series of on-deck incubation experiments using natural plankton assemblages which were amended with Fe (Martin and Fitzwater, 1988; Martin et al., 1989, 1990; Coale, 1991). The dramatic increase in phytoplankton biomass (as shown by Chlorophyll *a* concentrations) in amended treatments relative to controls was interpreted as the evidence that dissolved Fe concentrations in surface seawaters in HNLC habitats were too low to support a high phytoplankton biomass (Martin and Fitzwater, 1988; Martin et al., 1989, 1990; Coale, 1991). Subsequently, several groups conducted similar "grow-out" experiments in different regions of the Southern Ocean and consistently showed Fe-induced phytoplankton growth stimulation (deBaar et al., 1989; Buma et al., 1991). Based on these results and the inverse correlation between atmospheric dust and CO_2 concentrations in ice cores since the Last Glacial maximum, Martin speculated that increased terrestrially-derived dust (and thus Fe) deposition in HNLC regions during glacial periods may have stimulated phytoplankton production, which may have led to more efficient macronutrient utilization, a strengthening of the biological pump, and thus an enhanced removal of the greenhouse gas CO_2 (Martin, 1990b; Martin et al., 1991). However, although the bottle incubations clearly established a link between Fe and phytoplankton production, the interpretation of the data became a source of animated debate (Banse, 1990, 1991). Parallel to the stimulation of growth in Fe-amended treatments, an increase of Chl *a* in control treatments relative to levels found in ambient waters, a significant lag time and a floristic shift to atypical species (large diatoms) in response to Fe addition were also consistently observed, suggesting a possible "bottle effect" resulting from light limitation and exclusion of mesozooplankton grazers (Banse, 1990, 1991). The question of controls on HNLC primary production was not fully answered yet but the provocative idea of a deliberate large-scale addition of Fe to the ocean surface to enhance carbon sequestration in the deep ocean initiated by Martin (1990a) had a snowball effect on a scientific community already greatly concerned by anthropogenically-induced global warming. An international symposium, organized by the American Society of Limnology and Oceanography, was held in 1991 and took a position with regard to large-scale ocean manipulation and geoengineering. A resolution

was adopted to discourage Fe fertilization as a policy option to reduce anthropogenic gases emission (Chisholm and Morel, 1991).

During the 1990's, dubbed as "the iron age of oceanography", research on Fe limitation bloomed. Indeed, all scales of time and space were examined: from the study of biomolecular interactions at the cell level (Raven, 1988, 1990, Hudson and Morel, 1990; Sunda, 1991; Wilhelm, 1995) to modeling of biogeochemical cycles at the ecosystem level (Joos et al., 1991; Peng and Broecker, 1991), as well as from the study of rapid (millisecond) photochemical reactions driving Fe chemistry in surface seawater (Wells et al., 1991) to the examination of paleoceanographic records over a geologic time-scale (Kumar et al., 1995). Evidence acquired from these studies improved the understanding of the interactions between Fe and phytoplankton but also revealed the complexity of the marine biogeochemistry of Fe (Wells et al., 1995). Spatial surveys revealed strong horizontal gradients of Fe in marine systems, decreasing by 100 to 1000 times between coastal and offshore waters (Johnson et al., 1997), and a distinction was made between coastal and oceanic domain based on their respective external Fe sources. As suggested by Martin, offshore regions were shown to in part rely on low and sporadic atmospheric dust input (Martin et al., 1989; Duce and Tindale, 1991). However, upwelling of deep waters (Coale et al., 1996a; Loubere, 2000) and lateral transport of Fe from distant continental margins (Martin et al., 1993) were also shown to be act as sources of "new"(as opposed to regenerated) Fe sources. In these offshore regions, it was estimated that the largest reservoir of Fe was the biota itself, and that regenerated Fe input was supplied at a rate more than an order of magnitude faster than that of external input (Wells et al., 1995; deBaar and deJong, 2001). By contrast in coastal areas substantial Fe inputs derived from rivers and anoxic sediments result in higher dissolved and particulate Fe concentrations (Wells et al., 1995; deBaar and deJong, 2001). Studies on Fe chemistry also revealed that Fe was present in various forms (*i.e.* size-classes and chemical species) in seawater, although mainly in the particulate size-class due to its insolubility, and that photochemical processes altered the speciation of this trace metal in the euphotic layer (Geider and Laroche, 1994; Wells et al., 1995). From these findings arose the problem of defining the biological availability of the various forms of Fe

present in seawater (Wells et al., 1995). Concomitantly, discrepancies between theoretical (Raven, 1988, 1990) and observed (Bruland et al., 1991; Doucette and Harrison, 1991; Hutchins et al., 1991; Sunda et al., 1991) Fe use efficiency for phytoplankton revealed that metabolic Fe requirements vary widely among species, especially between coastal and oceanic species (Brand, 1991; Sunda and Huntsman, 1995). This discovery indicated that Fe controls not only phytoplankton production in HNLC regions, but also the species composition within algal assemblages in marine systems, and therefore the food web structure of such ecosystems (Bruland et al., 1991; Wells et al., 1995).

Since the 1990s, evidence from mesoscale fertilizations and the continuous improvement of analytical methods have demonstrated that the low bioavailability of Fe controls primary production and community structure in the HNLC regions (Martin et al., 1994; Coale et al. 1996b, 2004; Boyd et al., 2000, 2004; Gervais et al., 2002; Tsuda et al., 2003) as well as other aquatic systems such as oligotrophic gyres (Paerl et al., 1994; Achilles et al., 2003), some coastal marine systems (Hutchins et al., 1998, 2002; Wells, 1999; Eldridge et al., 2004) and some lakes (Twiss et al., 2000). However, although recent fertilization experiment have shown a significant increase of carbon export in the upper layer of the water column (Coale et al., 2004), no one has yet been able to determine how much carbon is exported to the deep ocean upon Fe addition. Insights gained from these various mesoscale experiments led to the conclusion that the "biological carbon pump" may be influenced by Fe but also by other processes, such as grazing (Hutchins et al., 1994; Hutchins and Bruland, 1994; Landry et al., 1997), and bacterial regeneration (Chisholm et al., 2001; Boyd et al., 2000; Coale et al., 2004). Moreover, other factors intrinsically linked to organismal physiology appear to influence the efficiency of the biological pump. For example, it has been shown that Fe limitation enhances the relative utilization of silicic acid, and thereby the biogenic silica content relative to the three other elements (C, N, P), in diatoms (Hutchins and Bruland, 1998; Takeda, 1998). As a result, under Fe-replete condition, the cellular content of biogenic silica decreases and diatom cells become less dense. These cells in theory will settle more slowly and carbon be exported at slower rates relative to those in Fe-depleted waters. It is therefore questionable as to whether or not Fe fertilization truly improves

the strength of the biological pump in such environments (Chisholm, 2000; Chisholm et al., 2001). Therefore, the second part of the Fe hypothesis (*i.e.* a significant increase in carbon transfer from atmosphere to sea floor as a result of Fe-enhanced primary production) has yet to be demonstrated (Chisholm, 2000; Chisholm et al. 2001; Coale et al., 2004).

1.3. The microbial ferrous wheel

As the term High-Nutrient, Low-Chlorophyll indicates, efforts were initially focused on the phototrophic component of the food web, the role of heterotrophic microbes in the marine Fe cycle had been ignored until the 1990's. However, at that time, the traditional food chain model had already been revised to generate the food web model by integrating the microbial loop and highlighting the role of heterotrophic bacteria in the pelagic carbon cycle (Pomeroy, 1974; Azam et al., 1983; Fuhrman, 1992). Despite their small size, marine heterotrophic bacteria account for a substantial amount of the particulate organic carbon in the oceans and are the key players of the remineralization of organic material, and therefore contribute to nutrient regeneration but also to CO_2 emission (Fig. I.1; Furhman, 1992). Heterotrophic bacterioplankton can indeed act either as a "link" (by recycling and transferring organic material to higher trophic levels) or a "sink" (by respiring the organic material) (Azam et al., 1983). In 1988, the discovery of the smallest and most abundant photosynthetic organisms known (*Prochlorococcus*), also contributed to change our view of plankton community structure in aquatic environments. Indeed, it was shown that this "hidden forest" of photosynthetic microbes (*i.e.* cyanobacteria) filling the upper 200 m of the oceans is responsible for as much as two-third of the photosynthesis in the seas and removes nearly as much carbon dioxide from the atmosphere as all terrestrial plants (Chisholm et al., 1988, 1992; Nadis, 2003). Thereby, the importance of the small component of the planktonic community began to be acknowledged. However, "*oceanographers knew about the microbial loop but not the microbial ferrous wheel*" (Kirchman, 1996). It was not until the mid-1990's that,

concomitantly with the emergence of novel methods for determination of the ambient speciation of Fe, the interest for this microbial component increased.

At this time, laboratory experiments performed by several groups on various prokaryotic marine isolates demonstrated that, under Fe starvation, both marine bacteria (Trick, 1989; Reid and Butler, 1991; Haygood et al., 1993) and cyanobacteria (Wilhelm and Trick, 1994; Wilhelm et al., 1996) produce, and subsequently release, Fe-binding ligands (siderophores) which are likely to influence Fe bioavailability in their immediate vicinity. In the 1990's, although research on Fe ligands production by marine isolate was just doing its first steps, studies on terrestrial and pathogen bacteria and fungi Fe metabolism was abundantly documented. It was known that, in response to Fe limitation under aerobic condition, microorganisms synthesized and subsequently excreted siderophores, *i.e.* low molecular weight (300 – 2,000 Da; Pattus and Abdallah, 2000) binding ligands with high affinity ($K_s = 10^{22} – 10^{50}$; Ratledge and Dover, 2000) for ferric Fe that are taken up as Fe complexes through cognate membrane transport systems (Neilands, 1995). Hundreds of terrestrial siderophores were already structurally characterized, and classified in three main structural groups: the catecholates, the hydroxamates and the mixed siderophores (*i.e.* both catecholate and/or hydroxamate and/or other bidentate groups such as hydroxyacids) (Winkelman, 1990) [Appendix I]. The few first structurally characterized marine binding-ligands presented the same structural groups than known terrestrial siderophores: hydroxamates (Takahashi et al., 1987), catecholates (Jalal et al., 1989; Okujo et al., 1994; Wilhelm and Trick, 1994) and mixed siderophore (Haygood et al., 1993; Reid et al., 1993) [Appendix I]. Most of the marine siderophores isolated since then, the amphibactins, aquachelins and marinobactins are cell-associated mixed siderophores characterized by a short structurally conserved polar hydroxamate-containing peptidic headgroup and a variable hydrophobic fatty acid appendage which contains 14 to 18 carbons and varies in the degree of saturation and hydroxylation (Martinez et al., 2003; Appendix I). Such siderophores appear to be widespread among marine α-, β-, and γ-*Proteobacteria* but are rarely produced by their terrestrial counterparts (Martinez et al., 2003). Based on the structural singularity of amphibactins and other cell-associated catechols, it has been suggested that marine

bacteria may have adapted to their freely diffusible environment by evolving Fe(III) scavenging strategies that limit diffusion of the newly produced siderophore away from the cell (Wilhelm and Trick, 1994; Martinez et al., 2003). Further laboratory experiments suggest that these siderophores affect Fe bioavailability to other marine planktonic organisms (Hutchins et al., 1999; Granger and Price, 1999). Based on these laboratory evidences, it has been suggested that marine planktonic organisms could use siderophores to mediate Fe acquisition in the Fe-depleted oceanic regions (Bruland et al., 1991; Wilhelm and Trick, 1994; Wilhelm, 1995) and thereby play an important role in Fe biogeochemistry.

Concurrently in the mid-1990's new electrochemical techniques were developed. These techniques were sensitive enough to allow measurement of low concentration of Fe (< 1 nM), but also to determine ferric iron (Fe(III)) organic complexation as well as its redox speciation were developed (Wu and Luther, 1994; Gledhill and van den Berg, 1994; Rue and Bruland, 1995; van den Berg, 1995; Wu and Luther, 1995). Several groups applied these highly sensitive methods to oceanic systems and revealed that organic ligands of unknown origin and chemical structures bind to more than 99% of dissolved Fe in natural oceanic waters, leaving less than 0.1 pM (10^{-14} M) as inorganic Fe species (Fe(III)') in the surface layer (Gledhill and van den Berg, 1994; Rue and Bruland, 1995, 1997; Wu and Luther, 1995; Notting et al., 1998; Witter et al., 2000a). Although the sources of these Fe organic ligands remain unknown, it is assumed that they are utilized for biological Fe acquisition and derive from bacterial siderophores (Tortell et al., 1999), porphyrin from phytoplankton (Rue and Bruland, 1997), grazing (Hutchins and Bruland, 1994) and byproducts of viral attacks (Wilhelm and Suttle, 2000). These organic ligands are commonly divided into two classes owing to their conditional stability constant: the stronger ligand class (L1) and the weaker ligand class (L2).

The "stronger" ligand class ($K_{Fe^{3+}/L1} \sim 1.2 \times 10^{23} - 5 \times 10^{22}$) is generally detected in surface water at concentrations lower than 0.5nM (0.3 – 0.44 nM; Rue and Bruland, 1995, 1997). Several lines of evidence suggest that these class-1 ligands may be bacterial siderophores released to facilitate Fe(III) uptake:

(i) they exhibit similar conditional stability constants to that of well-known terrestrial siderophores (Table I.1; Rue and Bruland, 1995, 1997; van den Berg, 1995; Gledhill et al., 1998; Witter et al., 2000a,b);

(ii) natural organic Fe ligands, present in excess in surface seawater, exhibit chelating groups (catecholate and hydroxamate) similar to those for naturally produced terrestrial siderophores (Macrellis et al., 2001; Martinez et al., 2003; Gledhill et al., 2004);

(iii) evidence of *in situ* production of hydroxamate containing siderophores has been obtained from coastal cyanobacterial mats (Estep et al., 1975),

(iv) the list of characterized siderophores isolated from Fe-depleted monoclonal culture of marine autotrophic (Wilhelm et al., 1998; Wilhelm and Trick, 1994) and marine heterotrophic bacteria (Martinez et al., 2000, 2001, 2003; Granger and Price, 1999; Butler, 1998; Drechsel and Winkelmann, 1997; Reid et al., 1993) is exhaustive.

(v) Furthermore, some field experiments have suggested that marine bacteria in HNLC region may be able to acquire Fe(III) bound to exogenous siderophores (Fe:DFB and Fe:DFE) presenting similar stability constants to class-1 ligands (Maldonado and Price, 1999). Bacteria also appear to contain significantly more Fe per biomass unit than phytoplankton despite slower uptake rates (when normalized to surface area) as compared to those of phytoplankton (Maldonado and Price, 1999). It has been therefore suggested that siderophore production may be the only way for heterotrophic bacteria to compete for Fe with phytoplankton in HNLC environments.

However, indirect evidence and recent laboratory studies complicate this assumption and suggest that class-1 ligands may have more than one biological source:

(i) Class-1 ligands appeared to have contributed to most of the dramatical ligand concentration increase (+ 400 %) within a day or two after Fe infusion during IronExII mesoscale experiment (Rue and Bruland, 1997). Although this observation agrees with a biotic origin, it appears quite surprising since siderophores production would be expected to decrease in response to Fe addition (Neiland, 1995). Indeed, the concept of Fe limitation of oceanic bacterial production is highly debated. Theoretical estimates of Fe requirements for heterotrophic metabolism suggest that heterotrophic bacteria require

Table I.1

Conditional stability constants recorded in the literature for the terrestrial siderophore Desferrioxamine B (DFB), bacterial (aquachelin) and algal (*Emilia huxleyi*) marine siderophores and naturally occurring Fe(III) binding ligands as determined by competitive ligand exchange and equilibration – cathodic stripping voltammetry method.

Model siderophore	Log $K_{Fe'L}$	Competing ligand	Reference
DFB	21.6	1N2N	Witter et al. (2000b)
DFB	>23	SA	Rue and Bruland (1995)
Aquachelin	22.2	SA	Barbeau et al. (2001)
E. huxleyi's siderophore	21.5	1N2N	Boye and van den Berg (2000)

Location	Log K_{FeL}	Competing ligand	Reference
Arabian Sea	21.6 – 22.5	1N2N	Witter et al. (2000a)
Atlantic Ocean	22.2 – 22.9	1N2N	Witter and Luther (1998)[a]
North Sea	20.3 – 22.1	1N2N	Gledhill et al. (1998)
North Atlantic ocean	20.6	1N2N	Wu and Luther (1995)[a]
Western Mediterranean Sea	21.5 – 22.0	1N2N	van den Berg (1995)
Equatorial Pacific (L_1) (Iron-Ex II)	22.7	SA	Rue and Bruland (1997)[b]
Equatorial Pacific (L_2) (Iron-Ex II)	21.8	SA	Rue and Bruland (1995)[b]
ALOHA station (L_1) (Central Pacific Ocean)	22.9	SA	Rue and Bruland (1995)[b]
ALOHA station (L_2) (Pacific Ocean)	21.5	SA	Rue and Bruland (1995)[b]
Station T4 (L_1) (Vertex V, subarctic Pacific)	23.1	SA	Rue and Bruland (1995)[b]
Station T4 (L_2) (Vertex V, subarctic Pacific)	21.5	SA	Rue and Bruland (1995)[b]

[a] Owing to the lower stability constant of the competing ligand 1N2N as compared to SA, the lower limit of the detection window of this method is higher. Therefore, when using 1N2N, the stability constants that are more likely to be representative of the stronger ligands (L_1) (Wu and Luther, 1995).

[b] Two-Initially published conditional stability constants ($K_{Fe'/L}$) were converted to $K_{Fe^{3+}/L}$ considering $K_{Fe'/L} = K_{Fe^{3+}/L} \times \alpha_{Fe'}$, with $\alpha_{Fe'}=10^{10}$, (Witter et al., 1998)

N1N2 = 1-nitroso-2-naphtol ($B_{Fe3+/1N2N}$ = 13.04, Witter et al., 1998); SA = salicylaldoxime ($B_{Fe^{3+}/2SA}$ = 20.98, Rue and Bruland, 1995);

more Fe (per carbon) for growth than autotrophs. Heterotrophic metabolism depends on the respiratory electron transport system (ETS) which contains more Fe-containing proteins (*e.g.* 60 % more Fe per mol of cytochrome-c) than does the photosynthetic ETS (Table I.2; Raven, 1988; Tortell et al., 1999). Lab experiments have demonstrated that Fe-limited bacteria exhibit low growth and growth efficiency (Tortell et al., 1996; Kirchman et al., 2003), especially when nitrogen is supplied as nitrate (the most abundant source of nitrogen in HNLC regions; Kirchman et al., 2003). In the field, HNLC assemblages are generally dominated by prokaryotic and picoeukaryotic organisms and the growth these organisms does not appear to be stimulated by Fe fertilization (Boyd, 2002; Hutchins et al., 2001). Although some studies in Fe-depleted regions have demonstrated that heterotrophic bacterial production is directly limited by Fe in the Southern Ocean (Pakulski et al., 1996; Arrieta et al., 2004), other field experiments suggest that bacterioplankton is only indirectly limited by Fe, being primarily limited by the paucity of dissolved organic matter as a consequence of the low primary productivity of Fe-limited phytoplankton (Kirchman et al., 1993, 2000; Hutchins et al., 1998, 2001; Church et al., 2000; Cochlan, 2001; Hall and Safi, 2001).

(ii) Recent examinations of Fe-organic complexes generated by the virus-mediated lysis of marine bacterioplankton have demonstrated that these complexes are highly bioavailable to both marine prokaryotes and eukaryotes (Poorvin et al., 2004) and have stability constants in the same range as L1 ligands (Poorvin, Sanders, Hunter and Wilhelm, unpub).

(iii) The bacterial origin of these class-1 ligands is further questioned since Fe(III)-binding ligands in exudates from eukaryotic phytoplankton cultures present similar stability constants to siderophores (Naito et al., 2001; Hasegawa et al., 2001; Boye and van den Berg, 2000). Some of these ligands are released upon Fe enrichment (Boye and van den Berg, 2000) similarly to what is observed during mesoscale fertilization.

Table I.2

Theoretical Fe requirements of *E. coli* growing aerobically in Fe-replete medium containing yeast extract as carbon substrate (adapted from Tortell et al., 1999, their Table 1).

Enzyme complex*	**Functions**	**[Complex]** [(a)] (μmol gProtein^{-1})	**Fe content** [(b)] (atom complex^{-1})	**[Fe]cell** [(c)] (10^{-20}mol.cell^{-1})	**QFe** [(d)] (μmol Fe molC^{-1})
NADH-Q reductase	Respiratory ETS	0.206	2 × 2Fe-2S 6 × 4Fe-4S	89.4	
Succinate-Q dehydrogenase	FAD + succinate → fumarate + $FADH^+$	0.335	1 × 2Fe-2S 1 × 3Fe-4S 1 × 4Fe-4S	46.7	
Cytochrome b_1	Respiratory ETS	0.335	1	5.19	
Cytochrome oxidase		0.124	2	3.84	
Aconitase	Citrate isomerisation to isocitrate	0.018	1 × 4Fe-4S	1.15	
Superoxide dismutase	Detoxification $O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$	0.530	1	8.22	
Catalase	Detoxification $2H_2O_2 \rightarrow 2H_2O + O_2$	0.011	1	0.17	
Total estimated		—	—	**155**	**43**
Total measured (McHugh et al., 2003)		—	—	**148**	**42**
Total measured (Archibald, 1983)		—	—	**216**	**61**

**** 94% of the cellular Fe is found in the respiratory chain***, associated with NADH-Q reductase, succinate-Q reductase, cytochrome b_1 and cytochrome oxidase complexes.

[(a)] [Fe] containing catalysts were obtained from published values & from measurements of catalytic activity and turnover. NADH-Q redase was determined from the difference between total flavin (0.541 nmol. mg protein $^{-1}$) minus flavin associated with succinate dehydrogenase, where one flavin equivalent exists per NADH-Q or succinate dehydrogenase equivalent. Succinate dehydrogenase was assumed to be equimolar with cyt b_1. Cyt b_1 and cyt oxidase were obtained from Aconitase concentration was determined from enzyme activity of *E.coli* (63.5 nmol *cis*-aconitase min^{-1} mg protein $^{-1}$), turn over of purified enzyme in yeast (50μmol *cis*-aconitase min^{-1}), and molecular weight (68500 g.mol^{-1}). Fe-SOD concentration was determined from enzyme activity for *E.coli* (5.6 U mg.protein^{-1}, turn over of purified bovine Cu/Zn-SOD (3300 U mg protein^{-1}) and molecular mass of Fe-SOD (40000 g.mol^{-1}) and Cu/Zn-SOD (32000 g.mol^{-1}). Concentration of catalase was obtained from *Corynebacterium diphtheridae*.

[(b)] Includes heme and non-heme (Fe-S clusters) Fe.

[(c)] Fe content normalized per cell assuming 1.55×10^{-13} g protein.cell^{-1}.

[(d)] Fe content normalized per cellular C assuming 0.17 pg C.μm^{-3} and a cell volume of 2.5 μm^3.

By contrast to the L1-ligand class, the "weaker" ligand class ($K_{Fe^{3+}/L2} \sim 3 \times 10^{21} - 6 \times 10^{21}$) has been detected throughout the water column. These class-2 ligands generally occur at generally higher concentrations than class-1 ligands, reaching up to 1.5 nM (Rue and Bruland, 1995, 1997). It has been suggested that these class-2 ligands are derived from phytoplanktonic intracellular molecules (porphyrin complexes) released by grazing or cell lysis (Wells and Trick, 2004). Recent empirical evidences have lead to the hypothesis of a partitioning of Fe availability to prokaryotic and eukaryotic planktonic groups based on ligand composition: eukaryotic phytoplankton being the most efficient to acquire Fe bound to the class-2 ligands pool, while the prokaryotic component preferentially use Fe bound to class-1 ligands (Hutchins et al., 1999). This hypothesis remains a source of debate due to the large size of most naturally occurring organic ligands. Recent observations suggest that the major fraction of organic Fe- binding ligands is not soluble but colloidal (> 1 kDa) in nature, therefore ambient colloidal complexes may reduce Fe availability to all organisms (Wells and Trick, 2004).

Indeed, if the importance of Fe speciation versus Fe concentration is considered, the identification of the sources of organic Fe ligands in seawater as well as the relative bioavailability (*i.e*. the portion and chemical speciation of Fe that can be absorbed, transported and utilized physiologically) of these organic complexes remains to be elucidated. It is now clear that Fe bioavailability is one of the key controls of ocean carbon cycling and atmospheric CO_2. It has been demonstrated that marine heterotrophic bacterial size-class plays an active role in the marine Fe biogeochemical cycle, accounting for as much as 50% of the biogenic Fe in oceanic systems (Tortell et al., 1996) and for up to 50 – 70% of the Fe uptake in Fe-depleted seawaters (Tortell et al., 1996; Maldonado and Price, 1999). Gaining insight on the degree of Fe bioavailability to marine heterotrophic bacteria is therefore of crucial importance owing to the fact that it can impact their growth efficiency, and thereby their function as a "sink" or a "link" within the microbial loop (Azam et al., 1983). Unfortunately, presently available tools are inadequate.

2. Microbial bioreporters

2.1. Current diagnostic tools to assess Fe status of microbial cells

Although modern analytical methods allow for the measurement of complexed Fe in seawater (Wells et al., 1991; van den Berg, 1995; Wu and Luther, 1994) they do not provide any information regarding the biological availability of the various organic species of Fe to bacterioplankton in the complex mixture that is seawater. On the other hand, enrichment bioassays are globally impaired by "bottle-effects" which lead to difficulties in distinguishing between the effects of carefully controlled nutrient amendments and that of uncontrolled changes (*e.g.* reduced turbulence, exclusion of grazers, adsorption of possible Fe sources to the bottle wall) and generally indicate which particular nutrient will become the limiting factor rather than which factor is presently limiting (Beardall et al., 2001). To overcome these problems, several molecular (*e.g.* ferredoxin:flavodoxin ratios) or physiological indices (*e.g.* variable chlorophyll fluorescence to maximum chlorophyll fluorescence, F_v/F_m) have been used to diagnose nutrient status of planktonic cells in natural assemblages (reviewed by Geider and La Roche, 1994). However, such indicators are generally specifically designed for photosynthetic organisms and cannot be applied to heterotrophic bacteria. Moreover, the few diagnostics indices applicable to heterotrophic bacteria, such as carbohydrate-degrading ectoenzyme fingerprints (Arrieta et al., 2004) or ETS activity (Tortell et al., 1996), provide valuable insights with regard to the global physiological status of resident bacterial cells but do not necessarily reflect the level of bioavailable Fe sensed by the cells in their environment.

2.2. Whole-cell biosensors: definition and principle of the method

A novel bioanalytical tool has been recently developed to complement chemical analyses in the field: the whole-cell biosensor, also called bioreporter. A bioreporter can be defined as a biologically based measurement device or system which produces a measurable signal in response to the environmental change (Daunert et al., 2000). In

other words, a bioreporter consists in intact, living microbial cells, that have been genetically modified by introducing a "reporter gene" to connect the activity of a given promoter involved in a metabolic or regulatory pathway of interest to an easily recordable output signal (Bachmann, 2003). Such a device was initially developed to monitor promoter activity (King et al., 1990), but bioreporter applications have broadened over the past decade to become an independent research area extending from genetic engineering (*e.g*. to identify *cis*-acting elements involved in gene expression, Llamas et al., 2003) to environmental monitoring (*e.g.* to evaluate the risk of environmental contaminants and toxicants or to determine the bioavailability of a given chemical, Porta et al., 2003) (Daunert et al., 2000; Bachmann, 2003; van den Meer et al., 2004). Indeed, the use of bioreporters has evolved to constitute a valuable tool to study microbial ecology and gene expression in complex environments (Hansen and Sørensen, 2001).

The technical concept of cell-based sensing systems is the coupling of a sensing element to the coding region of a promoterless reporter gene through gene fusion. The sensing element generally comprises regulatory proteins and the operator/promoter sequence of chromosomal or plasmid DNA. It is the specificity of these sensing elements for the analyte of interest that confers the selectivity of the system, while the reporter protein generally sets the systems sensitivity and the detection window (Daunert et al., 2000; van den Meer et al., 2004). When this genetic construct is introduced into a living cell, the host organism provides the regulatory components necessary to regulate the expression of the reporter construct (Fig I.5; van den Meer et al., 2004). A bioavailable analyte can pass through the cell membrane through several routes: diffusion, active transport or passive transport. Once internalized, the analyte binds to a regulatory protein, which will modulate the transcriptional expression of the reporter gene from the target promoter. In the case of a repressor protein, the transcription of the reporter gene is repressed, no more reporter protein is produced, which result in the loss of signal. Therefore, the level of reporter gene expression in response to the target analyte is mirrored by the activity or concentration of the reporter protein. Upon calibration of the system, the concentrations of the target analyte in "unknown" samples can be inferred from the reporter signal.

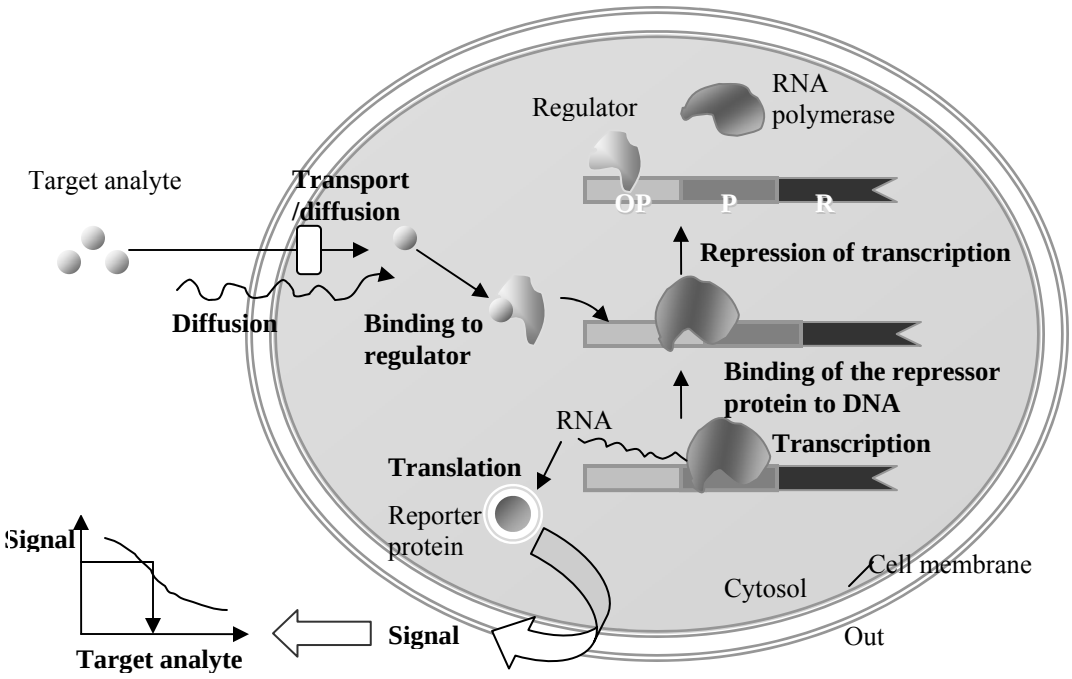

Fig. I.5. Schematic representation of the cellular events along the detection chain. A bioavailable analyte passes through the cell membrane and binds to a regulatory protein (here a repressor), which represses the transcription and translation of the reporter gene (adapted from Daunert et al., 2000; their Fig. 1). OP: operator; P: promoter; R: reporter.

Several reporter proteins can be used to generate the reporter signal; each of these reporters has advantages and disadvantages with regard to the experimental conditions and detection methods employed (Table I.3). The choice of the reporter depends on several factors such as stability, background activity, reporter gene expression efficiency, detection method, as well as the specific technical requirement of the methods (Daunert et al., 2000; van den Meer et al., 2004). The most commonly used reporter proteins are β-galactosidase, green fluorescent protein (GFP) and bacterial luciferase (*e.g.* from *Vibrio fisheri*, *Vibrio harvei*, and *Xenorabdus luminescens*) owing to the easily measurable and very specific signal produced by these proteins (Bachmann, 2003; van der Meer, 2004).

The whole-cell biosensor technology presents both advantages and drawbacks. The greatest advantages of these devices are their sensitivity, selectivity and stability which provide ideal conditions for environmental biomonitoring. Another advantage of

Table I.3

Advantages and disadvantages of reporter protein using in bacterial bioreporter technology (adapted from Daunert et al., 2000; their tables 2 and 3)**.**

Reporter protein	Detection method	Advantages	Disadvantages
Aequorin	BL	High sensitivity, easily measurable signal	Requires addition of substrate and the presence Ca^{2+}
α-amylase (amy)		Sensitive and stable	Endogenous activity, require separation of substrate and product,
β-galactosidase (β-Gal)	CR,HC,FL,EC,CL	Sensitive and stable, moderate linear range, does not require oxygen	Requires addition of substrate, endogenous activity.
Bacterial luciferase (luxAB)	BL	High sensitivity, no endogenous activity in most cell lines, real time/online bioassay	Requires addition of substrate, heat labile (depending on variants: <30°C, <37°C, <45°C), requires oxygen, high energy cost
Bacterial luciferase (luxCDABE)	BL	*Idem* than luxAB but does not require addition of substrate	*Idem* than luxAB but does not require addition of substrate.
Chloramphenicol acetyl-transferase (CAT)	RI, FL	No endogenous activity	Requires addition of substrate, utilization of RI, require separation of substrate and product, narrow linear range.
Green fluorescent protein (GFP)	FL, FC	Does not require addition of substrate or cofactor, spectral variants, no endogenous homolog in most systems, stable at biological pH, easy detection, real time/online bioassay	Moderate sensitivity, requires posttranslational modification, requires oxygen, interference with background fluorescence of host cell, cost of equipment, narrow linear range.
Ice nucleation protein	droplet freezing assay	Sensitive and stable, no endogenous activity	Long incubation time, background, tedious detection method, affected by growth phase

BL: bioluminescence; CL: chemiluminescence; CR: colorimetric; EC: electrochemical; FC: flow cytometry; FL: fluorescence; HC: histochemical; RI: radioisotope.

the bioreporter technology is their ability to provide physiologically relevant information on the effects of a target analyte. Indeed, they provide information that could not be acquired from cell-disrupting methods (Daunert et al., 2000). Since bioreporter systems are based on living components, they reflect the bioavailability of the target analyte in a given sample rather than its total concentration (Daunert et al., 2000; Bachmann, 2003; van der Meer, 2004). Additionally, they generally detect a group of compounds with similar properties rather than a single analyte (van der Meer, 2004). This method appears therefore to be a very valuable complement to chemical analyses in complex environments such as marine systems. Moreover, bioreporters are generally cost- and time-effective as they reproduce by themselves and do not require tedious purification procedures (van der Meer, 2004). Last, owing to their small size, they offer a strong potential for future miniaturization and portability, which allows on-site environmental monitoring (Daunert et al., 2000; van der Meer, 2004).

The drawbacks of the bioreporter technology are directly linked to the method. One of the main concerns is the environmental relevance of a biosensor constructed from a single bacterial strain (Hansen and Sørensen, 2001). Indeed, the choice of the reporter strain influences the relevance of the system as an environmental biosensor. Using a bacterial strain that is not native to the target environment is therefore questionable. Since bioreporter technology is limited to culturable strains and only a minority of bacteria can be cultured, the bioreporter response may not be representative to the resident bacterial population. Another disadvantage of the bioreporter technology resides in the possible loss of specificity of the system due to interferences from intracellular molecules and extracellular nutrients (Daunert et al., 2000). The choice of the reporter gene and host strain may also impact the natural sensitivity of the system (*i.e.* detection window) and selectivity (*i.e.* range of compounds detected) (van der Meer, 2004). The sensitivity of the method is also affected by the target analyte itself. For instance, the target analyte needs to be transported into the cells and to bind to the regulatory protein so that the signal can be generated, a stable target analyte is a prerequisite as otherwise it may be lost before interacting with the regulatory system of the host (van der Meer, 2004). The sensitivity of the system may also be influenced by the mode of transport of the analyte. Diffusion of

the target analyte may alter the sensitivity of bioreporters because it is a slow and unspecific process (van der Meer, 2004). Therefore, for environmental applications of bioreporters, the selection of each component of the biosensors system must consider factors such as incubation time, the type of organisms that will be transformed, and environmental factors (Heitzer et al., 1998).

2.3. Fe-dependent bacterial bioreporters

The only Fe-dependent marine bioreporter organism reported to date is the diatom *P. tricornutum* (Falciatore et al., 2000). The response of this eukaryotic microbial bioreporter is not specific to Fe, although under the appropriate conditions it can be considered Fe-dependent (Falciatore et al., 2000). In this particular case, a calcium dependent reporter protein (aequorin) was used. The level of expression of this reporter protein depends on cytosolic calcium concentrations, which are generally altered by signal transduction. The signal can be therefore induced by different sources of stress. It was shown that changes in ambient Fe level, within micromolar range, impact intracellular calcium levels, suggesting that diatoms may use calcium-mediated regulatory mechanisms to control Fe-metabolism. In this specific case, no quantitative assessment of Fe bioavailability was possible. Although interesting as an illustration, such a bioreporter would not be suitable for environmental monitoring.

Several Fe-dependant prokaryotic bioreporters have been developed to date, either for the study of *cis*-acting genetic elements (Althaus et al., 1999; Flores et al., 2003) or for environmental monitoring (Loper and Lindow, 1994; Loper and Henkels, 1997; Joyener and Lindow, 2000; Durham et al., 2002, 2003; Porta et al., 2003). They rely on the engineering of the cells to generate a transcriptional fusion between Fe-regulated promoter and various reporter genes. With the exception of the promoter *desA*, all promoters used in Fe-dependent bacterial bioreporters presently reported in the literature are directly regulated by the ferric uptake regulatory (FUR) protein (Table I.4):

(i) promoter of the *desA* gene which encodes for an enzyme (lysine decarbo-xylase) involved in the first step of synthesis of the siderophore desferrioxamine in

Table I.4

Bioreporters for Fe bioavailability.

Type	Gene Fusion (promoter-reporter)	Host strain	Environment	Detection window	Reference
Bacterial bioreporters					
Plasmidial	*desA::amy*	*Streptomyces coelicolor*	soil	N/A	Flores et al. (2003)
Plasmidial	*tonB::lacZ*	*E. coli KP1022*	lab	10^{-7} – 10^{-6} M Fe:NTA	Althaus et al. (1999)
Plasmidial	*Pvd::gfp*	*P. syringae* B728a	soil	10^{-7} – 10^{-4} M $FeCl_3$	Joyner and Lindow (2000)
Plasmidial	*Pvd::inaZ*	*P. fluorescens* PF5	soil	10^{-7} – 10^{-4} M $FeCl_3$	Loper and Henkels (1997) Loper and Lindow (1994)
Genomic	*pupa::luxCDABE*	*P. putida* WCS358	lab	10^{-8} – 10^{-6} M $FeCl_3$	Khang et al. (1997)
Cyanobacterial bioreporters					
Genomic	*isiAB::luxAB*	*Synechococcus* PCC7942	freshwater	pFe 21.1 – pFe 20.6	Durham et al. (2002) Porta et al. (2003)
Genomic	*irpA::luxAB*	*Synechococcus* PCC7942	freshwater	pFe 21.1 – pFe 20.6	Durham et al. (2003)
Eukaryotic bioreporters					
Plasmidial	*aequorin*	*P. tricornutum (Diatom)*	seawater	5 – 10 μM	Falciatore et al. (2000)

actinomycetes and is regulated by divalent metal dependent repressor DmdR, a FUR homolog regulator protein (Flores et al., 2003).

(ii) promoter of the *tonB* gene, which encodes for a membrane protein (TonB) involved in energy transduction for Fe-siderophore transport (Althaus et al., 1999),

(iii) promoter of the *pvd* gene, involved in the synthesis of pyoverdine (Loper and Lindow, 1994),

(iv) promoter of the *pupA* gene, encoding for an outer membrane protein (pseudobactin uptake protein A) involved in the transport of pseudobactin (Khang et al., 1997),

(v) promoter of the *isiAB* gene, which encodes for an Fe-dependent photosystem I-associated chlorophyll-binding protein and the redox catalyst flavodoxin (Durham et al., 2002),

(vi) promoter of the *irpA* gene, encoding for a membrane protein likely involved in Fe acquisition. (Durham et al., 2003).

The FUR system is nearly ubiquitous and well conserved among heterotrophic bacteria. Almost all bacteria use FUR homologues to regulate their Fe uptake system (Braun et al., 1998; Hantke, 2001). This protein plays a key role in Fe homeostasis by mediating the Fe-responsive transcriptional regulation of a variety of genes, including those involved in Fe homeostasis such as Fe uptake (Braun et al., 1998; Hantke, 2001; McHugh et al., 2003). Therefore, FUR-based bioreporters appear relevant for environmental studies. Although it has been shown that some genes can be induced by FUR when Fe is not limiting (McHugh et al., 2003), in the case of genes involved in Fe acquisition this protein generally acts as a positive repressor by repressing transcription upon interaction with its cofactor for DNA binding, the ferrous iron ion (Fe^{2+}) (Hantke, 2001; Andrews et al., 2003). It has been suggested that the binding of the cofactor Fe^{2+} to FUR increases the affinity of FUR for its cognate DNA-binding site of about 1,000 fold (Andrews et al., 2003). By contrast, in Fe-depleted conditions, due to the paucity of intracellular Fe^{2+}, the FUR protein loses its cofactor and thereby its affinity for its DNA binding site, which results in the derepression of the Fe uptake genes. Various reporter proteins have been used in these prokaryotic Fe-dependent bioreporters, namely: α-

amylase (Amy), ice nucleation protein (Ina), bacterial luciferase (lux), and the green fluorescent protein (GFP) (Table I.4). Advantages and disadvantages of these reporter genes, related to their assay conditions and the method employed for signal detection, are listed in Table I.3.

Owing to the easy, non-invasive and fast detection method as well as the high sensitivity of the bacterial luciferase, this reporter protein appears to be the most appropriate choice for environmental monitoring when compared to other reporter proteins previously used in the field (Ina). The portability and cost-effectiveness of the devices used for the detection of the bioluminescent signal are also two major advantages when compared to that used to analyze the fluorescence signal of GFP (*e.g.* flow-cytometers and fluorometers), another reporter protein commonly used in environmental monitoring (Hansen and Sørensen, 2001). Moreover, the lack of structural metal in the luciferase enzyme (Meighen and Dunlap, 1993; da Silva Nunes-Halldorson and Duran, 2003) is a significant advantage for the study of metal availability, as it won't interfere with the metal homeostasis of the host cell. Presently reported *lux*-based microbial bioreporters utilized in freshwater systems have demonstrated the effectiveness of the luciferase protein as a reporter gene for Fe bioavailability monitoring (Durham et al., 2002, 2003; Porta et al., 2003).

However, luciferase markers also present some significant limitations with regard to their application to Fe bioavailability monitoring, which include their dependence on the substrate concentration, the physiological state of the host microbial cell, and the presence of stimulating and inhibiting factors. First, when only the *lux* AB genes are used as marker, addition of exogenous aldehyde substrate (usually decanal) is required to allow the production of the bioluminescent signal. This may be a significant draw back since it can not only introduce Fe contamination in the samples to assess but also can be toxic to the bioreporter cells upon prolonged exposure (Porta et al., 2003). The utilization of the full *luxCDABE* operon appears therefore preferable since it also encodes for the enzymes (transferase, LuxD, and synthetase reductase, LuxCE) involved in the regeneration of the substrate, and therefore overcomes the problem of substrate addition (Fig. I.6). However, the use of the full *lux* gene cassette comes along with a strong

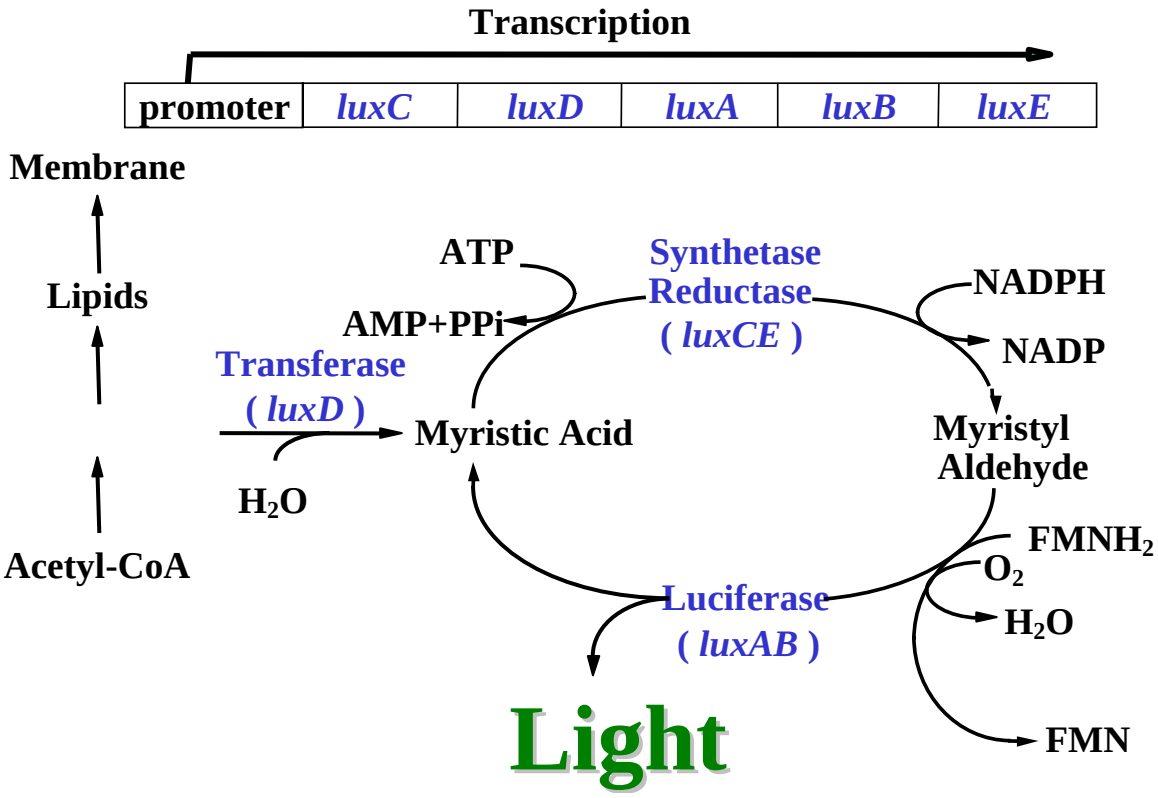

Fig. I.6. Biochemical and molecular reactions involved in bacterial bioluminescence (taken from Heitzer et al., 1998; their Fig. 1). The α and β subunit of the luciferase are encoded by two adjacent genes in the lux operon: *lux* A, *lux* B. These two subunits, when assembled, compose the luciferase enzyme which catalyzes the mixed oxidation of a long-chain aldehyde (myristyl aldehyde) and $FMNH_2$ to generate blue-green light emission (490 nm), FMN and water and the corresponding fatty acid (myristic acid). The full operon also comprises the genes (*lux* C, D, and E) encoding for the enzymes that regenerate the aldehyde substrate. The whole reaction generates enough energy (60 Kcal mol^{-1}) to support the light production (Meighen and Dunlap, 1993). However, the reaction is also energy consuming. Depending of the efficiency of the reaction (quantum yield, q.y.), one emitted photon would cost between 6 (q.y. = 1) and 20 (q.y. = 0.3) ATP molecules (Hasting and Nealson, 1981; Makemson and Hastings, 1986; van den Meer et al., 2004).

a strong physiological cost. The emission of one light quantum requires both reducing power and free energy under the form of one $FMNH_2$, one NAD(P)H and one ATP. Although the reaction generates enough energy (60 Kcal mol^{-1}) to support the light production (Meighen and Dunlap, 1993), it is nevertheless an energetically costly process. It has been estimated that light emission would require 6 to 7 ATP molecules for each photon produced (Hastings and Nealson, 1981; Makemson and Hastings, 1986), assuming 100% efficiency for the reaction (*i.e*. one light quantum per reacted molecule of $FMNH_2$) and that complete oxidation of NAD(P)H and $FMNH_2$ via the electron transport chain generate 3 and 2 ATP molecules (van den Meer et al., 2004). However, owing to the fact that bacterial luciferase generally has a quantum yield lower than 100%, one emitted photon could cost as much as 20 ATP (van der Meer et al., 2004). Although it has been shown that luciferase can support the growth of cytochrome-deficient bacteria (Makemson and Hastings, 1986), light emission remains a costly process and could cause a metabolic burden to the host microbial cells (van der Meer et al., 2004). Owing to the fact that Fe starvation impact electron transport systems and the metabolic activity of bacteria (Tortell et al., 1996; Kirchman et al., 2003), the efficiency of the bioluminescent response of the bioreporter cells in Fe-depleted environment may be significantly altered. Indeed, it has been reported that Fe may exert a control on luminescence emission in several photobacteria strains. It has been shown that an increase in exogenous Fe can repress light emission in *Vibrio harveyi* (Makemson and Hastings, 1982) or delays induction of luciferase synthesis in *Vibrio fisheri* (Haygood and Nealson, 1985). Although the way in which Fe impacts luminescence is poorly understood, it has been suggested that Fe could interfere with a regulatory protein involved in the regulation of *luxICDABEG* transcription (Haygood and Nealson, 1985, Dunlap, 1992). As such, the observed inhibition of light emission by Fe may reflect the regulation of the promoters controlling the *lux* operons in these naturally luminescent bacteria. Since these promoters are not present in bioreporter systems, one may assume that luminescence signal should not be affected by exogenous Fe. However, such an assumption has yet to be demonstrated. The use of a bioluminescent marker for Fe availability assessment in Fe-deficient environment, such as oceanic systems, may be indeed questionable. However,

since cellular requirements for Fe are most likely to differ between strains due to adaptative traits and their Fe-acquisition strategies (Granger and Price, 1999), such caveats in the method may be overcome by choosing a host strain with low requirements for Fe. Moreover, it seems evident that the utilization of *lux*-based bioreporter for environmental monitoring (*e.g.* Fe bioavailability assessment) must be preceded by an extensive laboratory characterization, during which the detection window and the signal background levels can be tested and thereby the environmental relevance of the bioreporter system assessed.

3. Research objectives

The research described here consisted of the development of a heterotrophic luminescent bacterial bioreporter system to monitor Fe bioavailability in oceanic systems. The central hypothesis of the present work is that such an Fe-dependent *lux*-based bioreporter systems is sensitive and selective enough to assess Fe bioavailability to heterotrophic bacteria in complex systems such as seawater. To test this hypothesis, we will examine the following assumptions:

(i) the expression of the bacterial luciferase operon (*luxCDABE*) is not affected by exogenous Fe within an environmentally relevant range of concentrations in this trace metal.

(ii) changes in the physiology or growth of bioreporter cells due to changes in Fe bioavailability do not affect the sensitivity of the signal,

(iii) the bioreporter system can be standardized using chemically defined culture medium.

Once these assumptions will be addressed, we will be able to use Fe-dependent bioreporters to compare Fe bioavailability between different environments and under different conditions.

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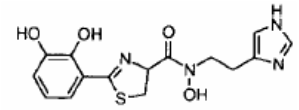
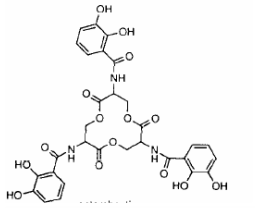
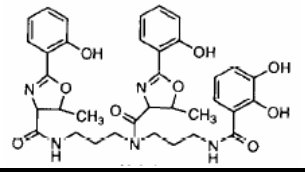
APPENDIX (PART I)

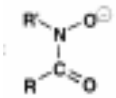
Appendix I –
Structures and stability constants of characterized terrestrial and marine siderophores as well as natural porphyrin ligands.

Catecholate Siderophores (bacteria only)

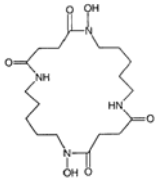
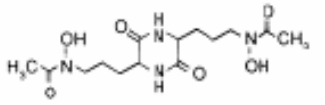
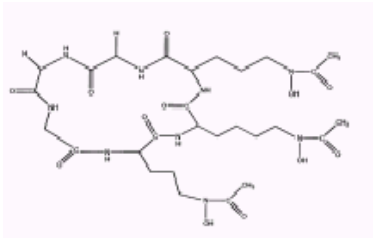
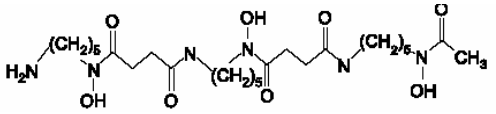
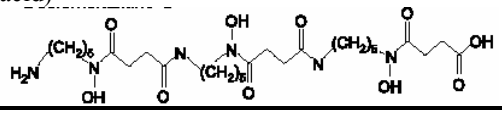
Siderophore / (producer)	Structure		Log K
Anguibactin (*Vibrio sp.*[a] *)			
Enterobactin (*E. coli, Salmonella typhimurium, Klebsiella pneumoniae*)		(cyclic trimer)	20.8^{b}, $49^{(PI)c}$
Vulnibactin (*Vibrio vulfunicus*[d]*)		(linear trimer)	

* marine organism
[a] Jalal et al., 1989; [b] Wu and Luther, 1994 (kinetic method; competing ligand:1N2N); [(PI)] [c] Lewis et al., 1995 (proton independent binding constant for EDTA = $10^{25.1}$); [d] Okujo et al., 1994.

Appendix I – Continued



Hydroxamate Siderophores (fungi and bacteria)

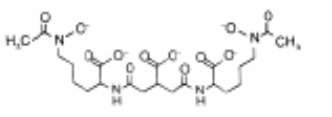
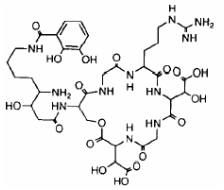
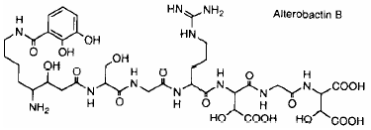
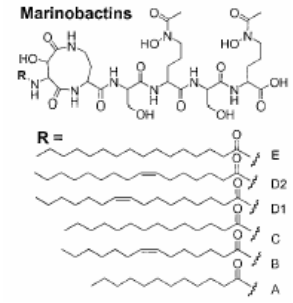
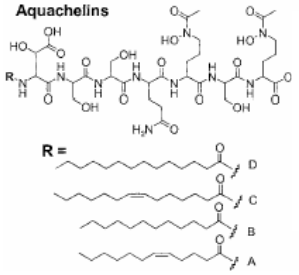
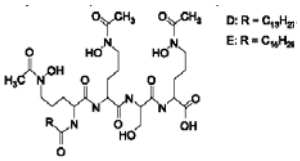
Siderophore / (producer)	Structure	Log K
Bisucaberin (*Vibrio salmonicida*[a]*, *Alteromonas haloplanktis*[b]*)	 (cyclic, dihydroxamic acids)	32.2[a]
Coprogen *eg*. Rhodotorulic acid (*Rhodotorula*, yeast)	 (tetradentate dihydroxamic acids)	
Ferrichrome (basidiomycetes fungi)	 (exocyclic, hexadentate trihydroxamic acid)	21.6[c], 29.1[(PI)d,e]
Ferrioxamines *e.g*. Desferrioxamine B (Actinomycete fungi)	 (linear acyclic, hexadentate trihydroxamic acid)	21.6[c] - 30.99[(PI)d,e]
e.g. Desferrioxamine G (*Vibrio* sp.[f]*)		

* marine organism

[a] Winkelmann et al., 2002; [b] Takahashi et al., 1987; [c] Witter et al., 2000b (CLE, CSV method; competing ligand: 1N2N; in seawater); [(PI)d] Lewis et al., 1995; [(PI)e] Reid et al., 1993 ([(PI)]proton independent binding constant for EDTA = $10^{25.1}$); [f] Martinez et al., 2001.

Appendix I – Continued

Mixed Siderophores (Mycobacteria and Bacteria)

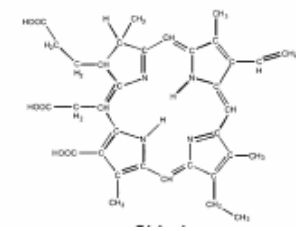
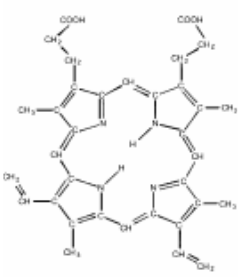
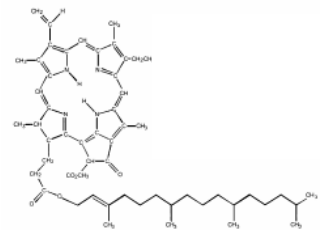
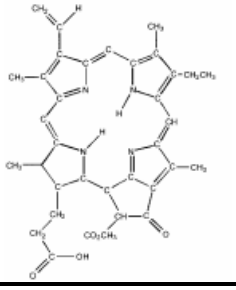
Siderophore / (producer)	Structure	Log K
Complexone (excreted) *e.g*. Aerobactin (*E. coli, Vibrio sp.* [a]*)	 (2 hydroxamates, 1 α-hydrocarboxylic acid)	22.5[(PI)b]
Alterobactin *e.g.* Alterobactin A (*Alteromonas luteoviolacea*[b]*)		20.7[b], 23.9[d], 49-53[(PI)b]
e.g. Alterobactin B (*Alteromonas luteoviolacea* [b]*)	 	20.4[b], >24[d], 49-53[(PI)b]
Marinobactins[e](membrane associated and excreted) (*Marinobacter* sp. DS40M6*)	 (2 hydroxamates, 1 β-hydroxy-aspartic acid, 6-aa peptide headgroup, fatty acid appendage)	
Aquachelins[e] (membrane associated and excreted) (*Halomonas aquamarina* DS40M3*)	 (2 hydroxamates, 7-aa peptide headgroup, fatty acid appendage)	22.2[f]
Amphibactins[e](not excreted) (*Vibrio* sp. R-10*)	 (Tris-hydroxamates peptidic group, Acyl appendage)	

* marine organism

[a] Haygood et al., 1993; [b] Reid et al., 1993 (proton dependent stability constant); [(PI) b] Reid et al., 1993 (proton independent binding constant for EDTA = $10^{25.1}$); [e] Martinez et al., 2003; [f]Barbeau et al., 2001 (CLE/ASV, competing ligand: SA), [d]Witter et al., 2000b (CLE/CSV, competing ligand:1N2N; in seawater).

Appendix I – Continued

Porphyrins Ligands (autotrophs only)

Siderophore / (producer)	Structure	Log K
Chlorin e6		
Protoporphyrin IX		22.4
Phaeophytin		22.2
Phaeophorbid a		

Structures taken from Weaver et al., 2003; Stability constants taken from Witter et al., 2000b (CLE/CSV, competing ligand:1N2N; in seawater).

Part II

CHARACTERIZATION AND FIELD TRIALS OF A BIOLUMINESCENT BACTERIAL REPORTER OF IRON BIOAVAILABILITY

Part II

CHARACTERIZATION AND FIELD TRIALS OF A BIOLUMINESCENT BACTERIAL REPORTER OF IRON BIOAVAILABILITY

This chapter is a revised version of a paper by the same name published in the special issue *"Novel Techniques for Chemical Characterization in Marine Systems"* of the journal *Marine Chemistry* in 2003 by Cécile E. Mioni, Amanda M. Howard, Jennifer M. DeBruyn, Nathan G. Bright, Michael R. Twiss, Bruce M. Applegate and Steven W. Wilhelm.

Mioni, C.E., A.M. Howard, J.M. DeBruyn, N.G. Bright, M.R. Twiss, B.M. Applegate and S. W. Wilhelm. Characterization and field trials of bioluminescent bacterial reporter of iron bioavailability. *Marine Chemistry* 83, 31-46.

My use of "we" in this chapter refers to my co-authors and myself. My primary contributions to this paper include (1) development of the problem relevant to my study of the heterotrophic bioreporter to assess Fe bioavailability in natural ecosystems, (2) identification and laboratory characterization of the heterotrophic bioluminescent reporter *Pseudomonas putida* FeLux, (3) characterization of the *E. coli* pFeLux, *E.coli* FeLux and *E.coli* Lux bioreporter strains, (4) laboratory and field data analyses, (5) most of the gathering and interpretation of literature, (6) pulling the various contributions in a single paper, and (7) most of the writing.

1. Introduction

Recent research has demonstrated clearly that Fe availability controls productivity and community structure in the High Nutrient Low Chlorophyll (HNLC) marine areas (Martin and Gordon, 1988; Martin et al., 1994; Coale et al., 1996a, 1996b; Boyd et al.,

2000), some coastal marine systems (Hutchins and Bruland, 1998) and some large lakes (Wurstburg and Horne, 1983; Twiss et al., 2000). Although Fe is the fourth most abundant element in the earth's crust, bioavailable Fe remains scarce in pelagic surface waters at near-neutral pH as a result of low input, biotic as well as abiotic adsorption, and rapid oxidization of the inorganic Fe(II) to the insoluble Fe(III)-complexes (Millero and Solongo, 1989; Kuma et al., 1992).

As the term HNLC indicates, most investigations have focused on the impact of Fe depletion on the phytoplankton component (*e.g.,* Hudson and Morel, 1990; Sunda et al., 1991; Sunda and Huntsman, 1995). However, recent improvements in the understanding of Fe chemistry have demonstrated that heterotrophic bacterioplankton in low Fe aquatic systems may also be directly or indirectly impacted. The application of electrochemical techniques has allowed for a reliable estimation of Fe-organic completion and redox speciation (Van den Berg et al., 1991; Wu and Luther, 1994, 1995; Rue and Bruland, 1997, 1995; Witter et al., 2000a,b; Powell and Donat, 2001). The results of these novel techniques have lead to the conclusion that more than 99% of the dissolved Fe in surface waters is bound to unknown organic ligands falling in two major classes (the stronger L1 and the weaker L2 ligand classes) that present stability constants similar to bacterial siderophores (Lewis et al. 1995, Rue and Bruland, 1995, 1997; Witter et al. 2000a,b). This finding and further observations during the mesoscale fertilization experiments have supported the hypothesis that these ligands could have a biogenic origin: as microbial Fe chelates (siderophores) produced by both photo- and heterotrophic prokaryotes to alleviate Fe stress (Wilhelm and Trick 1994; Butler 1998) or as the byproduct of viral (Wilhelm and Suttle, 1999) and grazing induced mortality (Hutchins and Bruland, 1994).

Traditional tools for the characterization of Fe bioavailability remain insufficient to determine whether the heterotrophic bacterial population is primarily limited by Fe availability or by dissolved organic carbon. While some experiments have shown that the bacterial community was responsive to Fe enrichment and that Fe requirements were high (Pakulski et al., 1996; Tortell et al., 1996), the response appears dependent on the chemical species of Fe that are provided (Granger and Price, 1999; Guan et al., 2001). Thus, the response to Fe-enrichment cannot be separated from the effect of enhanced

dissolved organic matter (DOM) production due to Fe-stimulation of the primary producers (Kirchman et al., 2000). Since the heterotrophic bacterial component is suspected to contribute to the production of the Fe-complexing organic ligands in surface waters, a better understanding of the bioavailability of different organic Fe pools remains crucial for a resolution of the biogeochemical machinations of the "microbial ferrous wheel" (Kirchman, 1996).

To assess the response of heterotrophic bacteria to Fe bioavailability in aquatic systems, we have developed a mobile genetic system that can be used as a luminescent reporter for the characterization of Fe bioavailability to aquatic prokaryotes. The well characterized, Ferric Uptake Regulator (FUR) - regulated bi-directional *fepA-fes* (ferric enterobactin uptake system – ferric Enterobactin esterase) promoter from *Escherichia coli* (Escolar et al., 1998) has been fused to the *Vibrio fischeri luxCDABE* reporter gene cassette on a transposable genetic element and harbored in *E. coli* SV-17. The resulting strain (denoted *E. coli* pFeLux) can subsequently be mated with other bacteria to introduce the reporter stably into the chromosome of the bacterium of interest. The structure and the regulation of the *fepA-fes* promoter system are well known. Through a single FUR-binding site (Escolar et al., 1998), it acts as a regulatory region for Enterobactin production and transport (Braun et al., 1998, Van der Helm, 1998). In the case of our reporter, de-repression of high-affinity Fe transport (displacement of the Fe - FUR protein complex from the promoter) results in light production (luminescence). As such, light production in these bacteria is linked directly to the Fe status of the cell. The present study reports on the characterization of the reporter after stable introduction into the chromosome of a halotolerant *Pseudomonas putida.* Laboratory work has been combined with a set of field experiments in an aquatic system (Lake Erie) where Fe availability is known to transiently limit primary production (Twiss et al., 2000). The results reported here demonstrate a differential availability of Fe in the presence of different chelators to the *P. putida* reporter strain. Moreover, the field studies suggest that much of the bioavailable Fe during the *in situ* study was located in the particulate (> 0.2 μm) size class, indicative of the significant role that the recycling of Fe plays in aquatic environments.

2. Material and methods

2.1. Construction of Fe bioreporters

All bacterial strains and plasmids used in this study are listed in table II.1. A 321 bp fragment containing the divergent promoters (*fepA-fes*) involved with high-affinity Fe uptake regulation in *E. coli* (Pettis et al., 1988) was amplified from *E. coli* K12 using the polymerase chain reaction and subsequently cloned into pCR2.1 according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The 321-bp fragment was then inserted upstream of a promoterless *luxCDABE* gene cassette in a miniTn5:*luxTcR* transposon to create a *fepA-fes::lux* fusion as described previously (Applegate et al., 1998; Hay et al., 2000). To facilitate construction of the lux fusion, the *fepA-fes* plasmid DNA was linearized with *SpeI*, dephosphorylated, and subsequently ligated into the unique *XbaI* site of the transposon, 5' to the *luxCDABE* gene cassette (Fig. II.1).

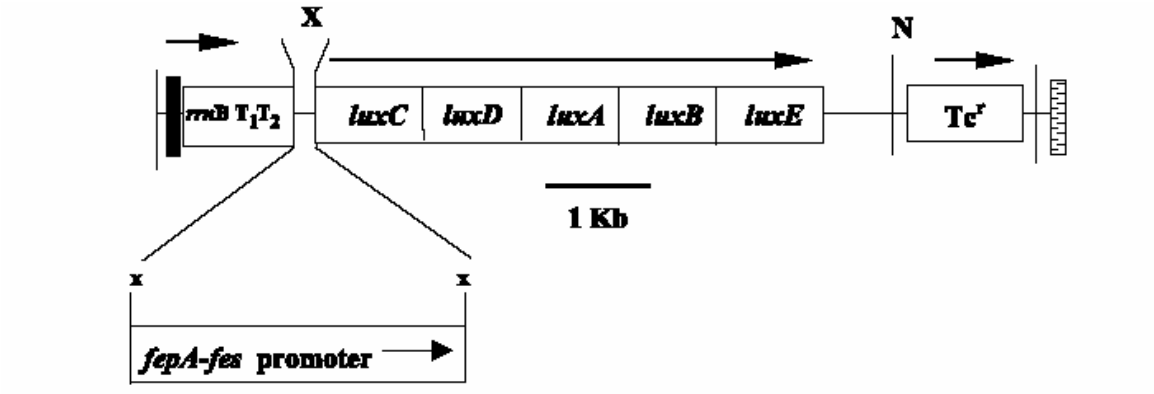


Fig. II.1. Physical and genetic map of the bioreporter system. Details of the construction of the mini-Tn5-*luxCDABE* reporter/vectors have been published elsewhere (Applegate et al., 1998; Hay et al., 2000). X, *XbaI* site; N, *NotI* site; *rrn*B T1T2, transcriptional terminators from *E. coli rrn*B; Tcr, tetracycline resistance gene.

Table II.1

Bacteria and constructs used in this study.

	Relevant genotype/characteristics	Reference
Plasmids		
pCR2.1	3.9-kb cloning vector for PCR products with 3' A overhangs; Ap^R Km^R	Invitrogen, Carlsbad, CA
pSWW1	pCR2.1 containing the 321 bp *fepA-fes* operator and divergent promoters	This study
pUTK83	Tn5*luxCDABE*Tc^R Mini Tn5 lux transposon in pUT unique *NotI XbaI* cloning site for chromosomal insertion of lux reporters, *E.coli* SV 17, amp, Tc^R on Tn	This study
pSWW2	pSWW1 linearizied with XbaI and inserted into the *XbaI* site of pUTK83	This study
pSWW3	Tn5*luxCDABE*Tc^R Mini Tn5 lux transposon containing *fepA-fes* 321bp DNA fragment fused to *luxCDABE* in pUTK83	This study
Bacterial Strains		
E. coli DH5α F'	Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_K-,m_K+) *phoA supE44 thi-1 gyrA96 relA1* (Tn5 Km^R F' episome)	Invitrogen, Carlsbad, CA
E. coli INVF'	F'φ 80*lacZ*M15 Δ (*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(rK mK^+) *phoA supE44 thi-1 gyrA96 relA1*	Invitrogen, Carlsbad, CA
E. coli SV17	λ *pir recA thi pro hsdR* M^+ RP4:2-Tc:Mu:Km $Tn7Tp^RSm^R$; mobilizing strain for pUT/mini-Tn5 derivatives	DeLorenzo et al 1993
E. coli Lux	*E. coli* containing a chromosomally inserted Tn5*::luxCDABE*Tc^R Mini Tn5, Km^R	Hay et al. 2000
P. putida FeLux	*P. putida* containing a chromosomally inserted Tn5 *fepA-fes::luxCDABE*Tc^R Mini Tn5, Tc^R	This study
E. coli FeLux	*E. coli* DH5α F' containing a chromosomally inserted Tn5 *fepA-fes::luxCDABE*Tc^R Mini Tn5, Km^R, Tc^R	This study
E. coli pFeLux	*E. coli*SV17 containing pSWW3	This study

This enabled transformation into *DH5α* Library Efficiency competent *E. coli* (Life Technologies, Gaithersburg, MD) with selection on LB agar plates containing tetracycline (Tc, 14 μg L^{-1}) and kanamycin (Km, 50 μg L^{-1}). Resistant colonies were then isolated and verified genetically using restriction endonucleases. Once verified the DNA was then digested with *Not I*, ligated and electroporated into *E. coli* SV-17 and plated on tetracycline. This step removed the pCR2.1 cloning vector and left the *fepA-fes* genetic element fused to the *lux* genes. The transformants were then patch inoculated onto kanamycin plates to ensure sensitivity and removal of the cloning vector. Isolates were further screened by restriction mapping to orient the 321 bp insert relevant to the *lux* genes. Directionality was also verified using PCR with a *lux* C 3' primer and each of the primers used to amplify the *fepA-fes* promoter region. A clone containing a *fes-lux* fusion was chosen for further study. Once chosen the clones were assayed for light production in the presence of Fe chelating agents using a Zylux FB-15 luminometer (Maryville, TN). Due to high background level of bioluminescence from the reporter strain based on the multi-copy plasmid format, a single copy chromosomal insertion was constructed. Chromosomal-based Fe bioreporters were constructed by mating the *E. coli SV-17* harboring the transposon with a halotolerant *Pseudomonas putida* and *E. coli* DH5α with a chromosomal kanamycin marker as previously described (de Lorenzo et al., 1993). Matings were then plated on pseudomonas isolation agar amended with 14-μg/mL tetracycline or LB containing Km (50 μg L^{-1}) and Tc (14 μg L^{-1}). Colonies were patch plated, evaluated for light production, and clones were chosen for further study.

Further verification of the bioreporter identity was determined by 16S rDNA sequence analyses. PCR was performed in triplicate, using universal eubacterial primers 46F (5'-GCYTAACACATGCAAGTCGA) (Kaplan et al., 2001) and 519R (5'-TTATTACCGCGGCKGCTG) (Lane, 1991). Using this primer set, the PCR amplification gives rise to a fragment of the 16S rDNA gene (using the *Escherichia coli* numbering system) approximately 473 bases long (Eldridge, 2004). PCR reactions were performed as described previously (Eldridge, 2004) with minor modifications: 25 μL PCR reactions were prepared in PuRe Taq Ready-to-go™ PCR beads tubes (Amersham Biosciences) using ~10 ng of template DNA. PCR products were directly cloned into the

TOPO-TA PCR 2.1 vector (Invitrogen) according to the manufacturer's protocol. Sequences were obtained from plasmid DNA extracted from 4 transformants by using Wizard Miniprep DNA Purification kit (Promega). Sequences of PCR-amplified 16S rDNA gene fragments were aligned in BioEdit Sequence Alignment Editor (Hall, 1999) using ClustalW alignment (Thompson et al., 1994). Sequences were then aligned to previously characterized sequences using the NCBI - Basic Local Alignment Search Tool (BLASTN, ver. 2.2.9) program (Altschul et al., 1997). Sequences from various *Pseudomonas putida* strains produced the most significant alignments (Score bit 901, identity : 98%).

2.2. Laboratory characterization of the Fe-dependent bioreporters

Study organisms – Three bioluminescent reporters were examined. The Fe-dependant bioreporters *P. putida* FeLux (chromosomally integrated construct) and *E. coli* pFeLux (plasmid construct) and *E. coli* FeLux (chromosomally integrated construct) were used to evaluate the effect of heterologous siderophores on Fe availability by monitoring endogenous bioluminescence. The recombinant *E. coli* Lux (described as *E. coli* # 82, Hay et al., 2000), containing the *luxCDABE* cassette integrated into the chromosome at a neutral site and lacking the *fepA-fes* promoter, was used as a control to demonstrate that the response of the *luxCDABE* system itself was independent of bioavailable Fe. No control strain is available for *P. putida* at this time.

Culture conditions – The first set of laboratory experiments were performed by growing the bioreporters overnight (~ 8 to 12 h) with shaking (1,000 rpm) at 37 °C in 250 mL acid-washed polycarbonate Erlenmeyer flasks filled with a Fe-replete medium: a modified recipe of the Luria Bertani (Miller) medium. For 1 L of medium, 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl were dissolved in Milli-Q water, and the pH adjusted to 7.5 with NaOH. After microwave sterilization (Keller et al., 1988), tetracycline was added to 50 μg mL^{-1}. No attempts were made to deferrate the medium since variations in the concentration of Fe contaminant found in 8-hydroxyquinoline can

result in variations in residual Fe concentrations and thus, variations between assays (Schwyn and Neilands, 1987; Hersman et al., 2000).

When an optical density at 600 nm (OD_{600}) of 0.1 (1 cm pathlength) was reached, 40 mL aliquots of the initial culture were transferred in 50 mL sterile polycarbonate tubes. The 40 mL of culture samples were either amended, to final concentrations ranging of from 0 nM (unamended control) to 200 nM of the appropriate chelator, or up to 25 nM of inorganic Fe ($FeCl_3$). Sub-samples (10 mL) were collected in triplicate in 15 mL sterile polycarbonate tubes and incubated at 25° C in a temperature-controlled shaker during the experimental period (6 hours). Fe availability, estimated by changes in the luminescence of the bioreporters, was evaluated at 0, 2, 4 and 6 h by measuring the light produced by 1 mL of culture. Growth was followed in the cultures during the incubation period by measuring the optical densities (OD_{600}) of each sample (at 0, 2, 4 and 6 h). Light production (relative light units per second; Rlu s^{-1}) was normalized per OD_{600} for each triplicate at each time-point. Reported values are the means of three replicate cultures for the time-point for which the signal was the strongest (t = 4 h for *P. putida* or t = 6 h for *E. coli*).

Fe chelators tested – Four well characterized hydroxamate-type Fe(III) chelates were tested, as described above: two trihydroxamates, the actinomycete siderophore desferrioxamine B (DFB, desferrioxamine mesylate, Sigma) and the basidiomycete siderophore ferrichrome (Sigma); one linear dihydroxamate-containing siderophore produced by the yeast *Rhodotorula pilimanae*, Rhodotorulic acid (RA, Sigma); and one non-metabolizable synthetic chelator 2,2'-dipyridyl (DP, Acros Organic) (Table II.2). Stock solutions of each chelator (50 μM) were prepared in acid-washed polycarbonate flasks. Chelators were dissolved in 1 mL of 50% ethanol and adjusted to final volume with Chelex-100 treated Milli-Q water (Price et al., 1988/89). Stock solutions were kept refrigerated until use.

Table II.2

Relative stability constants of Fe chelates used in this study.

	Ligand	Log $_{KFe^{3+}L}$	Reference
Trihydroxamates	DFB	21.6	Witter et al. (2000b)
	Ferrichrome	21.6	Witter et al. (2000b)
Dihydroxamate	Rhodotorulic acid	19.39	Nguyen van Duong et al. (2001)
Natural ligands	L1 class	18.6 – 22.9	Witter et al. (2000a,b)

2.3. Field testing

Stations and Sample Collection – Water was collected from the central basin of Lake Erie (Environment Canada Station 84, 81.65° W, 41.93° N) during a research cruise on the CCGS *Limnos* in July 2001. Data from the ship's CTD were used to collect temperature profiles to model the water column structure. Surface water (5 - 10 m) was collected with a trace metal-clean surface pumping system comprising a Teflon double diaphragm pneumatic pump (Husky 307™, McMaster-Carr™) and PFA Teflon™ tubing deployed off of the port side of the ship. Water was pumped directly into an on-deck Class-100 clean room facility. The system was allowed to flush for 30-60 min prior to each water collection. Water collected for manipulation was pre-filtered in-line through acid-cleaned 210-µm screening (Nytex™) to remove large zooplankton, etc.

Measurements of nutrient concentration were made at the National Laboratory for Environmental Testing (Environment Canada) using standardized techniques (NLET, 1994). Sample pre-processing (filtration) was completed on the ship. Samples were stored frozen prior to analysis. Total chlorophyll-*a* content of seston sampled from depth and collected onto 0.2 µm pore-size filters was quantified (Turner designs TD-700 fluorometer) using the non-acidification protocol (Welschmeyer, 1994).

To test the *P. putida* FeLux reporter in the field, 1 mL aliquots from overnight cultures of the bioreporter were inoculated into test water samples. Two sets of field trials

were carried out (July 13, 2001 and July 17, 2001). In the first trial, triplicate acid-cleaned polycarbonate tubes were filled with lake water and DFB was added to give final concentrations of 0, 10, 20, 30, 40, and 50 nM. Samples were incubated at ambient temperatures in reduced light conditions. Sub-samples (1.5 mL) were taken every 2 hours throughout a 24 h period to measure light production. In the second experiment, the same process was repeated with the addition of a filtered (0.2 μm) treatment. Due to a limitation in the available number of clean sample tubes, concentrations of DFB were added at 0, 15, 30 and 45 nM in the filtered water treatment.

Measurements of dissolved (< 0.2 μm) and total Fe – Samples for dissolved Fe analysis were collected as described above. Lake water was filtered through acid-cleaned 0.2 μm pore-size polycarbonate filters (Millipore) in a Teflon filtration ring (Savillex, Minnetonka, MN), and frozen in Teflon bottles until analysis. Water was acidified with 0.1 mL of concentrated ultraclean nitric acid (Baseline/Seastar, Sidney, BC) per 1 L of lake water and Fe content was determined by direct injection using Zeeman-corrected transversely-heated graphite furnace atomic absorption spectrophotometery (Perkin Elmer AAnalyst-800) using L'Vov platform graphite tubes. A certified freshwater reference material (SLRS-4; National Research Council of Canada) was diluted to the expected range of Fe concentrations in the sample and analyzed with the samples. Particulate Fe (> 0.2 μm) content in the lake water was determined by collecting seston from 0.3 L of lake water onto 0.2 μm pore-size polycarbonate filters (Millipore). Filters were digested in 1 mL of concentrated nitric acid in 8 mL Teflon jars (Savillex). Fe content in filter digests was determined as above.

Effect of chelators on Fe assimilation in freshwaters – To determine the effect of chelators on Fe uptake by the planktonic community, ethylenediaminetetraacetic acid (EDTA) and DFB were titrated into water samples and ^{55}Fe assimilation rates determined. Water was collected during a July 2000 cruise using an acid-clean Teflon-coated Go-Flo bottle suspended on a Kevlar line and triggered by a Teflon messenger. For uptake experiments, 0.1 μCi of [^{55}Fe]-$FeCl_3$ was added to triplicate polycarbonate

bottles (500 mL) for each concentration of the two chelates (50, 100 and 200 nM) as well as to a set of control bottles. Samples were incubated in an on deck incubator with solar influence reduced to *ca.* 37% by neutral density screening. After a 48 h incubation period, 100 mL were removed from each bottle and filtered onto a 0.2 µm pore-size polycarbonate filter (47 mm diameter). Filters were rinsed with a Ti/citrate/EDTA rinse (Hudson and Morel, 1989) modified for use in freshwater by omitting the NaCl (C.G. Trick, *pers. comm.*). Particulate radioactive fraction collected on filters was measured by liquid scintillation with background radioactivity measured and subtracted from all sample counts.

3. Results

3.1. Laboratory characterization

Fe-independent expression of the lux gene – To confirm that the *lux* gene itself was expressed independently from the Fe bioavailability, bioluminescence was measured after addition of the siderophore DFB and Fe in the *E. coli* Lux recombinant, containing the *lux* gene cassette but not the *fepA-fes* promoter (Fig. II.2A). No significant variation (*t*-test, $p > 0.05$ [150 nM DFB – 10 nM Fe], $p > 0.05$ [150 nM DFB – 25 nM Fe]) of the light production normalized per OD_{600} was observed over the experimental range of relative Fe concentrations. The ratio of luminescence between the Fe-limited medium (150 nM DFB) and the Fe-replete medium (25 nM Fe) was 0.933. A decline of about 20% (from 3.84 ± 0.45 d^{-1} to 3.14 ± 0.55 d^{-1}) in growth rate was observed for *E.coli* Lux when shifted to Fe-deficient media (150 nM DFB). However, no significant decrease of the growth rate was observed between the unamended control medium and the deficient medium (*t*-test, $p > 0.05$). The constitutive and stable expression of luminescence under the range of relative Fe concentrations suggests that the *lux* genes themselves are not regulated by the level of bioavailable Fe in the culture medium.

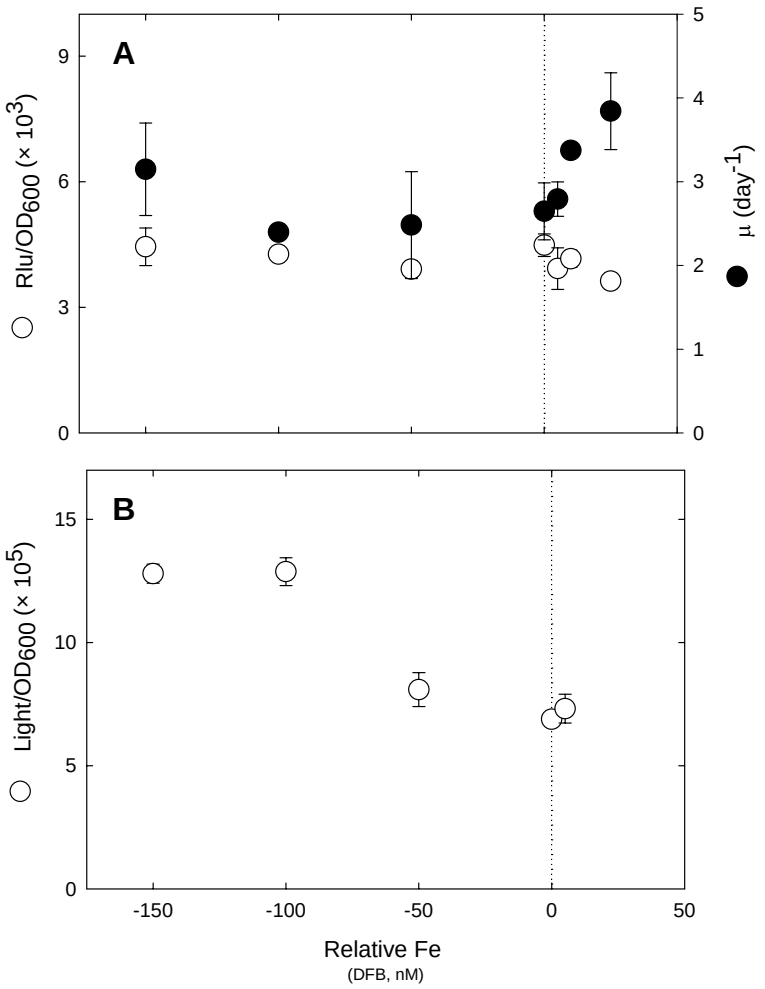


Fig. II.2. (A) Fe-independent expression of the *luxCDABE*. Cultures of *E. coli* Lux, containing the *lux* cassette, but not the *fepA–fes* promoter, were grown on LB medium supplemented with increasing amounts of DFB (50 – 150 nM, represented as ‘‘- relative Fe’’) or $FeCl_3$ (5 – 25 nM). The control medium (0 nM) was neither amended with DFB nor with Fe. The OD_{600} and bioluminescence (measured in RLU s^{-1}) were determined every 2 h over a 6 h experimental period. Growth rates (day^{-1}; ●) were calculated from linear regressions based of the OD_{600} data. The bioluminescence value correspond to the mean of light production normalized per OD_{600} at t = 6 h (○) (corresponding to the optimal activity). Error bars represent standard deviation of the means (n = 3). **(B) Induction of bioluminescence in the *E. coli* FeLux bioreporter upon DFB addition.** The bacterial culture was grown as indicated previously described for the bioreporter strain *E. coli* Lux. Bioluminescence values are means for the light production normalized per OD_{600}. Error bars represent standard deviation of the means (n = 3).

Effects of Fe-chelates on fepA-fes promoter activity in E. coli – Regulation of the promoter *fepA-fes* activity (demonstrated by *lux* gene expression) by bioavailable Fe was confirmed by repeating a similar experiment with the *E.coli* FeLux recombinant (Fig. II.2B). By contrast to the strain *E.coli* Lux, a significant variation of the activity of the bioreporter by the strain *E. coli* FeLux was observed (*t*-test [5 nM Fe – 150 nM DFB], $p < 0.001$). The bioluminescence expressed by *E. coli* FeLux was inversely related to Fe availability. Light production relative to the control increased dramatically with the concentration of DFB added (up to 1.87 fold between the control and 100 nM DFB), indicating that DFB chelated Fe in a form that was not available to *E. coli*. These data demonstrate that Fe availability regulates bioreporter expression.

Effects of Fe-chelates on fepA-fes promoter activity in P. putida FeLux – A similar set of experiments were repeated with the *P. putida* Fe sensor that we constructed for use in natural aquatic systems: *P. putida* FeLux. As with the *E. coli* FeLux, the lux expression of *P. putida* FeLux responded to shift in Fe availability in the culture medium (Fig. II.3). A significant variation in the activity of the bioreporter by the strain *P. putida* FeLux was observed (*t*-test [15 nM Fe - 100nM DFB], $p = 0.014$). Light production relative to the control increased markedly with the concentration of DFB added (up to 1.77 fold between the control and 100 nM DFB). Conversely, it decreased when Fe was added to the medium (~ 20% between control and 25 nM Fe). The increase of bioluminescence as a function of Fe availability follows a dose-dependent pattern as shown by the linear relationship with the relative Fe concentration between 100 nM DFB and 10 nM Fe ($R^2 = 0.93$):

$$y = -9270\,x + 9.53 \times 10^5$$

With y the normalized bioluminescence and x the relative Fe concentration. The slight decline in luminescence observed between 100 nM DFB and 150 nM DFB may be a consequence of a physiological decline of the bacteria under Fe starvation leading to a slowdown of the transcriptional activity of the promoter. Such a decline was not observed with the strain *E. coli* FeLux at similar concentration of DFB.

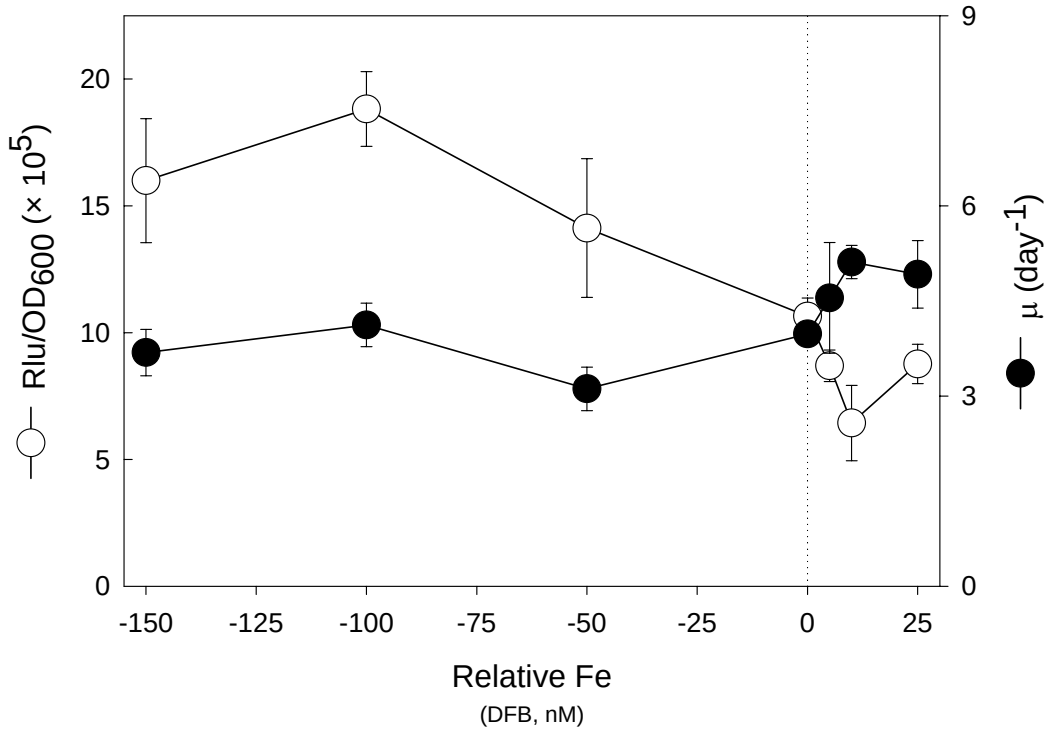


Fig. II.3. Induction of bioluminescence in the *P. putida* FeLux bioreporter upon DFB addition. The bacterial culture was grown as indicated previously described for the *E. coli* bioreporter strains. Bioluminescence values are means for the light production normalized per OD_{600} at t = 4 h (corresponding to the optimal activity). Error bars represent standard deviation to the means (n = 3).

The growth rate for the bioreporter in Fe-replete conditions (control, 3.98 ± 0.14 d^{-1}) is similar to previously reported growth rate for Fe-replete *Pseudomonas* sp. (3.59 ± 0.32 d^{-1}, Granger and Price, 1999). No significant change relative to the unamended control was observed in the growth rate upon titration of Fe by DFB (- 7%; *t*-test, $p > 0.05$) or upon enrichment with Fe (+ 25%, *t*-test, $p > 0.05$).

Influences of different chelators on Fe availability to the P. putida FeLux bioreporter – To assess the differential effects of Fe-binding ligands on the bioavailability of Fe, we challenged the bioreporter *P. putida* FeLux with various well - defined chelators (Fig. II.4). The addition of different ligands resulted in a variety of responses. The strongest bioluminescence was observed upon addition of DFB and ferrichrome. Relative light production increases linearly ($R^2 = 0.95$) between the 5 nM Fe and 150 nM of ferrichrome, with a similar slope to that calculated upon addition of DFB ($R^2 = 0.92$). The decline of light production at concentrations of ferrichrome higher than 150 nM is mostly likely due to a decrease in the metabolic activity of the cells upon Fe starvation. However, this decline is observed at higher concentrations (but similar Rlu) of ferrichrome (150 nM) relative to DFB (100 nM). Therefore, even though they possess identical Fe-affinity constants (Table 1.2), ferrichrome appears to not be as efficient as DFB in sequestering the initially bioavailable Fe from this strain. Unlike the DFB and ferrichrome treatments, the DP or RA treatments did not exhibit an increase of luminescence (Fig. II.4). Compared to the unamended control, the addition of DP or RA did not significantly influence light production (*t*-tests, $p > 0.05$). Within the range of our experimental concentrations, the synthetic chelator DP and the siderophore RA appear less efficient than DFB and ferrichrome at sequestering Fe from *P. putida* FeLux culture medium.

Transcriptional regulation of the FeLux reporter in different microbial hosts – Because the results described above suggested that heterologous chelators alter the availability of Fe to *P. putida*, further experiments were done to evaluate the impact of

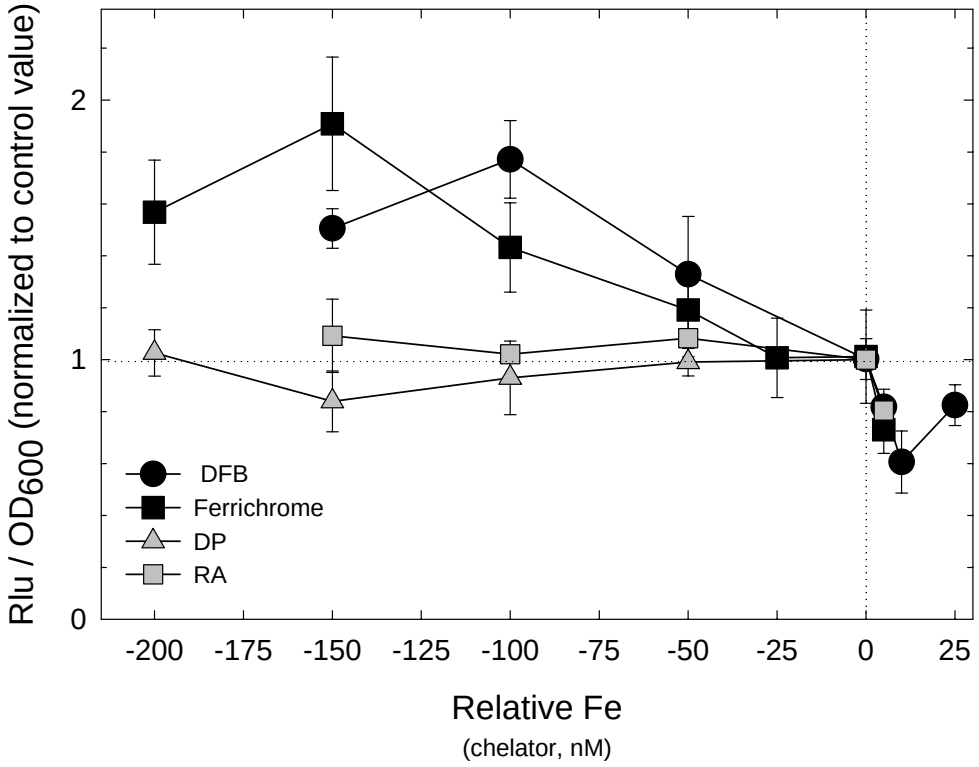


Fig. II.4. Bioluminescence induction in *P. putida* FeLux grown in LB medium supplemented with DFB (●), ferrichrome (■), DP (▲) and RA (■). OD_{600} and bioluminescence were determined for each triplicate over a 6-h experimental period as indicated previously. The data are plotted as the ratio of the light production at t = 4 h normalized per the correspondent OD_{600} (RLU/OD_{600}) between the treatment amended with 100 nM of chelator and the control (non-amended). Error bars represent standard deviation of the means (n = 3).

the same ligands on Fe nutrition of *E. coli* strains. Two *E. coli* bioreporters were used: *E. coli* pFeLux (parental strain), where the *fepA-fes:luxCDABE* construct is maintained as an extra-chromosomal element construct is maintained as an extra-chromosomal element (plasmid), and *E. coli* FeLux with the bioreporter chromosomally inserted. Data obtained for these two bioreporters (as well as the for *P. putida* strains) are compiled in Fig. II.5.

The addition of the heterologous siderophores yielded different responses dependent upon both the chelator tested and the bioreporter host strain. DFB addition resulted in the strongest increase in luminescence for each strain. In addition, the response of the three bioreporters to DFB addition compared to their respective control was about the same magnitude (~1.7 - 1.8). The expression of the bioreporter construct as a plasmid may explain the large variations observed for *E. coli* pFeLux relative to the strains with the construct in their chromosome, as excess multiple-copies of the promoter region within a cell may not be regulated by a limited abundance of Fe-FUR repressor. However, these data demonstrate that DFB-complexed Fe is not directly available to either *P. putida* or *E. coli*. As with *P. putida* FeLux, neither the addition of DP nor the addition of RA to *E. coli* pFeLux resulted in a significant variation in luminescence (relative to the unamended control). However, in contrast to *P. putida*, both *E. coli* strains did not exhibit any significant difference of luminescence between the treatment amended with 100 nM of ferrichrome and the unamended control (*t*-test, $p > 0.05$ for *E. coli* pFeLux, $p > 0.05$ for *E. coli* FeLux). These data suggest that ferrichrome can be used as an Fe source by *E. coli*.

3.2 Field data

Station description - As predicted for our July samplings, significant stratification of the water column (a thermocline at *ca.* 18 m where $Z_{max} = 26$ m) established conditions where Fe concentrations would be expected to be drawn down in the upper-mixed layer (Twiss et al., 2000).

Total dissolved phosphorus, 4.3 µg L^{-1} (0.14 µM), and total dissolved nitrate 202 µg L^{-1} (3.26 µM) concentrations in the upper mixed layer were accompanied by concentrations of chlorophyll *a* indicative of oligotrophic/mesotrophic conditions. During

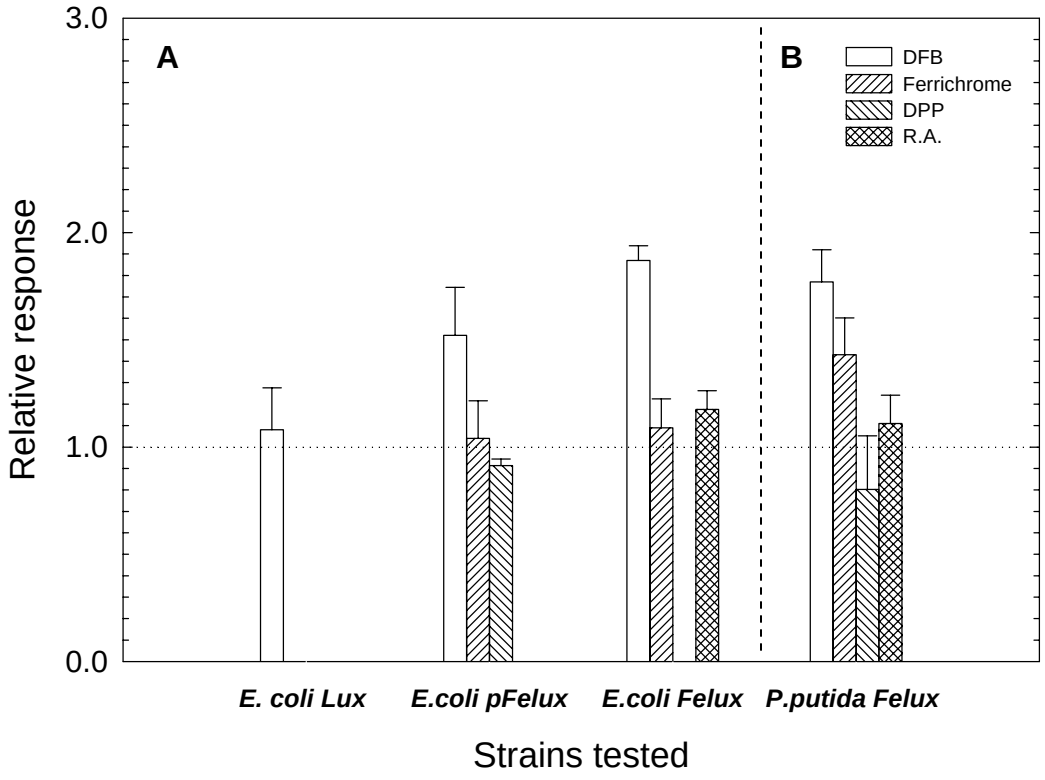


Fig. II.5. Intercomparison of the induction of bioluminescence among strains after the addition of 100 nM DFB, ferrichrome, DP or RA. Data are expressed as ratio of bioluminescence normalized per OD_{600} between the treatment amended with 100 nM of chelator and the unamended control (Light/OD_{600} [100 nM chelator] ÷ Light/OD_{600} [control]). Errors bars represent standard deviation of the means (n = 6) for each points. (A) Responses of *E. coli* control (*E. coli* Lux), *E. coli* parental strain (*E. coli* pFeLux) and *E. coli* bioreporter (*E. coli* FeLux) at t = 6 h. (B) Responses of *P. putida* bioreporter (*P. putida* FeLux) at t = 4 h.

experiments, the *in situ* chlorophyll-*a* ranged from a high of 1.95 (± 0.11) μg L^{-1} on July 12th down to 1.55 (± 0.04) μg L^{-1} on July 15th.

Dissolved Fe concentrations demonstrated a small range of variability over the study period in the surface waters (10 m) of the station 84 (Table II.3). Dissolved Fe ranged between 2.62 nM (July 18) and 6.77 nM (July 13) during the survey. However, dissolved Fe concentrations did not exceed 6 nM at the sampling sites for the assessment of the luminescent responses of the bioreporter on July 13 (3.98 ± 0.14 nM) and July 17 (3.21 ± 0.37 nM). Particulate Fe concentrations evaluated on July 18 (28.6 nM) were nearly an order of magnitude larger than the dissolved Fe concentrations.

Table II.3

Fe concentrations in Lake Erie, July 2001

Date, time	**Location and depth**	**Dissolved Fe** (nM)	**Particulate Fe** (nM)
July 13, 10:00*	St. 84, 10 m	3.98 (± 0.14)	ND
July 13, 16:00	St. 84, 10 m	3.26 (± 0.86)	ND
July 13, 16:30	St. 84, 10 m	6.77 (± 0.20)	ND
July 17, 10:00*	St. 84, 10 m	3.21 (± 0.37)	ND
July 18, 10:00	St. 84, 10 m	2.62 (± 0.65)	28.6 (± 3.20)
July 14, 10:00	Put-In-Bay (OH), surface	23.8 (± 0.49)	ND
SLRS-4 Standard (certified as 1844± 89)		1735	1941

Dissolved (< 0.2 μm) Fe concentrations (± S.D., *n* = 3) in Lake Erie were taken for bioreporter work (*) as well as other times during the cruise to demonstrate the consistency of Fe concentrations. Samples from a coastal harbor (Put-In-Bay, OH) are presented to contrast the neritic and pelagic environments. Particulate Fe samples were collected after the effects of filtration on bioavailability were noted (± S.D., *n* = 5). ND = no data.

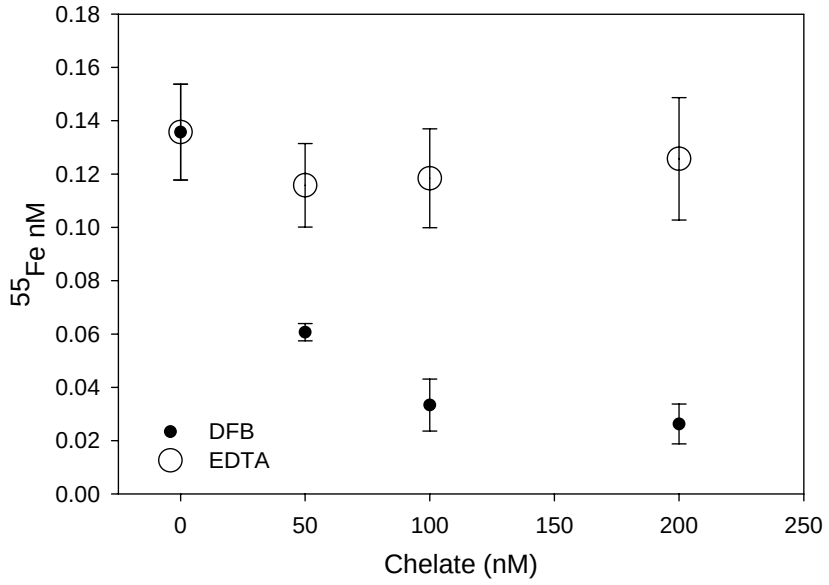


Fig. II.6. Effect of chelating agents on ^{55}Fe assimilation in freshwaters. Fe uptake into particles was estimated using the Ti/citrate/EDTA method as altered for use in freshwaters (see Methods and Materials). Results indicate that increasing concentrations of DFB inhibited Fe uptake by the biological community while the synthetic chelator EDTA had no effect.

Effects of DFB on Fe uptake in by the Lake Erie microbial community – To demonstrate the validity of using DFB as an Fe sequestering agent in this natural system, we have included information from a July 2000 cruise on Lake Erie where DFB as well as the synthetic chelator EDTA were added and Fe assimilation measured with the radiotracer ^{55}Fe. Addition of increasing concentrations of DFB resulted in a decrease in Fe uptake by the ambient community, indicating that DFB sequestered Fe in a form that was not available for uptake by biota in the sampled lake water (Fig. II.6). In contrast, EDTA, which has a lower affinity (and specificity) for Fe relative to DFB, did not affect Fe uptake by the community. These data support the laboratory observations of a differential efficiency among cationic chelators to sequester bioavailable Fe and demonstrate that Fe-DFB is not recognized as a Fe source by the biota sampled.

Assessment of Fe availability in pelagic Lake Erie – Addition of the fungal siderophore DFB into whole water (< 210 μm) from central basin of the Lake Erie induced a significant luminescent response at concentrations of DFB higher than 30 nM ($p > 0.05$, Fig. II.7A). Bulk light production increased by *ca.* 2-fold between 20 nM and 50 nM of added DFB. These data suggest that DFB efficiently decreases the bioavailability of Fe when added at concentration above 20 nM in whole water, subsequently leading to Fe stress in the microbial community. When a similar experiment was performed both in whole water and in filtered water (< 0.2 μm), the bulk increase in luminescence upon DFB addition was 2-fold higher in the filtered water than in whole water (Fig. II.7B). The onset of luminescence also occurred at lower concentrations of DFB in the filtered treatment, which suggests that a substantial fraction of the bioavailable Fe was removed by filtration. These results and those of the laboratory studies are considered below.

4. Discussion

Two significant conclusions are drawn from this study. The first is that genetically engineered microbial strains (bioreporters) can be used to rapidly (4 – 6 h) characterize the bioavailability of Fe under different conditions in both laboratory and field experiments, providing a necessary compliment to the analytical techniques that are currently in place for the characterization of Fe concentration and chemical speciation. This novel tool may allow for the determination of which factor (Fe or DOM) is the primary limiting resource of heterotrophic production in HNLC environments. Moreover, it may also provide insight on the influence of the chemical speciation of Fe on microbial community structure in a platform that is simpler to apply (the *lux* reporter system) than most other reporters (*e.g.* Loper and Lindow, 1994). The second conclusion from this study is that Fe bioavailability (at least in the central basin of Lake Erie) is strongly dependent on the presence of Fe in the particulate (> 0.2 μm) pool. Although others have suggested that the biota themselves may represent one of the most rapidly cycled pools of bioavailable Fe (*e.g.* Wells et al., 1995), we believe this represents some of the first data

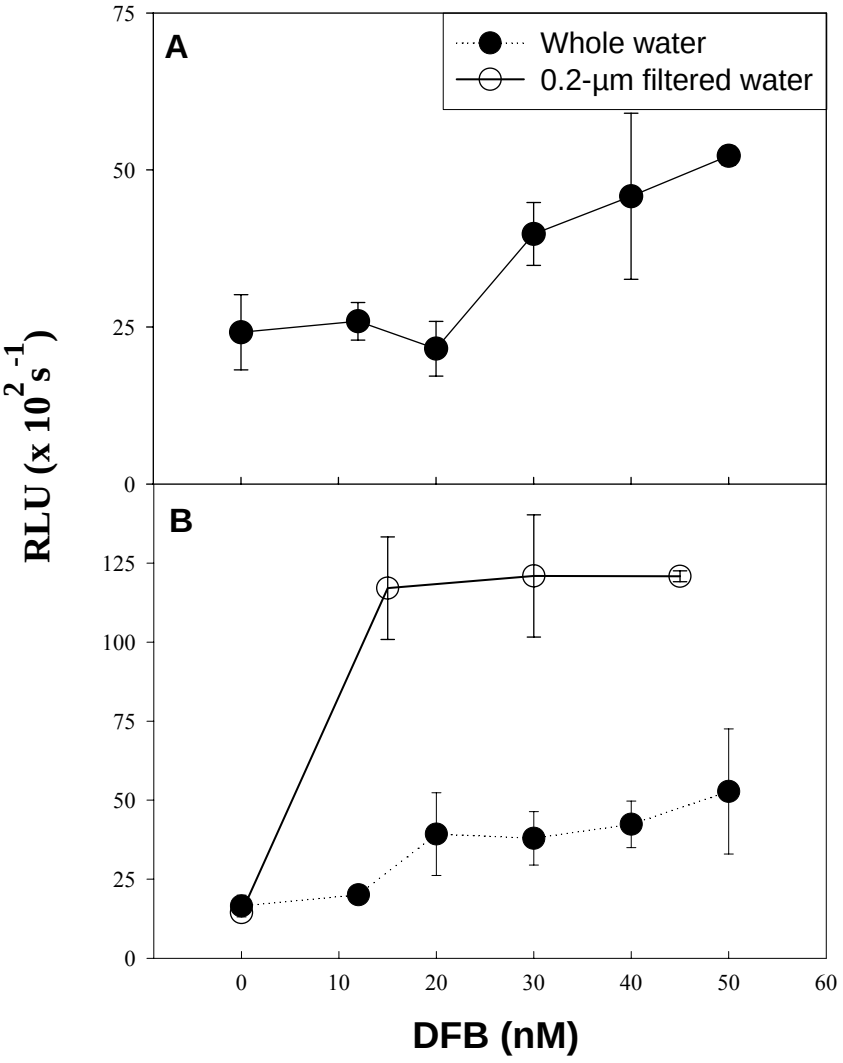


Fig. II.7. Response of the *P. putida* FeLux bioreporter to increasing concentrations of DFB in water from the central basin of Lake Erie. (A) Luminescence (RLU. s^{-1}) is shown versus the concentration of DFB added to whole water (●: < 210 µm) samples collected July 13, 2001 and amended with DFB and incubated at t = 6 h. Increased light production at concentrations >20 nM suggests that Fe stress occurred at DFB concentrations greater than this. (B) Luminescence (RLU. s^{-1}) is shown versus the concentration of DFB added to whole water (●: < 210 µm) and filtered water (○: < 0.2 µm) samples collected on July 17, 2001 and amended with DFB and at t = 6 h. The results clearly demonstrate that DFB additions to filtered water samples induce Fe deficiency faster than in whole lake water, implying a significant component of the bioavailable pool was removed by 0.2 µm filtration.

to truly addresses this hypothesis. These results are discussed below in addition to other perceived advantages (and limitations) of these bioreporters.

4.1 Characterization of Fe-L availability using bioreporters

Several studies in recent years have used xeno-siderophores to alter Fe assimilation by marine phyto- and bacterioplankton. As a result of these studies, contrasting information concerning the use of these chelates (particularly DFB) has arisen. DFB has been demonstrated to effectively reduce the available Fe to most members of the phytoplankton community (Wells et al., 1994; Wells, 1999; Hutchins et al., 1999a; Timmermans et al., 2001). And while some evidence suggests that phytoplankton communities in the sub-Arctic Pacific can access at least small amounts of DFB-bound Fe (Maldonado and Price, 1999), results from other studies suggest the flux of DFB-complexed Fe to the cell surface is probably not sufficient to satisfy cellular requirements (Hutchins et al., 1999b). Recently Martinez and colleagues (2001) have demonstrated the production of desferrioxamine G (a structural relative to DFB) by a marine *Vibrio* sp. isolated from a marine invertebrate larva. As such, they concluded that the production of this compound inferred its utility as a bioavailable Fe-chelate complex to the marine community. In the current study we have shown DFB reduces Fe availability to our reporter strains under both laboratory and field conditions. In both cases, the high-affinity Fe transport system (as denoted by the activity of the *fepA-fes* promoter) was derepressed upon nanomolar addition of the fungal siderophore DFB. In addition, DFB additions also reduced uptake of ^{55}Fe by the microbial community in pelagic Lake Erie. Thus, DFB sequesters a substantial amount of available Fe originally present in the environment. As a result, we are able to titrate the available Fe to a point where it is no longer bioavailable to our reporter strains.

As part of this work we have demonstrated the functionality of this mobile genetic construct in strains of both *E. coli* and *P. putida*, and that it can be used to characterize the bioavailability of various chelates to some microorganisms. A dose-dependent response of the bioreporter *P. putida* FeLux was observed during the laboratory assays upon enrichment in Fe and sequestration of Fe by addition of the siderophores DFB and

ferrichrome. This dose-dependent response was observed while the addition of chelators had no significant impact on the growth rate of the bioreporters. The minimal impact of Fe-stress on the growth rate of *P. putida* in this study is similar to data previously reported for the same species (Granger and Price, 1999). Therefore, the bioluminescence production by FeLux bioreporter may be sensitive to alterations in Fe bioavailability that do not immediately affect cellular growth rate over the experiment period but may affect heterotrophic metabolism. Additionally, the linearity of this dose–dependent response underlines the interest of using this type of bioreporter as a quantitative tool in aquatic systems as previously reported (Durham et al., 2002). The decline of the light, observed for the highest concentrations of siderophores tested on *P. putida* FeLux, suggests the effective range of use for this biological tool in characterizing Fe availability. Any physiological stress, such as deficiency in respiratory electron transport system ETS, can lead to a reduction in the metabolism of the bioreporters and subsequently reduced transcription (and therefore expression) of the *lux* reporter gene. Indeed, it has been estimated that heterotrophic metabolism, dependant on the maintenance of respiratory ETS, requires 60% more Fe per mol of cytochrome-*c* than does the photosynthetic ETS (Raven, 1988); subsequent studies have shown that respiratory ETS is a site primarily affected by Fe limitation (Tortell et al., 1996). However, the results of the field assays seem to indicate that the range of linear dose-dependant response is wide enough to use the *P. putida* FeLux in environmental samples.

The ability to cross-feed on exogenous siderophores is an important survival strategy for eubacteria in their natural environment. Previous studies have suggested that different strains of *P. putida* were able to use siderophores from other species (Loper and Henkels, 1999; Jurkevitch et al., 1992a, 1992b). In this study, the absence of luminescence increase following the addition of nanomolar concentrations of the weak chelator DP is consistent with the literature data: when used in a culture medium to titrate available Fe, DP is generally added at millimolar concentrations (Khang et al., 1997; Loper and Henkels, 1999). The experimental range of DP concentrations was probably too low to alter significantly the Fe availability to *P. putida* and, therefore to derepress the high-affinity transport systems. The absence of a response to the addition of RA in

this study may also indicate that this siderophore was not efficient at sequestering Fe away from *P. putida* FeLux. This may be due to a lower specificity and lower affinity of RA for Fe relative to DFB, or due to the ability of this strain to assimilate this xenosiderophore. Conversely, the dose-response observed after addition of ferrichrome suggests that this fungal siderophore is almost as efficient as DFB at sequestering Fe away from *P. putida* FeLux, leading to the derepression of the high-affinity transport systems. This finding seems surprising in consideration of previous work, demonstrating that a terrestrial *P. putida* was able to utilize Fe-bound ferrichrome via a Fe^{3+}-ferrichrome uptake system (Jurkevitch et al., 1992a, 1992b). However, the same authors observed that the rate of uptake of ^{55}Fe complexed to ferrichrome by *P. putida* St3 was very low compared to other siderophores such as pseudobactin (Jurkevitch et al., 1992a). It is therefore possible that the ability to use Fe – ferrichrome complexes varies among strains of the same species.

In contrast to the response of *P. putida* FeLux, no significant light was detected for both *E. coli* bioreporters after addition of ferrichrome. Like many bacteria, although *E. coli* does not produce ferrichrome, it does produce Fhu A receptor which will transport ferrichrome (Fecker and Braun, 1983). Moreover, while the expression of *fhu* A is also thought to be regulated by the FUR protein, leakiness in this regulation can lead to the expression of up to a hundred copies of the receptor on the surface of cells maintained in Fe-replete conditions (Hantke and Braun, 1998). This strain-dependent response to Fe-bioavailability highlights the importance of constructing genetically-mobile reporters so that the response of a variety of cells to Fe-speciation can be examined. In the case of our construct, we specifically chose a FUR-regulated promoter as the *fur* gene is well distributed throughout many prokaryotes and displays significant homology between several strains (Hantke and Braun, 1998; Hantke, 2001). As such it is predicted that this construct can be moved into other cultivable marine prokaryotes such as Vibrios and other Pseudomonads, thereby providing a broader selection of bioreporters to employ in attempting to model the bioavailability of Fe to native bacterial community.

4.2 Fe availability from the particulate size class.

Previous studies using DFB to reduce Fe assimilation have typically involved the addition of DFB (100 nM - Wells, 1999; 50 nM - Hutchins et al., 1999a; 100 nM - Hutchins et al., 1999b) well in excess (*ca.* 5 – 100 fold) of the total dissolved Fe concentrations. While our observations confirmed the ability of DFB to sequester Fe away from both our reporter strains and the natural community (*cf.* ^{55}Fe uptake data) in Lake Erie, we also noted that filtration removed a significant proportion of the bioavailable Fe. Assuming that dissolved Fe is not lost onto the filters (our protocols were essentially the same as those used in other Fe-clean studies) then this suggests that a significant proportion of the bioavailable Fe was associated with the particulate size class. Assuming that the availability to the reporter strains is an indication of the availability of Fe to the greater community and that there is a 1:1 relationship between the chelate:Fe complex and availability, then in the case of the whole water from the central basin of Lake Erie, somewhere between 20 and 30 nM Fe is biologically available to the planktonic community. The 1:1 relationship has been well established with respect to the Fe-binding kinetics of DFB, and this has further been shown to induce Fe-stress in a recently developed reporter for Fe bioavailability to freshwater cyanobacteria (Porta et al., 2003). This concentration of available Fe seems to closely match our estimates of total Fe (dissolved plus particulate) from our samples on July 18 (*ca.* 30 nM). As such, it supports previous suggestions that much of the Fe that is bioavailable in the system (either directly or indirectly) is associated with particulate (and most likely biological) material (Wells et al., 1995). Moreover, it stresses the need to examine the total Fe pool and not just the dissolved Fe pool as has been carried out in some studies of Fe bioavailability (*e.g.* Christian et al., 2002). As stressed elsewhere (Hutchins 1994; Wilhelm and Suttle, 2000), recycling of Fe by grazing and viral activity continuously supplies Fe back to the microbial community. Protozoan grazing in pelagic surface waters of Lake Erie actively recycles bioactive metals between particulate and the dissolved phase (Twiss et al., 1996) and viruses in this lake have been shown to be responsible for *ca*. 12 – 23 % of the microbial mortality (Wilhelm and Smith, 2000). As such, removal of biological particles from samples thereby reduces a significant source of

persistently remobilized Fe. Moreover, it has been demonstrated that organisms within the planktonic community possess mechanisms for accessing Fe associated with abiotic particles (*e.g*. Maranger et al., 1998). It is becoming increasingly clear from this and other studies (Hutchins et al., 1999b; Wells, 1999; Maldonado and Price, 1999) that the temporal component (*i.e.* the kinetics of interactions with biota) of a compound's bioavailability requires consideration equal to that given to its chemical structure.

5. Conclusion

As researchers and politicians continue to debate how Fe fertilization may allow for the use of the oceans as sinks for anthropogenically produced CO_2 (Chisholm et al., 2001, 2002; Neufeld et al., 2002; Johnson and Karl, 2002), it is surprising that we still have no clear concept of what represents the bioavailable component of the aquatic Fe pool. Obviously any understanding of the physiology and nutritional status of the different members of the planktonic community (including the heterotrophic bacterioplankton) remains limited by this lack of knowledge. As our knowledge of the identity of the "bioavailable pool" grows, we can hypothesize that this store of Fe represents a heterogeneous mixture of Fe-containing compounds and that the availability of these compounds is often on time scales outside (either too short or too long) our experiments. The tool described in the current text, when combined with the current arsenal of the modern day aquatic biogeochemistry, should allow for a better characterization and temporal scaling of the bioavailable Fe pools in different aquatic regions and lead to a better understanding of the true regulatory role Fe plays in these environments. Future studies, using different hosts as well as multiple ligands under different competitive regimes, should help us provide some clarity in these areas.

Acknowledgements

We thank Rick Bourbonniere, Melanie Eldridge, Shannon Pedigo, R. Michael McKay, Charles G. Trick and the Captain and crew of the CCGS Limnos for assistance. Leo Poorvin and Rachel Preis provided valuable comments on the manuscript. Supported by a UT-Center for Environmental Biotechnology Graduate Fellowship to C.E.M., a grant from the Natural Sciences and Engineering Research Council of Canada to M.R.T. and a National Sciences Foundation award (OCE-000298) to S.W.W.

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Part III

FIELD TEST OF A BIOLUMINESCENT REPORTER *P. PUTIDA* FELUX IN AN OCEANIC SYSTEM

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1. Introduction

The Pacific Ocean (from the Spanish, *Pacífico*, peaceful) is the oldest, the largest and the deepest of the five world's oceans. Its area exceeds that of all land surface of the planet, representing a third of the Earth's surface *ca.* 176.9 million km^2 (Chester, 2000). The Pacific Ocean is commonly divided geographically into five mains domains: the subarctic North Pacific (north to 40°N), the subtropical North Central Pacific (40°N-10°N), the Equatorial Pacific (10°N-10°S), the South Pacific (10°S-40°S) and the subantarctic Pacific (south to 40°S) (Fig III.1; deBaar and deJong, 2001; Gregg et al., 2003). However, the biogeochemical features of the Pacific basin vary greatly not only between these regions but also regionally within each of these domains. Over the past two decades, topical research cruises including international programs such as Tropical Atmosphere and Ocean (TAO) moorings or Joint Global Ocean Flux Study (JGOFS) and remote sensing monitoring (NASA ocean color mission) have contributed to improve our knowledge on the physical (*e.g.* light, temperature), chemical (*e.g.* nutrient availability) and biological (*e.g.* growth rate, zooplankton grazing) processes that drive the biological carbon pump in the Pacific Ocean (Le Borgne et al., 2002).

Initially, macronutrient (especially nitrate) bioavailability was used to classify geographic domains of the Pacific Ocean in term of primary production regions. Indeed, primary productivity, community size-structure and thereby the strength of the biological

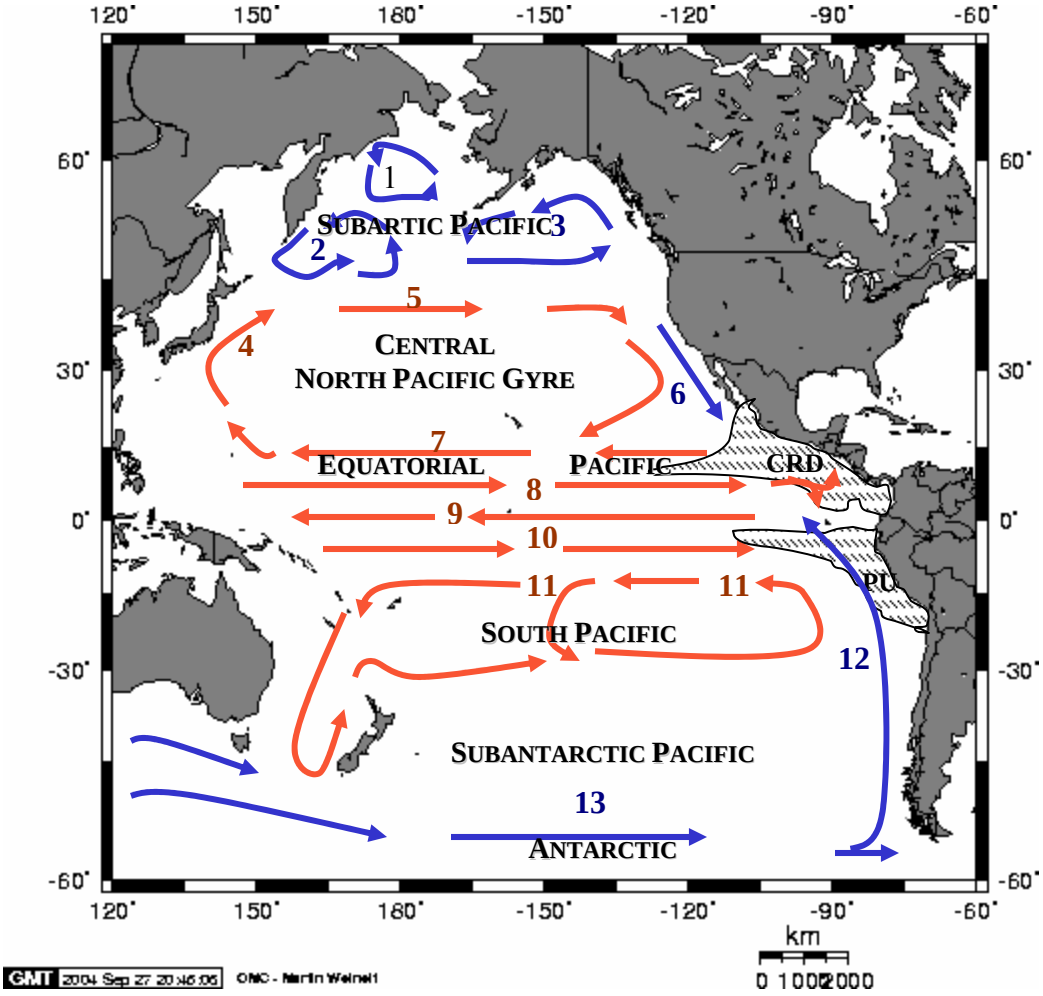


Fig. III.1. The main surface currents of the Pacific Ocean. 1: Bering Sea Gyre; 2:Western Gyre; 3: Gulf of Alaska gyre; 4: Kuroshio current; 5: Subarctic current; 6: California current; 7: North equatorial current (NEC); 8: North equatorial counter current (ECC); 9: Equatorial current; 10: South equatorial countercurrent (SEC); 11: South equatorial current; 12: Humboldt (or Peru) current; 13: Antarctic circumpolar current (west wind drift). The main coastal upwelling zones of the East Tropical Pacific (ETP) are shown: PU: Peruvian upwelling; CRD: Costa Rica Dome. The approximate oxygen minimum zone (< 1 µmol L^{-1}) of the East Tropical Pacific is shown in shaded region. (Adapted from de Baar and de Jong, 2001).

carbon pump are largely influenced by the availability of nutrients in the euphotic zone. On this basis, three main oceanic regions were identified (Dugdale and Wilkerson, 1992; Chester, 2000):

(i) Eutrophic or High-Nutrient High-Chlorophyll (HNHC) regions, where large concentrations of upwelled and riverine nutrients inputs (*ca.* NO_3 ~ 15-30 µM) sustain highly productive regimes. These HNHC conditions are found mainly in coastal upwelling regions which represent only 0.1% of the world's ocean surface, including the shelf off the western margin of South America (*e.g.* Peru and Costa Rica under certain conditions) and the western USA, but contribute for a significant fraction of the total global new production (*ca.* ~ 0.8 Pg C yr^{-1}; Chavez and Toggweiler, 1995). These areas are dominated by large-celled phytoplankton species with fast growth rate which may allow them to overcome zooplankton grazing (Minas and Minas, 1992) and specific nitrate uptake (V_{NO3} ~ 0.2 – 0.8 day^{-1}; Dugdale and Wilkerson, 1992) which indicates that there are performing close to their optimum.

(ii) Oligotrophic or Low-Nutrient, Low Chlorophyll (LNLC) regions, characterized by minimal nutrient supplies (*ca.* NO_3 < ~ 0.05 µM), and thereby low primary production (in term of "new" production), due to minimal water mixing or to a deep permanent thermocline and nutricline which prevents upwelling of the nutrient-rich underlying waters to the euphotic zone where the phytoplankton grow (Chester, 2000; Le Borgne et al., 2002). These oligotrophic regions coincide with the central Pacific ocean gyres spanning between *ca.* 10° and 40° north or south of the Equator, and the warm pool of the western Pacific. As a result, although these areas cover a surface that is ~ 1000 times greater than that of the HNHC regions, their contribution to the total global new production falls in the same range than these later (*ca.* ~ 0.5 Pg C yr^{-1}; Chavez and Toggweiler, 1995).

(iii) The High Nutrient, Low Chlorophyll (HNLC) areas which are characterized by macronutrient-rich (NO_3 ~ 15 – 30 µM; Chester, 2000) surface waters but chronically low chlorophyll standing stocks which are only twice as high as adjacent oligotrophic areas (*ca.* 0.3 – 0.4 µg L^{-1}; Whitfield, 2001). These HNLC conditions occur in 3 open-ocean areas of the Pacific basin: the subantarctic circumpolar waters in the Southern

Ocean, the eastern equatorial Pacific, and the Gulf of Alaska in the Subarctic Pacific. These three areas coincide with regional upwelling of nutrient-rich deep waters caused by divergence, and, also with strong seasonal deep mixing in the cases of both high latitude HNLC regions (Wells et al., 1995). These regions are generally dominated by small phytoplankton (picoplankton), including a significant portion of cyanobacteria (Whitfield, 2001; Boyd, 2002; Le Borgne, 2002). Phytoplankton assemblages generally exhibit slow specific uptake rates for nitrate ($V_{NO_3} < 0.05\ d^{-1}$; Dugdale and Wilkerson, 1992) which indicate that they are not performing at their optimum (Whitfield, 2001). Grow out experiments (deBaar et al., 1990; Martin and Gordon, 1988) and subsequently mesoscale fertilizations (Martin et al., 1994; Coale et al., 1996a; Boyd et al., 2000; Tsuda et al., 2003; Coale et al., 2004) performed by different groups have unequivocally demonstrated Fe is the primary limiting nutrient controlling primary production and community structure in these areas. Although studies initially focused on these three open-ocean resurgences, HNLC conditions are not constrained to offshore water. Indeed, on deck experiments have demonstrated that such HNLC conditions exist in coastal areas including both the Californian (Hutchins et al., 1998) and the South American (Hutchins et al. 2002; Frank et al., 2003; Eldridge et al., 2004) upwelling systems. Furthermore, other investigations suggest that Fe may also limit primary productivity in both Central North (DiTullio et al., 1993) and South (Behrenfeld and Kolber, 1999) Pacific gyres which were thought to be primarily limited by nitrogen.

Fe limitation is indeed widespread in the Pacific Ocean and it has been estimated that 40% of its surface waters display HNLC conditions (Whitfield, 2001). Concentrations of Fe in surface waters of the Pacific Ocean average ~ 0.26 nM, which is five times lower than the average concentration in Atlantic Ocean, *ca.* 1.3 nM (Donat and Bruland, 1995). However, Fe limitation is far from being homogeneous throughout the Pacific basin, and biogeochemical as well as physical forcings differ markedly from north to south but also from west to east. Due to its role in the global carbon budget, the equatorial region of the eastern Pacific is probably the most extensively documented. It has been estimated that the overall Equatorial Pacific contributes for most of the oceanic CO_2 emission to the atmosphere, outgassing 0.7 – 1.5 Pg C as CO_2 each year, most of

which originating in the HNLC areas (Le Borgne et al., 2002). The Eastern Tropical Pacific (ETP) is also under focus because this highly productive region supports one of the world's largest industrial fisheries (Daneri et al., 2000; Thorpe et al., 2000) and is periodically affeected by the El Niño-Southern Oscillation (ENSO) event which cause the breakdown of these fisheries (Lehody et al., 1997; Chavez et al., 1999).

Surface waters of the Eastern Pacific Ocean display significant variations in Fe concentrations both on a time- and spatial-scale. These features offer ideal conditions to study the variations of Fe bioavailability between water masses of distinct biogeochemical signatures supporting the growth of different planktonic assemblages. However, field studies reported to date for the eastern Pacific have only examined Fe biogeochemistry at a regional scale and were mainly localized in the known HNLC areas. We report here on the first field testing in oceanic system of the Fe-dependent bioreporter strain *Pseudomonas putida* FeLux (Mioni et al., 2003). Variations in Fe bioavailability were examined during the mature phase of the 2002/2003 El Niño event (October 2002) at the scale of the whole eastern Pacific basin, along a South-North northwest transect spanning between surface waters off coastal Chile (26.31°S, 75.03°W) and Mexico (17.51°S, 112.13°W). Simultaneous with monitoring Fe bioavailability, we manipulated Fe availability at each station *via* a series of Fe addition and removal experiments in size-fractioned seawater samples (*i.e.* in the presence of naturally occurring organic Fe-ligands complexes) by using the fungal siderophore desferrioxamine B (DFB). Results from this large scale comparative study enabled us not only to compare the levels of bioavailable Fe between various domains of the eastern Pacific basin but also to assess the degree of Fe limitation to the bacterial component. Furthermore, results of Fe manipulation experiments in presence (0.8 μm filtered water) and absence (0.2 μm filtered water) of the resident prokaryotic component enabled us to compare the degree of competition between members of the microbial components. To the best of our knowledge, this is the first report documenting the utilization of a Fe-dependent bacterial bioreporter in oceanic systems.

2. Oceanographic settings

To avoid scattering with too many references in the Discussion, we will first review the characteristics and physicochemical forcing of the region crossed by the Bride of Tabasco voyage.

2.1. ENSO phenomenon

El Niño event typically occurs near Christmas, hence the name *El Niño*, which means "Christ child" in Spanish (Philander, 1990). This phenomenon generally refers to anomalous warming of the sea surface temperatures (SST) in much of the tropical eastern and central equatorial Pacific (and eventually Chile) as the result of the eastward expansion of the warm pool (> 28° C), although it is now suggested that its impacts are much broader (Chavez et al., 1999; Glantz, 2001; McPhaden, 2004). During that period, the coastal upwellings off the western coast of South America are relaxed. El Niño events can last from 12 to 18 months and typically tend to recur every 3 to 7 years (Glantz, 2001; McPhaden, 2004). The counterpart to El Niño, La Niña, is generally characterized by cooler than normal sea surface temperatures across much of the equatorial eastern and central Pacific and an intensification of the coastal upwellings along the coast of southwestern America. The 2002/2003 El Niño event was of moderate strength (+1.8°C in average in November 2002) and reached its peak phase in October/December 2002 (Fig.III.2; McPhaden, 2004), which coincided with our study period. The largest SSTs anomalies were mainly concentrated in the Central Pacific (maxima > 2.5°C at 170°W) and affected the west coasts of America only weakly (McPhaden, 2004).

2.2. Southeast Pacific

During normal conditions in the southeast Pacific, the hydrography north of 45°S is under control of the Humboldt current, which originates from the Antarctic circumpolar water and transports the cold Fe-depleted, nutrient-rich subantarctic waters to the surface waters off southern Chilean coast (*ca.* ~ 40 – 45° S). From this point, the

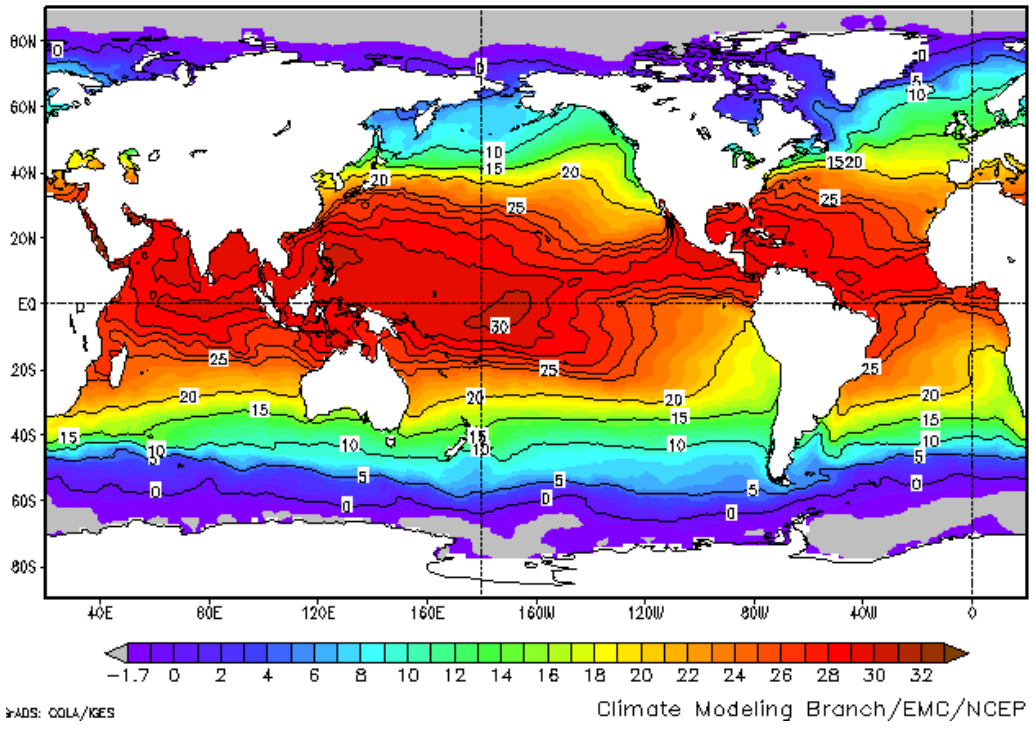


Fig. III.2. Monthly global sea surface temperatures for October 2002 (courtesy NOAA/olv2 satellite, ftp://ftpprd.ncep.noaa.gov/pub/cmb/sst/oimonth_v2/).

Humboldt Current runs up to northern Peru (Fig.III.1; Romero and Hebbeln, 2003) and is subsequently deviated to the west where it merges to the South equatorial current (SEC) (Hutchins et al., 2002). Although conditions appear favorable for intense primary production throughout the year in the surface waters along the whole Chilean coast (Thomas, 1999), there are strong regional variations (Daneri et al., 2000). The benthic diatom *Thalassiosira* spp. dominates south to 38°S and is associated with the cold, southern, non-upwelled Fe-depleted, nutrient-rich waters brought by the Humboldt current (Romero and Hebbeln, 2003). In northern and central Chile, intense blooms of long chain-forming diatoms (>20 μm), with predominance of *Chaetoceros* species, are mainly observed where nutrient-enrichment through coastal upwelling of nutrient-rich equatorial subsurface water occurs (*ca.* 34°- 38°S; Romero and Hebbeln, 2003; González et al., 2004). Five main upwelling cells are presently recorded in the northern and central coast off Chile: 23°S, 27°S, 30°S, 33°S and 35-38°S (Romero and Hebbeln, 2003). In contrast, primary production remains typically low between 22°S and 33°S off the northern Chilean coast (Daneri et al., 2000) and the planktonic assemblage is generally dominated by picoplanktonic species (Gonzalez et al., 2004). This low production area coincides with the narrowing of the continental shelf (*ca.* ~ 34°S). Nevertheless, local pulses of chlorophyll occur year-round at the Antofagasta upwelling (*ca.* 22-23°S; Marin and Olivares, 1999) as a result of localized increase in wind-driven coastal upwelling intensity and suffice to support short food chain dominated by small clupeiform fish species (González et al., 2004). Unfortunately, data presently available with regard to Fe concentrations are very scarce in this area. The only report available belong to Eldridge et al. (2004) who reported very low concentration of Fe (*ca.* 0.08 nM) during the 2000 La Niña episode in the northern part of the Chilean coast (Appendix III.1).

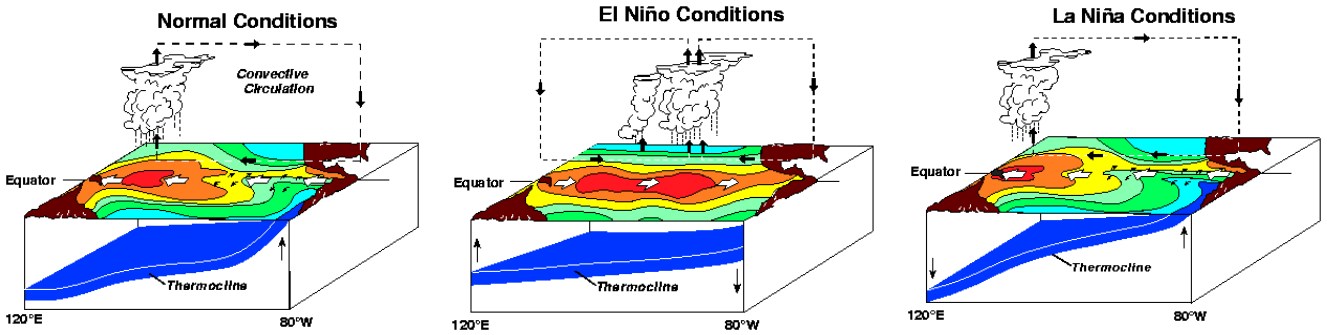
During an El Niño episode, the upwelling system off coast of northern Chile weakens and the thermocline flattens while the warm nutrient-poor equatorial waters move to the coast and the system switches to oligotrophic conditions with dominance of picoplanktonic species (Iriarte and González, 2004). However, the 2002/2003 El Niño did not appear to have affected the coastal upwelling system off coast of northwestern Chile and the planktonic assemblage in this area (*ca.* ~22°S) was dominated by large

microplanktonic species of chain-forming diatoms (*ca.* ~ 70% of the total phytoplankton abundance) (Iriarte and González, 2004). Therefore, the surface waters were probably Fe-replete.

2.3. Eastern tropical and equatorial Pacific

The eastern equatorial Pacific basin is an upwelling zone where the supply of major nutrients from underlying waters lead to intense biological production as well as a very distinct oxygen minimum zone (Fig. III.1; de Baar and de Jong, 2001). Two distinct zonal upwellings exist in the ETP: the countercurrent and the eastern equatorial ridges, which are associated with enhanced productivity (Fiedler and Filbrick, 1991). The eastern equatorial upwelling system is characterized by three upwellings cells: one at *ca.* ~ 5° N (Costa Rica dome/countercurrent) and two zonal cells located along *ca*. 0° and 10°N (Peruvian/Ecuador upwelling system) (Ichii et al., 2002). These coastal resurgences are adjacent to the oceanic equatorial pacific HNLC region located east to the Galapagos Islands. Under normal conditions, the Humboldt (or Peru) Current runs from the Peruvian coast to *ca.* 5°N and then flows westward to merge to the south equatorial current (SEC) as it moves toward the central equatorial Pacific (Fig. III.1; Ichii et al., 2002). The SEC flows westward across the Pacific basin, following the same direction than the trade winds. North of the upwelling system, the North equatorial Current also flows westward with the trade winds. The North Equatorial Countercurrent (NECC) flows eastward between the NEC and SEC where the trade winds weakens. The NECC is submitted to seasonal fluctuations and reaches a maximal strength in August-December, when the Intertropical convergence zone migrates northward in the eastern equatorial Pacific, and when the upwelling system is strong (Wyrtki, 1966). While progressing toward the America continent, the NECC splits: one part turns to the North to merge with the north equatorial current around a permanent structure located *ca.* 9°N, 89°W called the Costa Rica dome, the other part flows to the south and merge to the SEC (Ichii et al., 2002). Under such conditions, the upwelling off coast of Peru supplies macronutrients at a faster rate than Fe (Chavez et al., 1999) which results in a “mosaic” of Fe-repleted and HNLC

conditions (Fig. III.3; Hutchins et al., 2002; Frank et al., 2003; Eldridge et al., 2004). Fe concentrations reported for this area are effectively below the "threshold" concentration of Fe (< 0.5 nM, Martin, 1992) below which phytoplankton growth is severely limited (*ca.* 0.06 – 0.3nM, Appendix III.1). Furthermore, this Fe limitation appears to be not only spatial but also group-specific. Diatoms appear to be more severely Fe-limited than cyanobacteria while picoeukaryotes do not appear to be Fe-limited (Eldridge et al., 2004). Other studies suggest further that Fe availability controls the species composition within a given planktonic group, such as diatoms (Frank et al., 2003) or heterotrophic bacteria (Eldridge, 2004). The Costa Rican dome region appears even more severely limited than the Peru upwelling system in "normal" conditions (Frank et al., 2003). During El Niño events, the northeast trade winds weaken and the NECC intensifies out of phase with the SEC and transports warm oligotrophic waters to the ETP (Ichii et al., 2002). As a result, the NECC deepens the countercurrent thermocline and the SEC flattens the equatorial thermocline, and subsequently reduces the upwelling of nutrient rich waters along the Peruvian coast (Fig. III.3). As a consequence, this model predicts that the Fe input to the surface will decrease significantly, leading to low-nutrient, low-Fe conditions (Chavez et al., 1999). In such conditions, the productivity of the system is severely reduced (Lehody et al., 1997; Chavez et al., 1999). Owing to the fact that the SST anomalies were "*weak and short-lived*" in the eastern Pacific during the 2002/2003 El Niño event (McPhaden, 2004), such drastic conditions are not likely to have occurred during our study period. However, intermediate conditions between "normal" and typical "El Niño" may be expected. In contrast, in the Gulf of Papagayo, changes in the strength of trade winds due to a northward shift in the ITCZ result in an increase of precipitation over Costa Rica in May-October and the expansion of the cool water of the Costa Rican Dome (Ichii et al., 2002). Such changes appear to have occurred during October 2002 (McPhaden, 2004). In such conditions, integrated Chl*a* appears to increase in the water column of the Gulf of Papagayo and could explain the occurrence of apex predator (*e.g.* high Jumbo squid and yellowfin tuna) in this region during El Niño event (Ichii et al., 2002).



Physics	Upwelling-favorable trade winds; deep thermocline in west, shallow in east; cold tongue; strong undercurrent	Weak trade winds, reduced upwelling; enhanced rainfall; flat thermocline across basin, recovering in central and eastern Pacific before SST; no cold tongue; weak undercurrent.	Upwelling-favorable trade winds; shallow thermocline in central Pacific, enhanced cold tongue; strong undercurrent
Chemistry	Elevated macronutrients (NO_3, PO_4, $Si(OH)_4$, and CO_2); flux of CO_2 out of ocean; Fe limitation	No enhancement of macronutrients; oligotrophic conditions; flux of CO_2 close to zero, Fe limitation.	Elevated (higher than mean) macronutrients; flux of CO_2 out of ocean (stronger than mean); enhanced supply of Fe
Biology	Chlorophyll ~ 0.2 to 0.3 μg L^{-1} Picoplankton dominated ; Primary production (PP) ~ 75 mmol C m^{-2} day^{-1}; New production (NP) ~ 15% PP	Chlorophyll ~ 0.05 μg L^{-1} Picoplankton dominated ; PP ~ 35 mmol C m^{-2} day^{-1}; NP ~ 10% PP	Chlorophyll < 1 μg L^{-1} , higher in bloom; Diatoms important; PP ~ 80 mmol C m^{-2} day^{-1} reaching 160 in blooms; NP ~ 15% PP up to 50% in blooms.

Fig. III.3. Schematic of the relations between physical, chemical, and biological parameters in the East equatorial Pacific during normal, El Niño mature, and La Niña conditions. Parameters: ocean's temperature structure (color), surface winds (⇨), ocean upwelling (↑)/downwelling (↓), atmospheric convection (up and down black arrows and dashed cells) and cloud patterns (Taken from the TAO Project Office, NOAA/Pacific Marine Environmental Laboratory - http://www.pmel.noaa.gov/toga-tao/el-nino; comments taken from Chavez et al., 1999; their table 1)

2.4. Subtropical northeast Pacific

The northern part of the transect is under the influence of both the cold HNLC waters of the California Current, which flows southward along the California coast, and the lateral transport along isopycnal of Fe stored in the suboxic underlying waters of the equatorial Pacific (Fig. III.1; de Baar and de Jong, 2001). The lowest O_2 concentrations are found within a band spanning from 10° to 20°N (de Baar and de Jong, 2001). In October, however, the northwesterlies slacken, reducing the strength California Current. These "oceanic periods" are generally exacerbated by El Niño event. In this area, concentrations of Fe reported for offshore stations in "normal" conditions average ~ 0.4 nM and increase markedly with depth up to ~2 nM in the suboxic waters (Appendix III.1; Gordon et al., 1982; Landing and Bruland, 1987). This region is also characterized by horizontal gradient in Fe level, with ten times higher concentrations in coastal stations (1-5 nM within the 150 m surface layer) as compared to offshore stations (Landing and Bruland, 1987; Gordon et al., 1982). The last station of the transect was localized between these offshore and inshore stations. However, during the 2002 El Niño, this area was clearly impacted and bathed by unusually warm waters (Fig. III.2).

3. Material and methods

3.1. Study area

The Bride of TABASCO cruise (Bacterial Response to Irradiance Driven Energy – Translatitudinal Assessment of Biological Acclimation to Solar Conditions in the Ocean) occupied 14 stations from October 1 to October 17, 2002 along a South–North transect between 12°19′ S – 26°08′ N and 75°08′ W – 120°47′ W (Fig. III.4). The cruise track covered three sectors of the Eastern Pacific Ocean: the southeastern Pacific (Stations BT1 – BT3), the Equatorial Pacific (Stations BT4 – BT10) and the subtropical northeastern Pacific sectors (Stations B11 – BT13). Experimental locations comprised waters with different signatures which are known to be affected by El Niño.

Fig. III.4. The Eastern Pacific Ocean basin with sampling stations. Locations for dissolved Fe reports for the study area are identified. The annotated and fully referenced data set is available in Appendix III.1. References from which the data set was derived are: Eldridge et al. (2004), a; Franck et al. (2003), b; Landing and Bruland (1987), c; Gordon et al. (1982), d; Martin et al. (1994), e; Rue and Bruland (1997), f; Gordon et al. (1998), g; Martin and Gordon (1988), h. Coale et al., 1996b.

Table III.1

Sampling dates and local time, locations, and ambient parameters (T: surface temperature, S: salinity)

Station	Date	Time (am)	Location	T(°C)	S (‰)
BT01	01-Oct-02	05:53	26°3S, 75.0°W	15.24	34.41
BT02	02-Oct-02	05:34	21°5S, 77.0°W	17.00	35.00
BT03	03-Oct-02	05:35	17°0S, 79.0°W	17.28	35.16
BT04	04-Oct-02	05:29	12°2S, 81.4°W	17.99	35.28
BT05	05-Oct-02	05:27	08°2S, 83.5°W	19.80	35.25
BT06	06-Oct-02	05:29	04°2S, 85.0°W	20.39	35.17
BT07	07-Oct-02	05:34	00°0S, 86.0°W	24.06	34.08
BT08	09-Oct-02	05:30	05°4N, 92.4°W	27.42	33.52
BT09	10-Oct-02	05:30	08°0N, 96.0°W	28.19	33.28
BT10	11-Oct-02	05:30	10°2N, 99.4°W	28.58	33.33
BT11	12-Oct-02	05:30	12°4N, 103.1°W	28.85	33.53
BT12	13-Oct-02	05:30	15°1N, 105.5°W	29.12	33.98
BT13	14-Oct-02	07:15	17°5N, 111.1°W	28.69	34.40

*outlier, the fluorescence was 0.397 at the same station 13h later.

3.2. Sample collection

For all stations surface seawaters were collected before sunrise at ~ 6 a.m. (local time). Hydrographic data were collected using the ship's Seabird CTD. The characteristics of each station are summarized in Table III.1. Water samples were collected at 5 m depth with Niskin bottles mounted on the ships' CTD rosette for chlorophyll *a* (Chl *a*) measurements. Total chl *a* were determined from duplicate 50 mL samples collected on 0.2-µm pore-size polycarbonate filters (Osmonics) after extraction (~ 24 h) in 90% acetone. Chl *a* was quantified with a Turner Designs TD-200 fluorometer using the non-acidification protocol of Welschmeyer (1994). For bioreporter and Fe analyses, seawater samples were cleanly collected using 3 independent Go-Flo casts (10 L) at 10 m depth and subsequently homogenized in 20 L acid-cleaned polycarbonate carboys. A subsample (~ 200 mL) of unfiltered water was retained for measurement of total Fe concentrations and was stored frozen in acid-washed Teflon flask for future

analysis. Subsamples (~ 1 L) for the bioreporter assays were filtered with acid-washed (0.8 μm or 0.2 μm) polycarbonate filters and were processed immediately. All manipulations were performed in a Class-100 clean area to reduce potential Fe contamination.

3.3. Shipboard assessment of Fe availability using P. putida FeLux bioreporter

Culture conditions – P. putida pFeLux stock culture was maintained in Pseudomonas Isolation Agar (Remel) supplemented with 50 μM μL^{-1} of tetracycline (Tc). Prior to experiment, several colonies were screened in sterile and 0.2 μm filtered marine broth (Difco, marine broth 2216). The colony presenting repetitively the largest variations in light production relative to the control (no addition) after addition of desferrioxamine B (DFB) and $FeCl_3$ (1.5 nM) was selected for the field experiments. Cultures from the selected colony were maintained daily by transferring 1 mL of overnight grown broth culture into sterile 15-mL polycarbonate tubes filled with ~ 6 mL of sterile and 0.2 μm filtered marine broth 2216 (Difco) supplemented with 50 μM μL^{-1} of Tc. Maintenance cultures were grown in the dark in a temperature-controlled incubator (25°C) on an orbital shaker (2,200 rpm). Consistent with the results from preliminary growth tests, the following amounts of Chelex-100 treated and filter-sterilized nutrient stocks were added to the seawater in order to make sure that the level all nutrients but Fe were sufficient to support the growth of the bioreporters: NH_4NO_3, 20 μM; NaH_2PO_4, 10 μM; Glycerol, 1 μM and vitamin mix (ESAW recipe, Berges et al., 2001), 100 μL. The day prior to each station, 5-mL of overnight culture was transferred for acclimation to an acid-washed and microwave-sterilized 500 mL polycarbonate Erlenmeyer filled with 95 mL of filtered sterilized (0.2 μm) enriched seawater collected off coast of Chile. The resulting batch culture was subsequently incubated overnight at 25°C with shaking.

Manipulating bioavailable Fe – For the first set of experiments, seawater (~ 2 L) collected with Go-Flo bottles was supplemented as indicated above. This enriched seawater was then trace metal clean filtered (with either a 0.2 or a 0.8 μm polycarbonate filter). Acid-washed and microwave-sterilized Oakridge tubes were filled with 18 mL

aliquots of filtered seawater. Fe availability was manipulated by supplementing the seawater aliquots with either increasing concentrations of DFB or ferrichrome (up to 10nM) or $FeCl_3$ (up to 1.5nM) as described previously (Eldridge et al., 2004). The control treatment (0 nM) was neither amended with DFB nor Fe and is therefore representative of the level of bioavailable Fe in initial seawater sample. The experiment was initiated by adding 2 mL of the overnight-acclimated *P. putida* FeLux culture to each replicate. Each treatment was repeated in triplicate. Light production was measured at 2, 4 and 6 h using a luminometer (Zylux FB14). In parallel, at t = 4 h and t = 6 h, 950 µL of each treatment was fixed with 50 µL of glutharaldehyde (2.5 % v/v) and kept refrigerated until enumeration in the lab using epifluorescence microscopy upon treatment with acridine orange (Hobbie, 1977). Light production was normalized to reporter cells mL^{-1}. Reported values are the means of three replicate cultures for the time-point for which the signal was the strongest (t = 4 h).

Dilution experiments – To assess the level of bioavailable Fe in the seawater, a second set of experiment was performed at the station 12 (15°05N, 106°53W). Filtered (0.2-µm) and enriched (C, N, P, vitamins + 1.5 nM $FeCl_3$) surface seawater sample was serially diluted (100 %, 75 %, 50 % and 25 %) with a chelex-100 treated defined seawater medium modified from the ESAW medium recipe (Berges et al., 2001) to achieve a final volume of 18mL per replicate. Salts and macronutrients solutions of the ESAW medium were supplemented with glycerol (1 µM) to provide a carbon source for heterotrophic bacteria growth and were subsequently treated with Chelex-100 resin. After microwave sterilization, the synthetic seawater medium was amended with $FeCl_3$ (1.5 nM), and 50% of the vitamins (50 µL L^{-1}) and trace metal mix (500 µL L^{-1}) in the published ESAW recipe. To avoid competition with natural ligands presents in seawater EDTA was omitted. Two other treatments were tested by adding 5 nM DFB to undiluted (100 %) and the highly diluted (25 %) seawater. The assay was initiated by inoculating 2 mL of overnight-acclimated bioreporter culture in each replicate. Each treatment was performed in triplicate. Data were collected as described above.

3.4. Statistical analyses

Statistical analyses for all the data presented here were performed using SPSS (ver. 12) software. Analyses of variance and independent *t*-tests (two-tailed) were performed assuming equal variance on mean values. The homogeneity of variance was tested in each analysis using the Levene test. For incubation experiments, analysis of variance (one-way *ANOVA*) was used to establish the statistical significances of variation among different treatments. In parallel, multiple comparison tests were performed to determine where the differences lied. Dunnett's test was used to analyze the significance of the variations of the means of a set of amended treatments relative to the control treatment mean (Corston and Colman, 2003). The Tukey's honesty significant difference test (*Tukey-HSD* test) was used to establish the statistical significance of variations among a set of treatments means (Corston and Colman, 2003). For all analyses, a 95 % confidence interval (α = 0.05) was used. Unless stated, results were considered significant at $p < 0.05$.

4. Results

4.1. Station description

Surface water temperatures increase along the transect, from *ca.* 15 °C at the southern station (BT01, 5 am) to *ca.* 29 °C at station BT12 (Table III.1, Fig. III.5A). This increase was not linear and several irregularities can be observed. At *ca.* 21°S, surface temperatures stabilized at ~ 17.2°C, before to raise again north to 14.5°S. This local pool of cooler water coincides with the highest salinities (~ 35.15 ‰) observed in surface (Table III.1; Fig. III.5B) and may reflect the coastal upwelling cell located at *ca.* 22 – 23°S. Temperatures off the Chilean-Peruvian border (*ca.* 15° S - equator) were similar to that previously reported for this region (Eldridge et al., 2004). The other inflection was observed south to the equator (~ 1°S) above which temperatures increased dramatically up to ~10°N (~ 29°C). This increase of temperature is associated to a marked decrease in

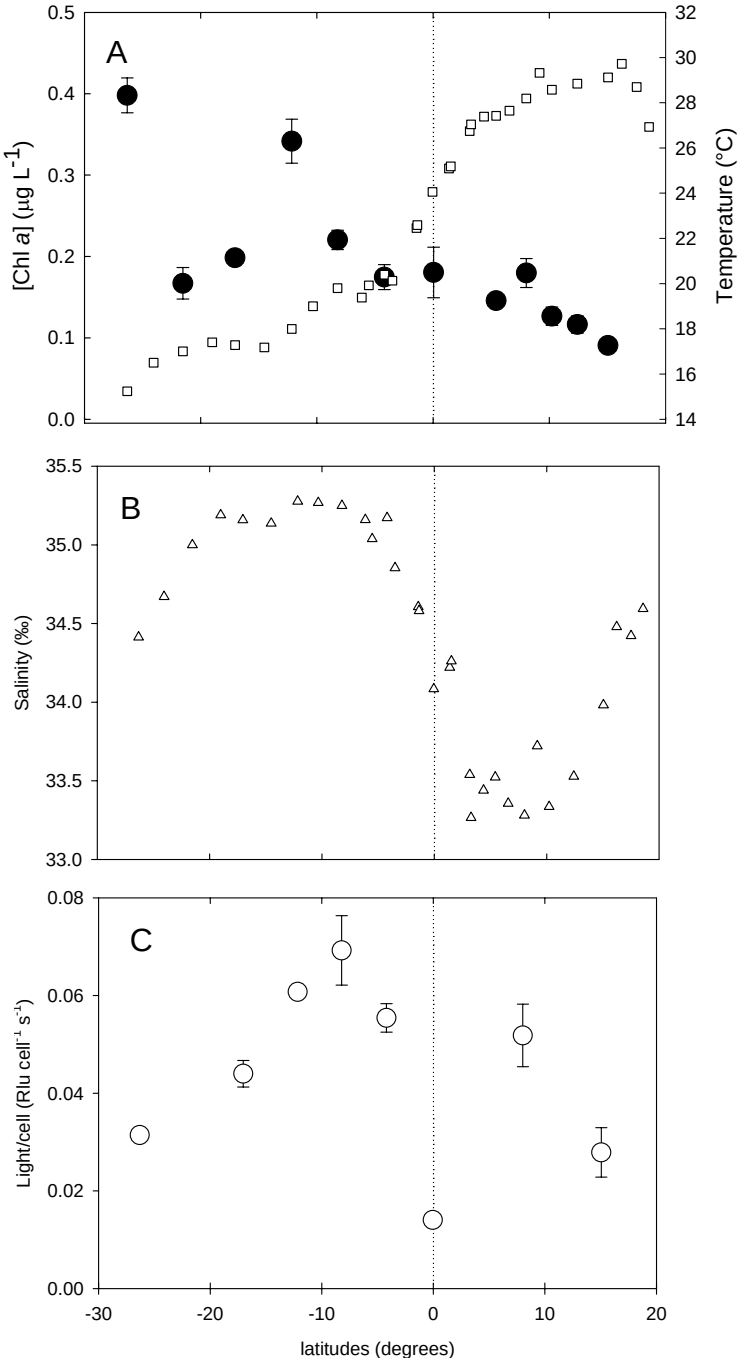


Fig. III.5. Evolution of biophysical parameters along the Bride of Tabasco transect. A: Chl*a* (●, courtesy Amy Baldwin and Wade Jeffrey) and SST (□); B: salinity; C: normalized bioluminescence in non amended 0.2 µm-filtered seawater samples. By convention, latitudes south of the equator are preceded of the sign "-" in the chart. The dashed line represents the equator.

salinity (~ 33.3 ‰, BT09). Above 10° N, temperature increased less dramatically (29.7°C at *ca.* 16°N).

Chl *a* concentrations were extremely low (< 0.5 µg L^{-1}) over the study area characteristic of oligotrophic system as expected during El Niño (Fig. III.5A).Two southern stations, surrounding a known local upwelling cell (*ca.* 22 – 23°S) off coast of Chile displayed slightly higher concentrations: BT01 (26 °S, 0.39 µg L^{-1}) and BT04 (14.5°S, 0.34 µg L^{-1}). Beside these two stations, the chlorophyll concentrations did not vary significantly (*ANOVA*, $p > 0.05$; mean ~ 0.19 µg L^{-1}) south of the equator (BT07). North of the equator, the chlorophyll level decreased from 0.18 µg L^{-1} (BT07, equator) to 0.075 µg L^{-1} (BT013, 15.1°N). This decrease coincided with changes observed in temperature and salinities. The evolution of these biophysical parameters suggests that the transect crossed several water masses and that stations north to the equator were affected by the 2002 El Niño episode. However, the oligotrophic waters of the warm pool did not appear to have reached the southernmost part of the transect (*i.e.* Chilean coast). These observations are consistent with previous reports (Fig. III.3; McPhaden, 2004).

4.2. Monitoring of bioavailable Fe levels using P. putida FeLux bioreporter

Light production by the bioreporter in response to Fe-stress increased by a factor 2 in the southern part of the transect, from the coast off Chile (station BT01; 0.031 ± 0.001 Rlu $cell^{-1}$ s^{-1}) to the central coast off Peru (BT04, 12.2°S, 0.061 ± 0.01 Rlu $cell^{-1}$ s^{-1}; Fig. III.5C). This increase in light production was significant (*ANOVA*, $p = 0.001$) and appears linear, suggesting that the bioavailability of Fe decreased northward gradually. Although light production appears to have increased at the station BT05 (North Peru, 0.069 ± 0.007 Rlu $cell^{-1}$ s^{-1}) as compared to station BT04, the difference between the means of the two stations was not significant (*t-test*, $p > 0.05$) due to the large standard deviation between triplicates for the station BT05. Except for the equatorial station (BT07), light production plateau to a maximal value of *ca.* 0.06 Rlu $cell^{-1}$ s^{-1} for the ETP stations located between central coast off Peru (BT05) and the Costa Rica Dome (BT09, 8.0°N), as no significant

variations were found between the means of these stations (*ANOVA, $p > 0.05$*). This result suggests that Fe bioavailability was the lowest in this region. Interestingly, a sharp and significant decrease (*ANOVA, $p < 0.001$*) in light production was observed within this zone at the equatorial station, displaying the minimal light production of the transect (*ca.* 0.014 ± 0.001 Rlu cell^{-1} s^{-1}). This low light production may reflect a local increase in Fe bioavailability, or may reflect a severe Fe-stress of the bioreporter cells resulting in a decrease in cellular energy which is necessary to sustain the luciferase reaction. Light production decreased significantly (*ANOVA, $p < 0.001$*) between the ETP stations and the northernmost station investigated (BT12; 0.028 ± 0.005 Rlu cell^{-1} s^{-1}) to reach a level similar but significantly lower (*t*-test, $p = 0.041$) to that observed at the southernmost station. This result suggests that Fe was more bioavailable north to the ETP system, in the region located between warm pool and waters affected by El Niño in less extent.

4.3. Response of P. putida FeLux to manipulations of bioavailable Fe

To gain more insight with regard to the degree of Fe bioavailability, we carried out Fe "removal" experiments by adding increasing concentrations of terrestrial siderophores that are not available to *P. putida* FeLux (DFB or Ferrichrome) to 0.2 µm or 0.8 µm filtered seawater samples. Simultaneously, we conducted Fe enrichment experiments by supplementing the samples with inorganic Fe. The rationale for these 0.2 – 0.8 µm pore sizes was to separate the dissolved Fe fraction (< 0.2 µm) from the particulate (> 0.2 µm), and therefore from the resident prokaryotic component (which predominates in the 0.2 – 1 µm size fraction) based on the size-fraction classification of Bruland and Rue (2001; Appendix III.2).

Response of the *P. putida* FeLux bioreporter to the manipulation of Fe bioavailability in 0.2 µm filtered seawater (dissolved fraction) at each station investigated is shown in Figure III.6. Two main groups of stations can be differentiated:

(i) the stations in which addition of siderophore (*i.e.* DFB and/or ferrichrome) resulted in an increase in light production, suggesting that this siderophore decreased

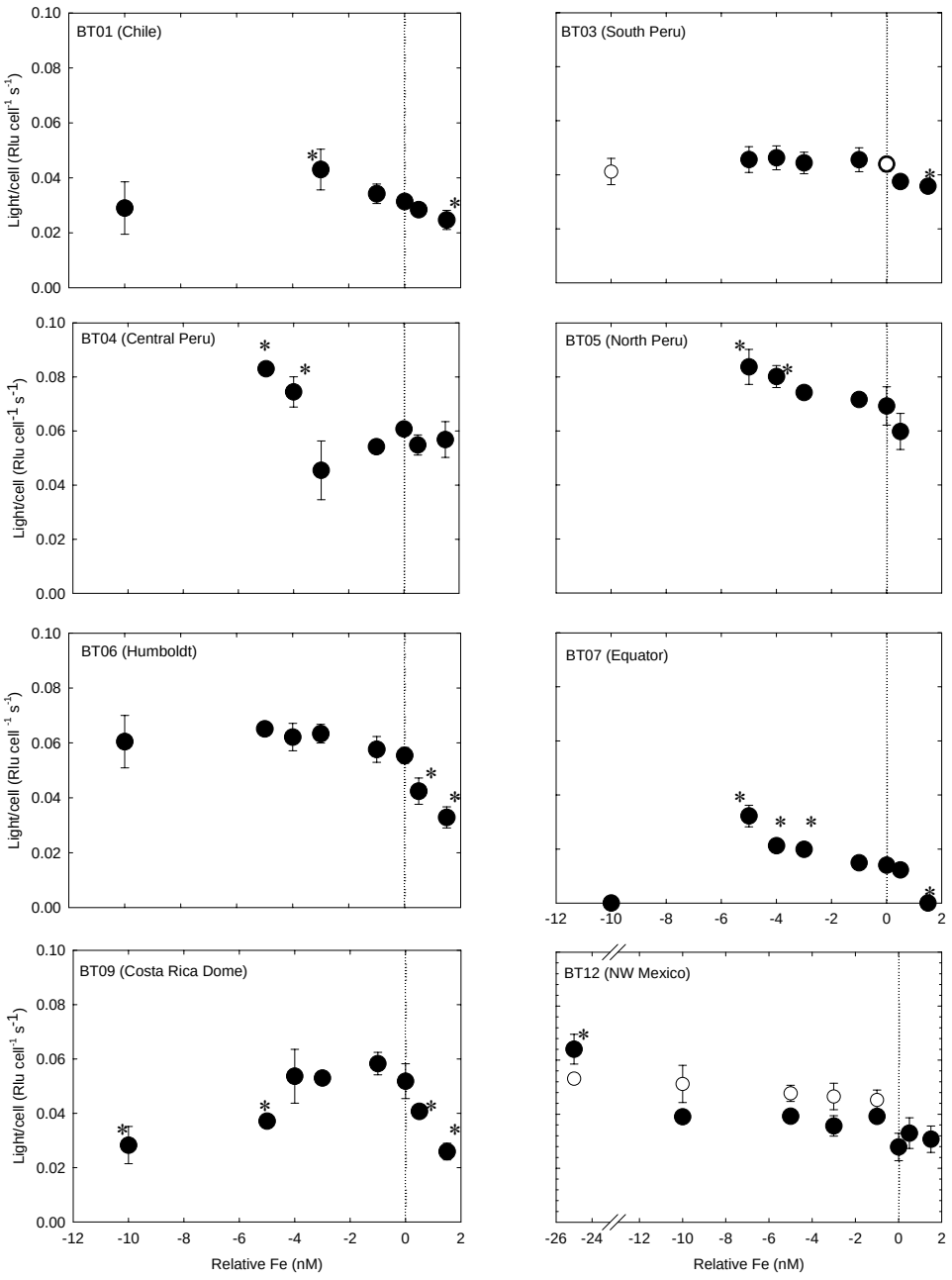


Fig. III.6. Response of the *P. putida* FeLux bioreporter to Fe manipulation along a S-NNW field track in East Pacific. Normalized luminescence is shown versus the concentration of relative Fe for 0.2 μm filtered samples amended with DFB (●), Ferrichrome(○) or $FeCl_3$. Increased light production after DFB addition suggests that this siderophore decreased the level of available Fe originally present in seawater. Addition of DFB in other stations did not result in an increase of luminescence possibly because the level of available Fe remained high despite addition of DFB or because stronger ligands than DFB (Log K_{FeL} > 21.6) were present in the water. Sample significantly different from the control are denoted by an asterisk.

the level of available Fe originally present in seawater (BT01, BT04, BT05, BT07, and BT12),

(ii) the stations in which addition did not result in an increase of luminescence, possibly because the level of available Fe remained high despite addition of DFB or because the bioreporter cells could not be pushed into further physiological stress or also because stronger ligands than DFB (Log K_{FeL} > 21.6) were present in the water (BT03, BT06, and BT09).

At the station BT01 light production increased linearly between the treatment amended with 1.5 nM of Fe and the treatment supplemented with 3 nM of DFB (R^2= 0.9998; *slope*: -0.0040). Light production in +3 nM DFB (*Dunnett's* test, p = 0.022) and + 1.5 nM Fe (*Dunnett's* test, p = 0.031) treatments was significantly higher and lower respectively as compared to that measured in the non-amended treatment. Light production appeared to decrease upon addition of 10 nM DFB but this decrease was not significant relative to the +3 nM DFB treatment (*t-test*, p > 0.05), suggesting that DFB additions greater than 3 nM were sufficient to remove most of the bioavailable Fe in the bioreporter cell environment. Above this threshold concentration, bioreporters cells may have been too severely starved to drive efficiently the luciferase reaction and subsequently generate higher amount of light. This threshold concentration falls in the range of concentrations reported for naturally occurring organic Fe-ligand complexes in seawater (*ca.* ~ 1.5 – 6 nM; Rue and Bruland, 1995, 1997; Witter et al., 2000). Taken together these results suggest that Fe bioavailability was high off coast of Chile and that the Fe species present in the dissolved fraction were at least partially available to the bioreporter cells. Moreover, the linear response observed upon DFB addition suggests that this siderophore removed the bioavailable Fe originally present in seawater in a "stoichiometric" manner. This observation further suggests that initially bioavailable dissolved Fe may have been bound to organic ligands with stability constant lower than that of DFB (Log K_{FeL} ~ 21.6; Appendix I).

By contrast to station BT01, the increase in the response signal upon DFB addition was not linear at the stations BT04, BT05, BT07 and BT12. Instead, the luminescent signals significantly increased upon addition of more than 3 nM DFB (Table III.2).

Table III.2

Statistical analyses for the Stations BT04, 05, 07 and 12. Threshold concentration of DFB ([DFB]) at which the luminescent signal increased significantly as compared to the control, *p*-value obtained for light production in control vs. threshold [DFB] treatment, and % of increase in light production relative to control.

Station	**Location**	**Threshold [DFB]**	***p*-value**	**Luminescence increase** (%)
BT04	Central Peru	4 nM	0.001	18.4
BT05	North Peru	4 nM	0.009	13.6
BT07	Equator	3 nM	0.005	29.6
BT12	NW Mexico	25 nM	< 0.001	56.4

As well, addition of Fe at nanomolar levels did not result in any significant decrease in bioluminescent signal at all these stations (with the exception of station BT07). This result suggests that the bioreporter cells did not perceive an increase of Fe bioavailability in the Fe-amended treatments. For the station BT12, by contrast to the DFB treatments, amendment of the seawater samples with Ferrichrome did not result in a significant alteration of the light production within the range of concentration tested (*Dunnett's* test, $p > 0.05$). This may indicate that the ligands present in the seawater sample had a higher stability constant than that of this terrestrial siderophore, therefore available Fe was not efficiently "removed". The proton independent binding constant for ferrichrome (*ca.* Log K = 29.1) has effectively been estimated to be 1 order of magnitude lower than that of DFB (*ca.* Log K = 30.99) (Appendix I). Taken together, these observations suggest that Fe originally present in the surface seawaters of these stations was bound to strong organic ligands (although with higher affinity for Fe than DFB: Log $K_{FeL\ (CLE/ASV)} > 21.6$) which may have been present in excess as compared to the amount of Fe added (*i.e.* $[L]_T > 1.5$nM), and that at least a fraction of the Fe-ligands complexes were bioavailable to the bioreporter cells.

By contrast to all the other stations, the bioluminescent signal did not increase significantly in response to the addition of DFB at the stations BT03, BT06, and BT09. However, a significant decrease in light production was observed upon addition of

inorganic Fe. When more than 5 nM of DFB were added to the sample, light production remained stable (BT06) or significantly decrease as compare to the control (BT09). At the station BT03, addition of Ferrichrome led to similar result than addition of DFB: no significant increase ($p > 0.05$) in luminescent response was observed over the range of concentrations tested. These results suggest that the bioavailable Fe originally present in the seawater sample may have been bound to organic ligands which were available to the bioreporter and presented higher stability constant than DFB (and thus, ferrichrome). At the station 9, the concentration of ambient bioavailable Fe may have been lower than in the 3 other locations, owing to the dramatical decrease in light production upon addition of both high (> 4 nM) DFB (*Dunnett's* test, $p < 0.001$ [$DFB_{(5\text{-}10nM)}$/control]) or subnanomolar Fe levels (*Dunnett's* test, $p = 0.011$ [$Fe_{(0.5nM)}$/control], $p < 0.001$ [$Fe_{(1.5\ nM)}$/control]). By contrast, the significant but small decrease of the luminescent signal upon Fe addition at the station BT03 (*Dunnett's* test, $p = 0.026$ [$Fe_{(0.5nM)}$/control]; $p = 0.038$ [$Fe_{(1.5nM)}$/control]) suggests that the bioreporter cells were close to the replete condition observed for station BT01. The linear decrease in bioluminescent signal observed upon Fe addition for stations BT06 and BT09 suggests further that if these presumably strong organic ligands were present in excess in the seawater sample and bound to the added Fe, the resulting complexes were readily available to the bioreporter cells.

4.4. Influence of the ambient bacterial community of Fe bioavailability

To investigate the impact of the small particulate fraction on Fe bioavailability, we conducted similar Fe removal/addition experiments at three stations: BT04, BT07, and BT09. Figure III.7 shows the response of the bioreporter cells grown in non amended filtered seawater. At all stations, the "small particulate Fe" treatments (< 0.8 µm) displayed significant change in luminescent signal relative to that observed for the "dissolved Fe" samples (< 0.2 µm). At the station BT04, the luminescent signal decreased slightly but significantly (*t*-test*;* $p = 0.049$) when bioreporter cells were incubated in 0.8-µm filtered seawater. In contrast, the 0.8 µm treatments displayed

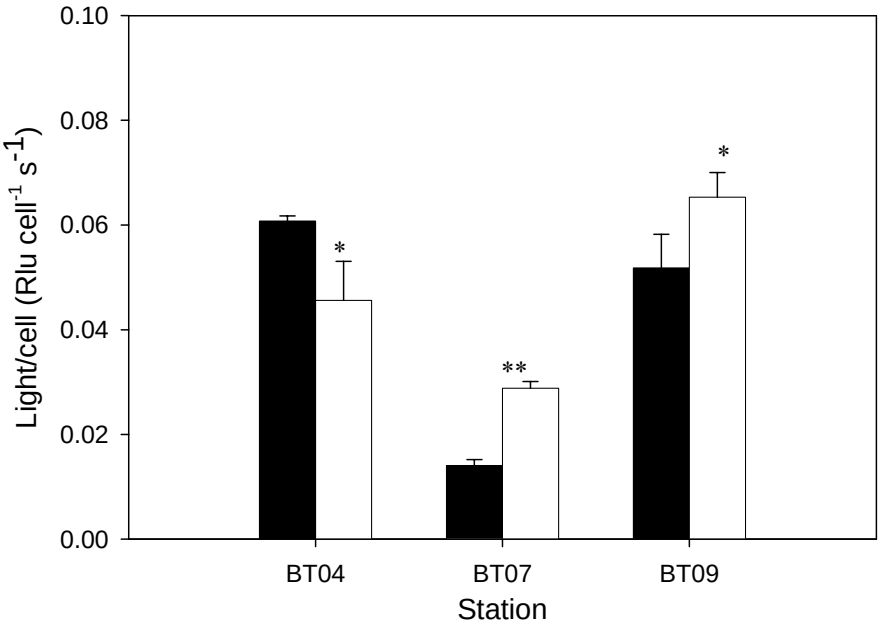


Fig. III.7. Response of *P. putida* FeLux when grown in dissolved (< 0.2 µm, black bars) and dissolved + particulate fraction (< 0.8 µm, white bars) of naturally occurring Fe. Asterisks indicate that the "Particulate + dissolved" sample was statistically different from the "dissolved" sample (*t-test*; *: $p < 0.05$; **: $p < 0.001$).

significantly higher luminescent signal for both stations BT07 (*t*-test, $p< 0.001$) and BT09 (*t*-test, $p = 0.042$). These changes were especially marked at the equatorial station (BT07) as the signal increased by a factor two between the 0.2 µm and the 0.8 µm filtered treatments. Although significant, variations in bioreporter response were less pronounced at both station BT04 (- 25%) and BT09 (+20%).

Response of the bioreporters to Fe manipulations in both 0.2 µm and 0.8 µm filtered seawater sampled at these three stations is presented in Figure III.8. Within the range of concentrations tested, addition of Fe or DFB to the 0.8 µm seawater sample did not significantly alter the bioluminescent signal relative to the control at all stations investigated (*Dunnett's* test, $p > 0.05$). However, differences appear when the < 0.8 µm treatments are compared to the < 0.2 µm treatment. The particulate fraction (> 0.2 µm) appears to buffer alterations in Fe bioavailability. In the current situation, contrasting

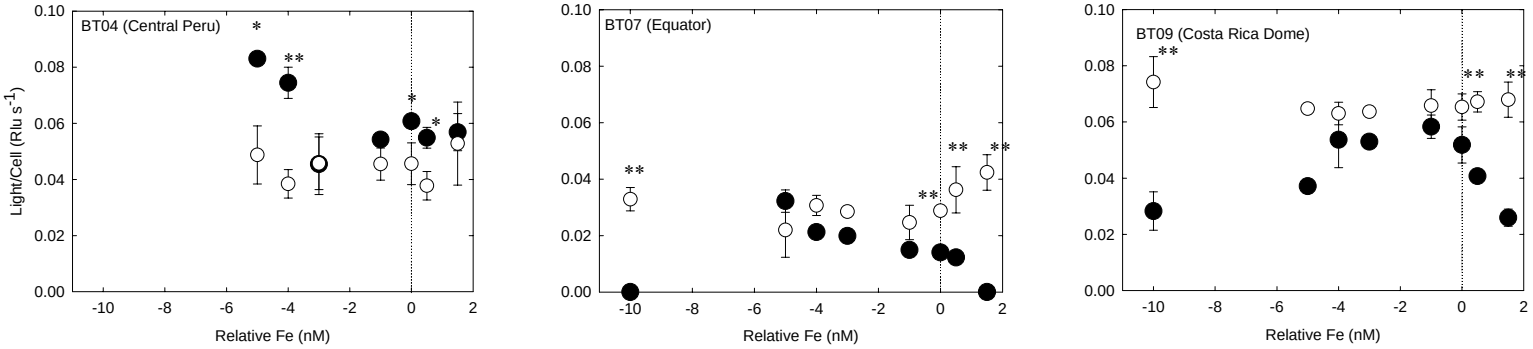


Fig. III.8. Manipulation of Fe availability at stations BT04, BT07, and BT09 in 0.2 µm (●) and 0.8 µm (○) filtered seawater samples. Samples statistically different from each other at comparable relative Fe level are denoted by an asterisk (*t-test*, *: $p < 0.05$; **: $p < 0.001$).

results suggest that the importance of the particulate pool is dependent on location (community structure, total Fe, nutrients, *etc*.).

4.5. Dilution experiment

To better characterize the response of the bioreporters at station BT12, a dilution experiment was performed. Filtered seawater sample (< 0.2 µm) was supplemented with 1.5 nM Fe and serially diluted with a synthetic seawater medium (BESAW, 1.5 nM $FeCl_3$). Figure III.9 clearly demonstrates that the dissolved fraction of Fe was not limiting in the undiluted surface seawater of station BT13 as addition of 5 nM of the siderophore DFB did not alter the luminescent signal. Light production in the 75% and 50% dilution treatments was not significantly different to that observed in the undiluted sample (*Dunnett's* test, $p > 0.05$). However, the luminescent signal in the 25% dilution treatment increased slightly (*ca.* 13%) but significantly (*Dunnett's* test, $p = 0.042$) relative to the undiluted treatment, demonstrating a decrease in Fe availability by dilution effect. For this 25% dilution treatment, addition of 5 nM of DFB led to a more dramatical increase in the bioluminescent signal relative to the undiluted treatment (*ca.* 50%; *Dunnett's* test, $p < 0.001$). This observation clearly confirms that Fe bioavailability was high at this station (dissolved Fe > 1.5 nM). Furthermore, owing to the fact that it is more than unlikely that total dissolved Fe concentrations be greater than 5 nM in this region, the lack of variation in bioluminescent signal upon DFB addition in the undiluted sample suggests that originally present dissolved bioavailable Fe may have been bound to strong organic ligands presenting higher stability constant than DFB to Fe(III).

5. Discussion

Chemical analyses have demonstrated that most of the dissolved Fe in seawater surface of the Eastern Pacific Ocean is chelated to organic ligands presenting similarities to siderophores and other intracellular cell ligands (*e.g.* porphyrin) that may be released *via* grazing or viral lysis (*e.g.* Rue and Bruland, 1995, 1997). This suggests that the

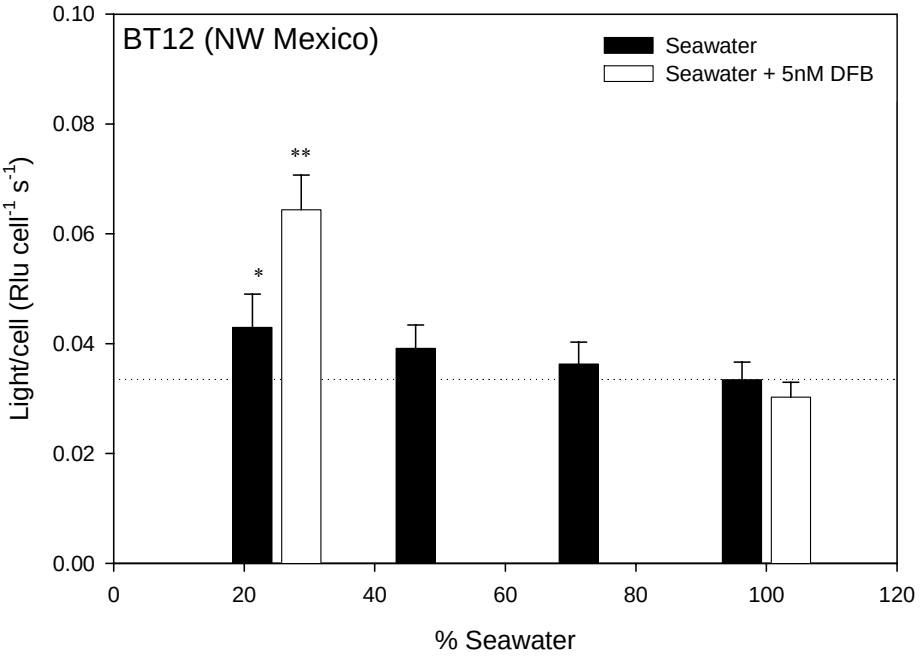


Fig. III.9. Response of the *P. putida* FeLux bioreporter to serial dilution of enriched seawater (< 0.2µm, 1.5nM Fe) with BESAW medium at the Station BT12. Normalized luminescence (RLU $cell^{-1}$ s^{-1}) is shown versus the corresponding seawater dilution (% seawater). No increase of light production was observed following addition of DFB in the undiluted seawater treatment while a significant increase was observed in the diluted treatment (25% seawater). These results confirm the hypothesis of a high ambient level of available Fe. The samples significantly different from the undiluted treatment are denoted by an asterisk (*Dunnett's* test, *: $p < 0.05$; **: $p < 0.001$). The dashed line indicates the level of bioluminescent signal in the undiluted treatment.

microbial component of the planktonic assemblage greatly influences Fe speciation in the marine environment through differing Fe acquisition mechanisms and thereby may exert a control on Fe bioavailability. As such, the microbial component plays a key role in Fe biogeochemistry in oceanic system. Because traditional analytical methods cannot resolve the bioavailable fraction of Fe from its total concentration, or eventually its speciation, various experiments have been performed to assess the bioavailability of Fe to the marine bacterioplankton. These includes size-fractionated Fe-uptake experiments coupled or not with manipulation of the bioavailable Fe (*e.g.* Tortell et al., 1996; Maldonado and Price, 1999; Weaver et al., 2003), estimation of bacterial production *via* measurement of leucine or thyminidine incorporation (Pakulski et al., 1996; Church et al., 2000; Kirchman et al., 2000). Results from these studies suggest that bacterioplankton are either directly or indirectly Fe-limited, where indirect limitation results from the reduced release of dissolved organic matter by Fe-limited phytoplankton (Pakulski et al., 1996; Church et al., 2000; Kirchman et al., 2000; Arrieta et al., 2004). Although these experiments provide valuable insight with regard to the Fe status of the global bacterioplankton population, they generally require addition of external components (*e.g.* ^{55}Fe) or do not provide information on the microbial response that allows us to assess the degree of Fe bioavailability *in situ*. We previously reported that biosensor technology could help to fill this gap (Mioni et al., 2003). Indeed, we demonstrated that the Fe-dependant bioreporter *P. putida* FeLux could be used in freshwater system to detect variations in Fe bioavailability at trace levels (Mioni et al., 2003). Taking advantage of the halotolerance of the host strain *P. putida*, we used the same bioreporter in this study to monitor changes in Fe bioavailability in the eastern Pacific Ocean. We assessed the variations in Fe biovailability along a NNW transect in the Eastern Pacific Ocean using an Fe-dependent bioluminescent reporter. To the best of our knowledge, this is the first report of the use of Fe-dependent bioreporter in oceanic system.

Several conclusions can be drawn from the present study: (i) the bioreporter *P. putida* Felux can be used in marine systems to monitor Fe bioavailability in a relatively short period of time (4 h), (ii) changes in Fe bioavailability to heterotrophic bacteria do not always correlate with gross changes in total phytoplankton biomass (as measured by

chlorophyll) (iii) manipulation of Fe bioavailability by using xenosiderophores (*e.g.* DFB and ferrichrome) in conjunction to bioreporter analyses provides valuable information with regard to the degree at which Fe is bioavailable in environmental samples, and (iv) the small particulate pool (0.2 - 0.8 μm) may act as either a sink or a source of bioavailable Fe depending on the location. These points will be developed in the text that follows.

5.1. Assessment of Fe bioavailability in oceanic systems by using bioreporters

Recent report on the southeast Pacific region suggest that the Chilean coast were not affected by the 2002 El Niño episode and that the plankton assemblage was not limited by Fe owing to the fact that long-chain forming diatom dominated the assemblage (Iriate and Gonzáles, 2004). Research performed in the coastal region of the ETP during normal or La Niña condition have demonstrated that Fe was a limiting factor of the phytoplankton growth (Hutchins et al., 2002; Frank et al. 2003) and that different populations within the same phytoplankton assemblage can be distinguished based on their Fe-status (Eldridge et al., 2004). However, little is known with regard to the heterotrophic bacterioplankton in this zone, especially during an El Niño event. A mesoscale experiment (IronEx II) in oceanic waters of the Equatorial Pacific (*ca.* ~ 10° further west from our transect) reported an increase in bacterial abundance in response to Fe enrichment, suggesting that the heterotrophic bacterial component was directly limited by Fe at this location (Cochlan, 2001). In contrast, grow out experiments performed in the Central Equatorial Pacific suggest that carbon limitation controls bacterioplankton growth in this region (Kirchman and Rich, 1997) although Fe was not directly tested in this study and trace-metal clean techniques were not applied. Previous reports for the Peru upwelling suggests that autotrophic bacterioplankton are Fe-limited rather than Fe starved in this region (Eldridge et al., 2004) and that the natural Fe gradient correlates with shifts in the bacterial community within this zone (Eldridge, 2004), suggesting that bacterioplankton are in some extent affected by the variation in Fe bioavailability. Surface seawater temperatures measured at the stations located off coast of Peru during the Bride of Tabasco voyage were similar to that reported by Eldridge et al. (2004).

Therefore, one may assume that the impact of the 2002 El Niño event on the Peruvian upwelling system was minimal and conditions similar to that reported by Eldridge et al. (2004) prevailed during our voyage. Indeed, dramatic changes in seawater temperatures and salinities were only observed for the tropical stations located north of the equator, suggesting that only the northern part of the transect was affected by El Niño.

Bioreporter analyses clearly show a decrease in dissolved Fe bioavailability toward the equator, either from the Chilean coast to the Central coastal area off Peru or from the coastal waters northwest off Mexico to the Costa Rica Dome. Manipulations of Fe bioavailability clearly suggests that Fe was not limiting in the surface waters off Chile and the northern station. In contrast, stations located within the Peruvian coastal system displayed the highest bioluminescent signal without significant variations between stations, suggesting that Fe bioavailability was the lowest in this region. Bioavailable Fe levels estimated from bioreporter analyses correlate pretty well with the actual dissolved Fe concentrations reported in the literature for the study area (Fig. III.10). The peak in

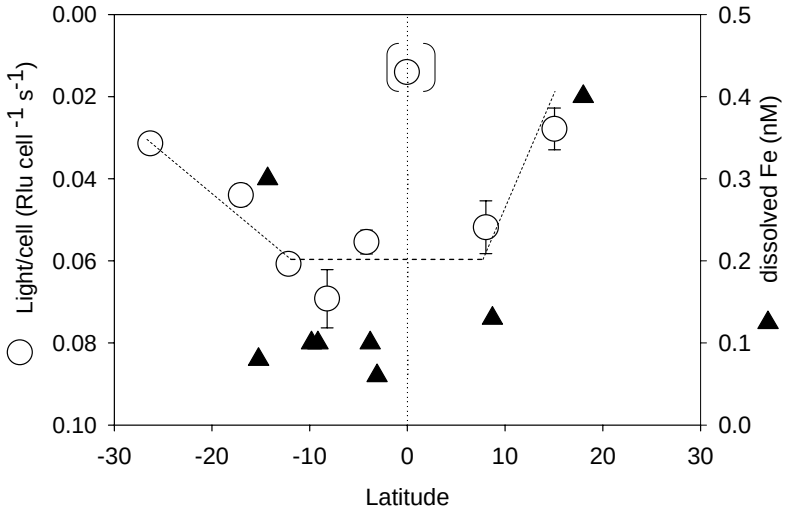


Fig. III.10. Comparison of the bioavailable level of dissolved Fe estimated by using *P. putida* Fe Lux to dissolved Fe reported for the study area. The annotated and fully referenced data set is available in Appendix III.1. Note that the Y-axis for the normalized bioluminescent signal values is inverted to facilitate the comparison with Fe concentrations. Vertical dashed line represents the Equator.

Fe bioavailability observed at the equatorial station suggests that bacterioplankton were not limited by Fe in this region. Although no Fe concentrations are presently available for this location, bacterial production was the highest at this station (*ca.* 0.04 - 0.05 pMol Leu mL^{-1} L^{-1}; Pakulski, pers. com.) suggesting that resident bacterioplankton growth was not as much limited as in the surrounding stations of the ETP. Taken together, these results suggest that *P. putida* FeLux is sensitive and selective enough to detect variation in Fe bioavailability at trace levels in complex environment such as seawater. Moreover, the decoupling observed between Fe bioavailability (as measured by the heterotrophic bacterial reporter) and Chl *a* suggests that either other factors than Fe may be involved in controlling phytoplankton biomass (*e.g.* limitation in macronutrient within the oligotrophic waters of the warm pool, in light, or control exerted by temperature, grazing pressure, etc.) or that Fe speciation influences the plankton community composition. Previous studies have effectively demonstrated that the ability to acquire Fe from various organic Fe complexes varies greatly between taxonomic groups and even between species (Granger and Price, 1999; Hutchins et al., 1999; Guan et al., 2001; Weaver et al., 2003).

By coupling bioreporter analyses to manipulation of Fe bioavailability, we were able to gain more insight with regard to the pool of bioavailable Fe. Stations investigated in the present study could be classified in three main groups:

(i) Fe-sufficient station with bioavailable Fe chelated to weak organic ligands ($\text{Log } K_{\text{Fe-L}} < \text{Log } K_{\text{Fe-DFB}}$) as illustrated by station BT01. Interestingly, this station was located at proximity to a known upwelling cell supplying the surface seawater with nutrient rich deep waters (Romero and Hebbeln, 2003). The concentration in L2-class (weaker) ligand is known to increase with depth up to concentration ~ 3 nM (Rue and Bruland, 1995, 1997), corresponding to the concentration at which the bioluminescent signal saturated. One may therefore assume that upwelled waters at this station may have been a significant source of "new" Fe.

(ii) Fe-sufficient station with bioavailable pool of Fe chelated to strong ligands as illustrated by stations BT07 and BT12. The station BT07 was located in equatorial seawater where picoplankton is known to dominate the plankton assemblage (Coale et al.,

1996b; Cochlan, 2001). Our observations suggest that Fe was not limiting at the equator and that Fe chelated to strong organic ligands (Log $K_{Fe\text{-}L}$ > Log $K_{Fe\text{-}DFB}$) that could derive from resident plankton community were readily available to the bioreporter at this location (BT07). This observation is in agreement with the bacterial production rates estimated for this station. The equatorial station exhibited the highest bacterial production rate (in term of Leucine incorporation, Pakulski, pers. comm.). Since siderophores are only produced under Fe-limiting conditions, the Fe-ligands originally present in the surface waters of this station are more likely to be derived from grazing or viral lysis (*vis a vis* Poorvin et al. 2004). Station BT12 was located in the most oligotrophic waters (in term of chl *a*) at the edge of the warm pool where picoplankton is expected to dominate the biological biomass. Unfortunately, Leucine incorporation was not assessed at the station BT12, and therefore bacterial production is not known at this site. Results from the dilution experiment performed at the station BT12 demonstrated however that Fe bioavailability was high at this station. Removal/addition experiments suggested further that Fe originally present at this station may have been chelated to strong organic ligands (Log $K_{Fe\text{-}ferrichrome}$ > Log $K_{Fe\text{-}L}$ > Log $K_{Fe\text{-}DFB}$) and present in excess (> 1.5 nM).

(iii) Stations displaying Fe stress (but not Fe-starvation) and characterized by bioavailable Fe chelated to strong ligands (Log $K_{Fe\text{-}L}$ > Log $K_{Fe\text{-}DFB}$) more or less available to the bioreporter cells (BT03, BT04, BT05, BT06, and BT09). These stations cover both the Peru upwelling system (BT03, BT04, BT05, BT06) and the oligotrophic warm pool brought by El Niño (BT09) which are known to be characterized by Fe-limiting condition (Chavez et al., 1999; Hutchins et al., 2002; Frank et al., 2003; Eldridge et al., 2004). Resident planktonic species may be expected to be adapted to such limiting conditions, and thus better competitors in term of Fe acquisition may be expected. Within this group of stations, one can distinguish two subgroups:

- The Peru upwelling stations (BT03, BT04 and BT05) where bioreporters did not sense an increase of Fe bioavailability upon Fe enrichment and sensed or not a decrease in Fe bioavailability only upon addition of high amount of DFB. These observations suggest that strong ligands which were not recognized by *P. putida* FeLux may have been present in excess (> 0.5-1.5 nM), buffering the subnanomolar addition of Fe. Other strong Fe-

ligands were available to the bioreporter, "buffering" more (BT03 and BT05) or less (BT04) efficiently the removal of Fe through DFB addition. Striking coincidence, the station BT04 was also characterized by both the highest phytoplankton biomass (Chl *a*; Fig.III.5) and one of the lowest rate of Leucine incorporation observed along the transect (*ca.* < 0.02 pMol Leu mL^{-1} L^{-1}; Pakulski, pers. comm.). Previous studies have shown that heterotrophic bacteria are not very successful in using strong organic ligands derived from phytoplankton (*e.g.* porphyrin) (Weaver et al., 2003). Moreover, binding constants reported for porphyrin-type ligands is about one order of magnitude higher than that of DFB (Appendix I). Indeed, it is possible that such porphyrin-type ligands contributed for at least a fraction of the strong organic ligands present in excess (*ca.* > 1.5 nM) in the surface waters of station BT04, and that these ligands sequestered the inorganic Fe added to the Fe-amended treatments. In contrast, stations BT03 and BT05 were both characterized by low Chl *a* concentrations (Fig.III.5). Leucine incorporation rates were also greater than in station BT04 at these two stations (0.03-0.04 pMol Leu mL^{-1} L^{-1}; Pakulski, pers. comm.). Bacterioplankton were indeed more likely to exert a control Fe bioavailability at these stations.

- Stations BT06 and BT09 can be distinguished from the previous subgroup by the presence of strong ligands that were readily available to the bioreporters (as shown by the linear-like decrease in light production upon Fe addition).

Taken together, our results indicate that the bioavailability of dissolved Fe is dependent on the location (structure of community, total Fe, etc.) Although our results do not resolve the debate between indirect or direct Fe limitation, it clearly indicates that the pool of bioavailable Fe to heterotrophic bacteria such as *P. putida* FeLux differs between regions and that subtle differences exist between bioavailable Fe pools which would appear at first sight similar. Our results highlight indeed that Fe availability is tightly linked to Fe speciation in marine systems. This observation is in good agreement with previous reports suggesting a feedback relationship between the plankton community and the bioavailable Fe pool. The structure of the organic ligands derived from marine biota influences Fe availability to the planktonic community (Hutchins et al., 1999; Weaver et *al*., 2003) and, as a consequence, may influence the community composition (Eldridge,

2004). Although organic binding-ligands enhance in overall Fe bioavailability by increasing its solubility in surface oceanic waters they also compartmentalize the bioavailable Fe pool and thereby influence the competitive success of the various component of the planktonic community (Hutchins et al., 1999; Weaver and al., 2003; Griffin et al., 2004).

5.2. Impact of the small particulate fraction on Fe bioavailability

Previously, we reported that at least a fraction of particulate Fe is bioavailable to bacterioplankton (Mioni et al., 2003) in aquatic ecosystem. In the present study, the relationship between the particulate Fe pool and the bioavailable Fe pool appears more complex. Our results suggest that the small particulate size-class (0.2 - 0.8 μm) can effectively influence Fe bioavailability by acting as a source (station BT04) but also as a sink (stations BT07 and BT09). Moreover, the particulate fraction appears to buffer artificially induced (through DFB or Fe addition) variations in Fe bioavailability at all station investigated, indicating that it plays a key role in controlling the bioavailable Fe pool in marine systems. Therefore, our results highlight the complexity of the feedback relationships between Fe bioavailability and the particulate size-class.

Since the heterotrophic bacterioplankton was included in this small particulate size class, our results may reflect the level of cooperative or competitive production of siderophores by the resident bacteria. Recent model suggest that cooperation is advantageous, even when relatedness is low, in the case of a global competition (*i.e*. when DFB is added at high level in seawater sample containing several bacteria subpopulations) (Griffin et al., 2004). Such a cooperative siderophore production may explain why bioavailable Fe level sensed by bioreporter cells remained stable in the 0.8-μm filtered treatments when the system was pushed into further Fe-limiting conditions. However, siderophore or ligand production may also affect negatively Fe bioavailability as Fe additions to the system were not perceived by bioreporter cells, probably because Fe chelated to organic ligands that were not recognized by *P. putida* FeLux.

Although early reports claimed that only inorganic species of Fe were bioavailable to aquatic microbes (Rich and Morel, 1990; Morel et al., 1991), several lines

of evidence suggest that particulate Fe could be an important source of Fe to microbial organisms in oceanic environments. Most of Fe in surface seawater (up to 99% in the equatorial Pacific) is in the particulate size-class and most of the dissolved Fe fraction is in fact colloidal in nature (Sunda, 2001; Wells, 2003). Photochemical processes at seawater pH have been shown to increase the solubility of otherwise inert Fe crystalline oxides (Wells et al., 1991), and indeed its bioavailability to microorganisms. Other studies suggest that Fe hydroxide can support Fe uptake due to their fast dissolution kinetics (Kuma and Matsunaga, 1995) and that such hydroxides can adsorb to cell surface where they subsequently undergo bioreductive dissolution (Sunda, 2001). This mode of Fe acquisition may be used for example by the cyanobacteria *Trichodesmium* (Sunda, 2001). Siderophores also increase the solubility and dissolution kinetics of Fe oxides over a wide range of pH and may therefore increase the bioavailability of Fe in aquatic systems (Kraemer, 2004). Moreover, most microbial organisms are capable of phagotrophy which could be used to acquire Fe from the particulate fraction (Sunda, 2001). Experiments in the Pacific Ocean on the autotrophic flagellate *Ochromonas* sp. have demonstrated that this organism could acquire Fe directly from the particulate fraction by ingesting bacteria (Maranger et al., 1998).

In summary, in the current situation, contrasting results suggest that the importance of the particulate pool is dependent on location (community structure, total Fe, nutrients, etc.) in a similar fashion than what is observed for the dissolved (< 0.2 μm) Fe pool.

6. Conclusion

The present study validates the use of the Fe-dependent bioreporter *P. putida* FeLux in oceanic systems. Result presented here highlight the complexity of the bioavailable pool in marine environment and suggest that the speciation of Fe as well as the biological component itself influence Fe bioavailability to the bacterioplankton. A decoupling between Fe bioavailability and phytoplankton biomass suggest that other

factors than the total level of bioavailable Fe (*e.g.* the structure of the organic-binding ligands) influence the community structure.

In the current study, bioreporter analyses provide valuable insights on variations in bioavailable Fe but do not yet allow us to quantifying the concentration of bioavailable Fe. In consideration to this problem, future studies should include calibrations of the bioluminescent signal to assess the level of bioavailable Fe in the complex marine system.

Acknowledgements

We do not thank El Niño for coming during the cruise and making our interpretations more difficult than they are already. We thank (in alphabetic order, they all helped a bunch!) Amy Baldwin, Sherif Ghobrial, Wade Jeffrey, Jara Meador, Dean Pakulski, Leo Poorvin, and the Captain and crew of the RV/ *Nathaniel B. Palmer* for assistance. This work was supported by a UT-Center for Environmental Biotechnology Graduate Fellowship to C.E.M., a grant from the Natural Sciences and Engineering Research Council of Canada to M.R.T. and a National Sciences Foundation award (OCE-000298) to S.W.W.

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APPENDIX (PART III)

Appendix III.1 –

Iron in the surface waters of the Pacific Ocean (adapted from deBaar and de Jong, 2001; their table A2). Stations of the Brides of Tabasco cruise are indicated between brackets for corresponding published sampling sites.

°Lat, °Long.	Depth Range (m)	Fe range (nM)	Mean [Fe] (nM)	Reference	Footnote
Subtropical North Pacific Gyre					
~35°N, 122-139°W	0-100	0.01-0.16	~0.06	Martin & Gordon (1988)	1
36°N, 123°W	0-80	0.20-0.70	~0.40	Gordon et al. (1982)	2
~37°N, 124°W	25-100	0.14-1.19	__	Landing and Bruland (1987)	3
Eastern North tropical Pacific					
18°N, 108°W (BT13)	15-100	0.01-0.57	~0.40	Landing & Bruland (1987)	4
18°N, 108°W (BT13)	0-60	0.13-0.73	~0.40	Gordon et al. (1982)	5
19°N, 105°W	15-250	0.66-5.71	~2.00	Landing & Bruland (1987)	6
8.7°N, 90.6°W (BT08)	5-15	N/A	0.13	Frank et al. (2003)	7
Equatorial Pacific Ocean					
0-1.5°N, 92°W	0	0.01-1.50	__	Martin et al. (1994)	8
1°N, 92°W	20-150	0.03-0.22	~0.1	Gordon et al. (1998)	9
~0°N, 91-93°W	20-150	0.02-0.22	~0.1	Gordon et al. (1998)	9
0.5°N-2°S, 89°W (BT07)	20-150	0.02-0.22	~0.1	Gordon et al. (1998)	9
3.1°S, 86.3°W (BT06)	~7	N/A	0.06	Eldridge et al. (2004)	10
3.8°S, 85.3°W (BT06)	5-15	N/A	0.10	Frank et al. (2003)	7
4-7°S, 105-110°W	0	0.015-0.037	0.021	Rue & Bruland (1997)	11
5°S, 90°W	0	<0.05-0.14	0.07±0.03	Gordon et al. (1998)	9

Appendix III.1 – Continued

°Lat, °Long.	Depth Range (m)	Fe range (nM)	Mean [Fe] (nM)	Reference	Footnote
Eastern South tropical Pacific					
9.2°S, 80.5°W (BT05)	~7	N/A	0.1	Eldridge et al. (2004)	10
9.8°S, 81.6°W (BT05)	~7	N/A	0.1	Eldridge et al. (2004)	10
15.2°S, 76°W (BT03/04)	~7	N/A	0.08	Eldridge et al. (2004)	10
14.3°S, 78.4°W (BT03/04)	5-15	N/A	0.30	Frank et al. (2003)	7

[1] VERTEX-V (June 1984), stations 1, 2, and 4 from inshore to offshore with decreasing deep water values; station T3 was not reported by Martin and Gordon (1988) due to 6 outliers; however remaining 11 data points appear consistent.

[2] First ever journal publication of reliable dissolved Fe concentrations.

[3] CEROP-I and VERTEX-I at almost same time; two duplicates at VERTEX-I where five other samples were not analysed.

[4] VERTEX-II site; in 1981 first ever reliable Fe data as presented at AGU meeting (Landing and Bruland, 1981), several duplicates.

[5] First ever journal publication of reliable dissolved Fe concentrations; duplicates at 6 sampling depths with 5 extra Chelex-100 extraction based replications.

[6] Shallow nearshore site.

[7] Surface waters collected in context of Zn and Fe enrichment and uptake experiments (September 2000). Costa Rica Upwelling Dome (08.7°N, 90.6°W); Humboldt current region (03.8°S, 85.3°W) and Peruvian upwelling region (14.3°S, 78°W). Zinc concentrations are also provided for their Humboldt and Peru stations (0.06 nM and 0.1 nM respectively).

replicated?

[8] Surface water section in context of IRONEX I experiment (October 1993). Equatorial Pacific.

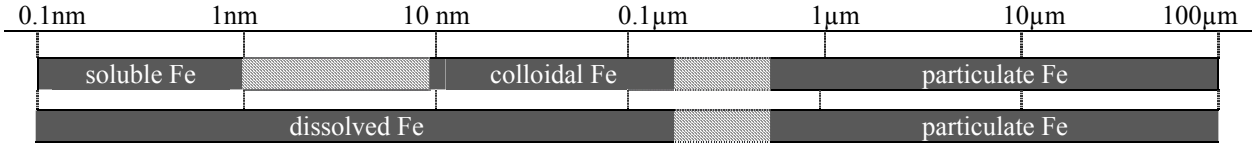
[9] Surface water values represent the initial and outside patch ambient conditions before the actual IRONEX I experiment (October 1993); their appendix B. at their page 1029 provides 10 datapoints of which seven reported as below the 0.05 nM detection limit; their text at their page 999 provides a mean value of 0.07 nM for only 8 samples. Also given are dissolved Fe values for meridional transect of three stations (Plumex 1, 4, 6) east of and near-equatorial 4 stations (Plumex 9, 11, 14, 16) west of Galapagos Islands. One more station Plumex 8 (1°N, 92°W) to northeast of islands shows very similar values.

[10] Surface waters collected in context of their Fe/DFB enrichment experiments. Their stations Bio-4 (03.1°S, 86.3°W), Humboldt current region; Bio-5 (09.16°S, 80.47°W) and Bio-6 (09.81°S, 81.57°W), Peruvian upwelling; and Bio-7 (15.23°S, 76.02°W), Shallow nearshore site. Not replicated, dissolved Fe (<0.2μm).

[11] Ambient dissolved Fe outside the enriched patch of IRONEX II experiment (May 1995; 3.5°S, 105°W), in context of organic complexation study.

Appendix III.2 –

Various chemical forms and species of Fe which can exist in dissolved and particulate phases (adapted from Bruland and Rue, 2001).



Fe(III)'
$Fe(OH)_2^+$
$Fe(OH)_3^0$
$Fe(OH)_4^-$

FeL_1

Fe-Humic

Alumino-silicate clays

Fe(II)'
Fe^{2+}
$FeCO_3^0$

Intracellular Fe

Extracellular Fe
(cell lysis products)

Fe-colloid

Extracellular Fe
Detritus

Part IV

TRACKING A NATURAL SHIFT IN BIOAVAILABLE FE IN A MARINE SYSTEM: A FIRST ESTIMATE USING A HETEROTROPHIC BACTERIAL BIOREPORTER

Part IV

TRACKING A NATURAL SHIFT IN BIOAVAILABLE FE IN A MARINE SYSTEM: A FIRST ESTIMATE USING A HETEROTROPHIC BACTERIAL BIOREPORTER

This chapter is a lightly revised of a paper by the same name to be submitted in the journal *Global Biogeochemical Cycles* in 2004 by Cécile E. Mioni, Sara M. Handy, Michael J. Ellwood, Michael R. Twiss, R. Michael L. McKay, Philip W. Boyd, Steven W. Wilhelm.

My use of "we" in this chapter refers to my co-authors and myself. My primary contributions to this paper include (1) development and optimization of the bioreporter calibration method in the synthetic seawater medium BESAW, (2) processing of the seawater sample in the laboratory (3) laboratory and field data analyses, (5) most of the gathering and interpretation of literature, (6) pulling the various contributions into a single paper, and (7) most of the writing.

1. Introduction

Fe is an essential micronutrient for microorganisms since it serves as cofactor for a wide variety of enzymes and catalyzes redox reactions (Raven, 1990; Geider and Laroche, 1994; Tortell et al., 1999). Although absolute requirements vary, it has been estimated that all living microorganisms require a minimum effective Fe concentration of ~10^{-8} M for growth (Braun and Hantke, 1997). Accumulated data demonstrate however that such concentrations of Fe are rarely reached in most oceanic systems. Minimal inputs from aeolian and deep water upwelling sources combined with rapid removal rates due to

particle scavenging results in total dissolved Fe concentrations ranging from averaging 0.02 nM to 1 nM in most pelagic marine systems (Johnson et al., 1997; Measures and Vink, 2001; Takata et al., 2004).

In situ perturbation experiments have demonstrated that the low ambient concentrations of Fe control phytoplankton growth, community structure and ecosystem function in large areas of the world's ocean, such as the Equatorial Pacific (Coale et al., 1996), the Subarctic Pacific (Tsuda et al., 2003) and the Southern Ocean (Boyd et al., 2000; Gervais et al., 2002; Coale et al., 2004). In these regions, mesoscale "enrichment" experiments have triggered phytoplankton blooms upon addition of Fe to the system. This enhanced growth of marine phytoplankton results in a switch in the ecosystem from recycling- to export-dominated planktonic communities (Bishop et al., 2004; Buesseler et al., 2004). Although Fe-deficient surface seawaters are generally dominated by regenerated picoplanktonic communities, accumulated data from laboratory and field bottle experiments suggest that both autotrophic (Wilhelm, 1995; Achilles et al., 2003) and heterotrophic (Palkuski et al., 1996; Hutchins et al., 1999a, 2001a; Weaver et al., 2003, Arrieta et al., 2004) bacterioplankton components may be Fe -limited as well. In fact, although the larger surface area to volume ratio should give them an advantage (Eldridge et al., 2004, and ref. therein), empirical models suggest that bacteria should be disadvantaged as compared to phytoplankton in such conditions, owing to their slower uptake kinetics and their significant higher requirements for this trace metal (Maldonado and Price, 1999). Therefore, the high abundance of bacterioplankton in most High Nutrient – Low Chlorophyll (HNLC) regions may be puzzling if only total dissolved Fe concentrations are considered. Indeed, it has been suggested that changes in the chemical speciation, more than the total ambient Fe concentration, control the speciation of dominant planktonic groups (Hutchins et al., 1999b, 2001a; Wells and Trick, 2004). Unfortunately, there are no analytical tools currently available to identify and quantify the fractions of Fe in seawater that are biologically available to either phytoplankton or bacteria (Gerringa et al., 2000; Wells and Trick, 2004).

Here we describe a new approach to assess Fe bioavailability in seawater: a heterotrophic bacterial reporter that responds specifically to Fe stress by producing a quantifiable bioluminescent signal. We have used a *Pseudomonas putida* strain which was genetically engineered to produce light (luminescence) when the cells native high-affinity Fe transport systems are derepressed (Mioni et al., 2003). This enables us to detect variations in bioavailable Fe levels *in situ.* In the present study, we describe the initial laboratory characterization of this *P. putida* Fe-dependent bioreporter in a chemically defined and modeled synthetic seawater medium (BESAW). We also present a set of field measurements performed during the late austral summer (February 2003) in the subantarctic Pacific region during the "FeCycle" mesoscale experiment. The FeCycle mesoscale experiment was designed to develop an unperturbed Fe budget by following a marine surface water patch in a HNLC region labeled with SF_6 tracer. The study site was characterized by subpolar HNLSiLC (High Nutrient – Low Silicic acid - Low Chlorophyll) waters representative of the majority of the Southern Ocean surface waters (~ 65%; Coale et al., 2004). During austral summer in this region, surface waters are typically depleted (< 1μM) of silicic acid as well as Fe; indeed it has been suggested that diatom stocks remain low due to a silicic acid – Fe colimitation (Boyd et al., 2001; Hutchins et al., 2001b; Nelson et al., 2001).

The austral summer provides optimal conditions to study the mechanisms which may control HNLC assemblages. At this period of the year, this HNLSiLC community is mainly dominated by prokaryotic and picoeukaryotic organisms (Boyd et al., 2001; Boyd, 2002; Hutchins et al., 2001b). As such, the microbial loop is more likely to drive the ambient community and concentrations of organic Fe-binding ligands (*e.g.* siderophores) can be expected to be high level. By coupling biogeochemical data with the bioreporter data, we have been able to demonstrate the response of this community to coincidentally observed natural variations in ambient Fe availability.

2. Material and methods

2.1. Strain and growth conditions

Pseudomonas putida FeLux (Mioni et al., 2003) stock cultures were maintained on Pseudomonas Isolation Agar (PIA) supplemented with 50 μg mL^{-1} of tetracycline (Tc) at 30°C. Prior to the experiment, several colonies were screened. The colony with the largest dynamic range of light production relative to the control (no addition) after an addition of Fe (1.5 nM) or Deferrioxamine B (DFB, 1.5 nM) was selected for the field experiments. Cultures from the selected colony were maintained in sterile marine broth 2216 (Difco) supplemented with 50 μg mL^{-1} of Tc at 25°C in sterile 50 mL polycarbonate tubes on an orbital shaker. Preacclimation of the bioreporter cells prior to experiments was performed in BESAW medium. BESAW medium is a completely defined medium modified from the marine synthetic medium ESAW recipe (Berges et al., 2001) and optimized for heterotrophic bacteria growth (Appendix IV.1). Final free ferric Fe concentrations (expressed as pFe, the –log [Fe^{3+}]) as well as the concentration of other free trace metals were inferred using the chemical equilibrium software MINEQL (Appendix IV.2). To reduce residual Fe, all nutrient stocks and water were treated with Chelex-100 resin (Price et al., 1988/89). All culture materials were soaked in dilute HCl (5 %) and rinsed with Chelex-100 treated Milli-Q water prior use. All manipulations were performed under a class-100 laminar flow hood with respect of both aseptic and trace-metal clean techniques.

2.2. Laboratory characterization of P. putida FeLux as an Fe bioreporter

Reagents and medium for the characterization of the bioreporter were prepared prior to the day of the experiment. Sterile aliquots Fe-free BESAW (18 mL) were dispensed into acid washed and microwave sterilized Oakridge tubes. Tubes were then supplemented with Fe from a 1000-time concentrated Fe:EDTA premix. The concentration of $FeCl_3.6H_2O$ was altered in each premix to create an increasing range of bioavailable Fe. The final concentration of EDTA was maintained constant for all

treatments (100 μM). For preacclimation, 5-mL of culture grown in marine broth 2216 (Difco) were transferred to an acid washed and microwave sterilized 500 mL polycarbonate Erlenmeyer containing 95 mL of sterile Fe-deficient BESAW (pFe 20.2, Appendix IV.2) and incubated overnight in the dark at 25° C with shaking. The experiment initiated by transferring 2 mL of an overnight-acclimated culture to each replicate tube (n = 3 for each treatment). The optical density at 600 nm (OD_{600}) and light production were measured every 2 hours over an experimental period of 12 hours using a spectrophotometer (Biomate 5, Thermospectronic corp.) and luminometer (FB-15, Zylux corp.). For each time point, light production was normalized to OD_{600}.

In a separate experiment, Fe-replete bioreporter cultures were used to calibrate the OD_{600} value relative to cell concentration. Cells were preconditioned as described above and transferred to a fresh BESAW medium of pFe 17.2. Every 2 h, cells were sampled to measure the luminescence and optical density at 600 nm (Fig. IV.1A). In parallel, 1 mL of culture was fixed with glutaraldehyde (2.5 % v/v) and enumerated by epifluorescence microscopy after treatment with acridine orange (Hobbie et al., 1977). Since the OD_{600} values correlated strongly (R^2 = 0.988) with the density of bioreporter cells (Fig. IV.1B), light production per OD_{600} values were converted to light production per reporter cell mL^{-1} using the empirically determined linear function:

$$y = -3.46 \times 10^6 + 3.61 \times 10^8 x$$

where Y is the number of bioreporter cell mL^{-1} and X the corresponding OD_{600} value. Reported values are the means of triplicate cultures (± S.D.) from the time-point for which the signal was the strongest (t = 12 h). Due to the high reproducibility of the data, calibration data will be presented here as the mean of the data obtained from three experiments performed in identical conditions (n = 9 per treatment).

2.3. Site selection and survey

The FeCycle mesoscale experiment was conducted during late austral summer (January 30, 2003 – February 12, 2003) in the vicinity of the NIWA's Southern mooring

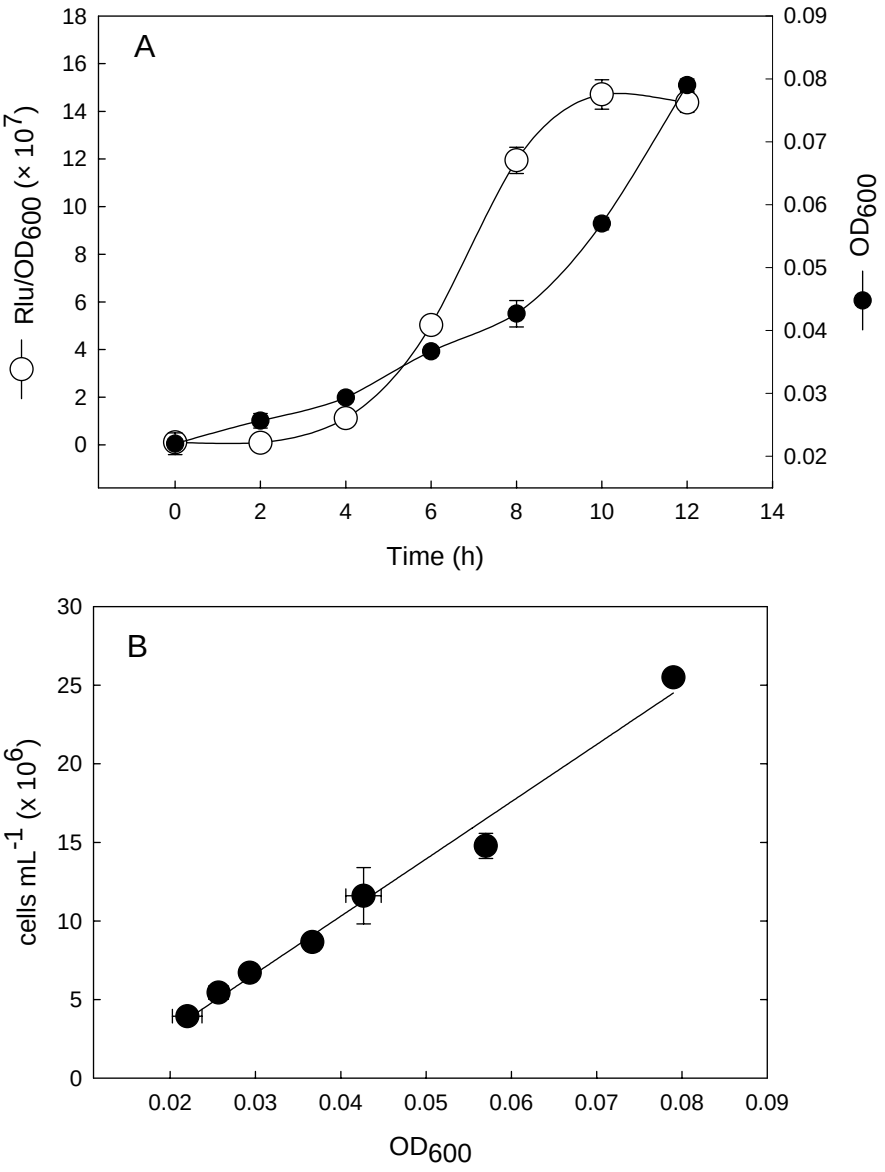


Fig. IV.1. Laboratory characterization of the *P. putida* FeLux bioreporter in the trace metal buffered BESAW medium. A: Typical growth curve and light production of *P. putida* FeLux in BESAW medium (pFe 17.2). Error bars represent the standard deviation between triplicates when larger than the symbol. B: Typical calibration curve of OD_{600} and cell concentration in Fe-replete BESAW medium with resulting linearization (R^2 = 0.988; *slope* = 3.61×10^8, *y-intercept* = -3.46×10^6). Error bars represent the standard deviations of triplicates when larger than the symbol size.

site (~46°30S, 178°30E) on board of the RV *Tangaroa*. The study site location was selected based on a pre-release oceanographic survey (*ca.* XTB section, CTD and underway seawater sampling) and local remote-sensing (SeaWiFS, SSH, SST, mooring data). The FeCYCLE site (46°16S, 178°70 E) was chosen to be representative (shallow mixed layer, low Fe and chlorophyll *a* concentrations) of subpolar HNLC subantarctic waters. Once the study site was selected, surface waters were labeled over a 50-km^2 area using the chemically inert tracer sulfur hexafluoride (SF_6) in a similar manner to that used during the SOIREE voyage (Boyd et al., 2000; Bowie et al., 2001). However, unlike the SOIREE experiment, the labeled patch was not infused with Fe. SF_6 concentrations were analyzed during daily underway mapping of the patch. The evolution of the patch is described elsewhere (Boyd et al., in prep.).

2.4. Field sample collection

All seawater samples were collected at the center of the patch of SF_6-labeled patch using a trace-metal clean pumping system (Almatec). Polyethylene tubing and pump were acid washed prior to the cruise and seawater was pumped through the sampling system for several hours prior to collect samples. In-line polyethylene filters (20 μm and 5 μm) were used to remove large particulate matter. Waters were homogenized in acid-cleaned container. Filtered (0.2 μm) mixed-layer seawater samples for bioreporter assay experiments were collected in acid-cleaned polycarbonate flasks and stored frozen (-20 ºC) until use.

Seawater samples for Fe chemistry were collected using the trace metal clean pumping system. Total dissolved Fe concentrations (< 0.2 μm) were determined on Teflon distilled HNO_3 (t-HNO_3) acidified (pH < 1.8) samples using a combination of the solvent extraction techniques described by Danielsson et al. (1978) and Bruland et al. (1979). Briefly, 250 g of seawater was buffered to a pH of 4.5 with purified ammonium acetate buffer in a 250-mL Teflon separation funnel. Purified ammonium pyrrolidinedithiocarbamate (APDC) / sodium diethyldithiocarbamate (DDC) solution was

then added followed by 5 mL of cleaned chloroform. The sample was extracted by shaking for 3 minutes. After allowing the phases to separate, chloroform was drained and the sample extracted a second time with 5 mL of chloroform. Following extraction, chloroform primary and secondary extracts were pooled, acidified with 75 mL of t-HNO_3, shaken, and left to react for one hour. Metals back-extracted from the chloroform phase were diluted to 1 mL with Nanopure water, shaken, and left overnight before analysis by graphite furnace atomic adsorption spectrometry using a Perkin-Elmer 4100 ZL with Zeeman background correction.

Hydrographic data (temperature, salinity, density (σ_t), pressure, fluorometry, oxygen and PAR) and nutrient samples were collected using the ship's Seabird CTD. Nutrient concentrations in samples were determined for dissolved phosphate (PO_4), orthosilicic acid ($Si(OH)_4$), nitrate (NO_3) and nitrite (NO_2) using by the New Zealand Institute of Atmospheric and Water Research as previously described (see Boyd et al. 2000). Total chlorophyll *a* (chl *a*) was determined from duplicate 50 mL samples collected on 0.2-μm pore-size polycarbonate filters (Osmonics) after extraction (~ 24 h) in 90% acetone. Chl *a* was quantified with a Turner Designs TD-700 fluorometer using the non-acidification protocol of Welschmeyer (1994).

2.5. Experimental manipulations of available Fe.

To ensure that Fe availability was growth limiting to the community during these experiments, we carried out two on-deck amendment experiments as previously described (Eldridge et al., 2004). DFB was purchased from the Sigma Chemical Company and dissolved into sterile, Chelex-100 treated water (Price et al., 1988/89) from a Millipore BioCell water purification system. Aliquots of the DFB stock were added to sample bottles to generate a triplicate series of DFB concentrations from 1 to 10 nM DFB. In parallel, +Fe bottles were amended with $FeCl_3$ (dissolved in 0.01 M Ultrex HCl) to final concentrations ranging from 0.5 to 3.0 nM added Fe. In combination with control bottles (bottles with no DFB or Fe added) this resulted in concentrations of Fe ranging

from 10 nM < ambient (+10 nM DFB) to 5.0 nM > ambient (+5.0 nM Fe). Sealed bottles were placed in on-deck (temperature ranged from 16.3 – 20.3 °C across stations), flowing seawater incubators lined with a 3.2 mm-thick sheet of spectrum-correcting blue Plexiglas® to simulate the light intensity and spectral quality of the 50% incident depth in the water column (Laws et al., 1990; Wells, 1999). All subsampling occurred after sunset to reduce light exposure effects, and to guarantee a full solar day exposure per 24 h period. Samples were collected at only the 72 h time point to reduce the potential for contamination during subsampling and the influence of bottle effects previously observed after this time point (Eldridge et al., 2004). Total chl *a* was determined for each treatment from samples collected onto 0.2, 5.0, or 20.0 μm pore-size polycarbonate filters (Osmonics) after extraction (~ 24 h) in 90% acetone. Chlorophyll was quantified with a Turner Designs TD-200 fluorometer using the non-acidification protocol of Welschmeyer (1994).

2.6. Fast repetition rate fluorometr.

Photosynthetic efficiency (the ratio of variable fluorescence to maximal fluorescence, F_v/F_m) was measured by analyzing 50 mL aliquots of samples in the dark chamber of the FAST^{tracka} fast repetition rate fluorometer (FRRF; Chelsea Instruments Ltd.). All samples were analyzed ~ 2 hrs after local sunset to avoid diel variability, and were dark-adapted at room temperature for at least 15 min. prior to analysis. Data were analyzed using the FAST^{tracka} FRRF post-processing program version 1.4 (Chelsea Instruments, Ltd.).

2.7. Determination of in situ bioavailable Fe level

Acclimated *P. putida* FeLux bioreporter cells (BESAW medium, pFe 20.2) were used to estimate available Fe in each seawater samples. The following Chelex-100 treated (except vitamins) and filter-sterilized nutrient stocks were added to filtered (0.2 μm) seawater samples to ensure that the level all nutrients but Fe were sufficient to support the growth of the bioreporters: 20 μM NH_4NO_3, 10 μM NaH_2PO_4, 1 μM Glycerol, and

100 µL vitamin mix (ESAW recipe, Berges et al., 2001). Enriched seawater (18 mL) was dispensed into each acid washed and microwave-sterilized Oakridge tubes. Triplicate samples were either unaltered (control treatment) or supplemented with $FeCl_3$ (1.5 nM final concentration). Amendment of the seawater samples was performed at least 24 h before the addition of preconditioned bioreporter cells. At time zero, 2 mL of acclimated *P. putida* FeLux bioreporter cells were added to each replicate. OD_{600} and light production were measured every 2 hours over a period of 12 hours using a luminometer (FB-15, Zylux corp.) and a spectrophotometer (Biomate 5, Thermospectronic corp.). At each time point, light production was normalized to OD_{600} and then converted to light production per reporter cells.mL^{-1}. Bioavailable Fe levels (estimated as $pFe = -\log[Fe^{3+}]$) were predicted from estimated cellular luminescence using the calibration curve established from BESAW medium treatments with known pFe. Calibration treatments were run in parallel to seawater treatments for each experiment.

2.8. Statistical analyses

Statistical analyses for all the data were performed using SPSS (ver. 12) software. Analyses of variance and independent *t*-tests (two-tailed) were performed assuming equal variance on mean values. The homogeneity of variance was tested in each analysis using the Levene test. For incubation experiments, Analysis of variance was used to establish the statistical significances of variation among different treatments. In parallel, multiple comparison tests were performed to determine where the differences lied. Dunnett's test was used to analyze the significance of the variations of the means of a set of amended treatments relative to the control treatment mean (Corston and Colman, 2003). The Tukey's honestly significant difference test (*Tukey-HSD* test) was used to establish the statistical significance of variations among a set of treatments means (Corston and Colman, 2003). For all analyses, a 95 % confidence interval was used. Results were considered significant at $p < 0.05$ unless otherwise stated.

3. Results

3.1. Laboratory characterization

To characterize the response of the bioreporter to Fe availability, preconditioned *P. putida* FeLux cells were incubated for 12 h in the trace-metal buffered synthetic seawater medium BESAW, supplemented with various concentrations of Fe to obtain a calculated range of pFe 20.2 to pFe 15.9 (Figure IV.2). The Fe chelating agent ethylenediaminetetraacetic (EDTA) was used to buffer free ferric Fe concentrations in BESAW culture medium. It has previously been demonstrated that, unlike other common xenobiotic ligands such as nitriloacetic acid (NTA), EDTA does not promote intracellular Fe incorporation in *Pseudomonas* species (Meyer and Hohnabel, 1992). The use of this Fe chelating-agent ensured therefore that the bioluminescence signal could be directly related to the computed level of available Fe (III) in the environment of the bioreporter cells.

Bioluminescence per cell increased linearly between treatment pFe 15.9 ($[Fe^{3+}] = 10^{-15.9}$ M) and treatment pFe 18.6 ($[Fe^{3+}] = 10^{-18.6}$ M). The dose-response signal saturated for treatments of Fe (III) concentrations below $10^{-18.6}$ M. All environmental samples tested were within the linear range of the calibration curve. A linear regression performed using the regression curve fitting of the Sigma Plot software (ver 9.0, SPSS corporation) in the linear region of this calibration curve provided the following equation:

$$y = 0.0701x - 0.684 \ (R^2 = 0.935).$$

3.2. Study site description

Dissolved Fe and macronutrients within the patch – The FeCycle site had a shallow surface mixed layer-pycnocline boundary (~ 40 – 50 m, Ellwood, 2004) characteristic of Austral summer thermal stratification in this region (Hutchins et al., 2001b; Boyd et al, 2001). Ambient dissolved Fe concentrations were low (< 70 pM) in

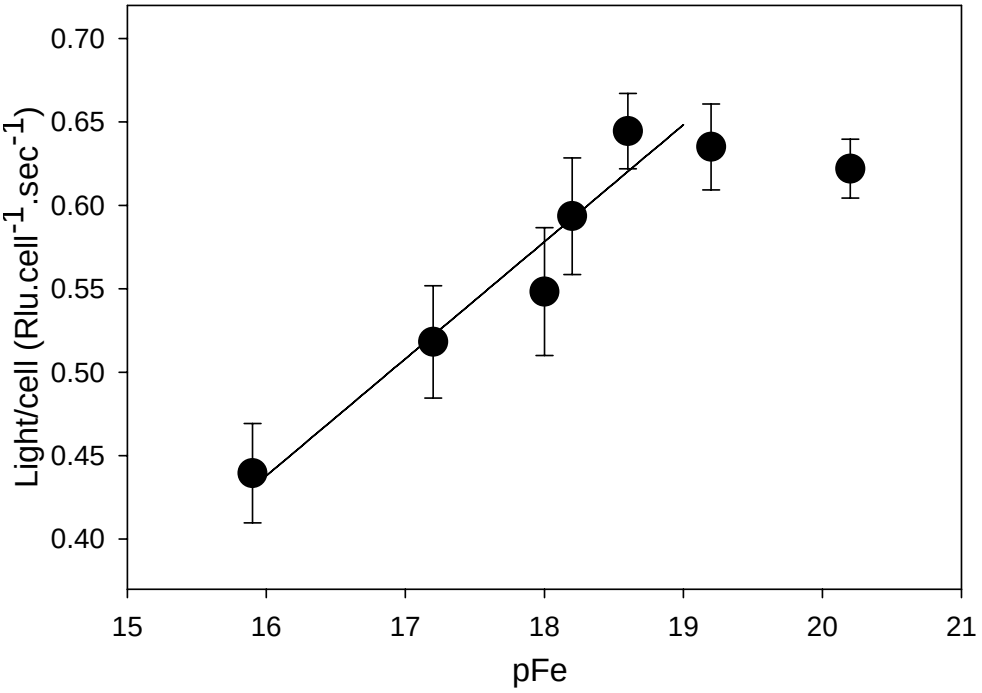


Fig. IV.2. Dose-response characterization of the Fe bioreporter *P. putida* FeLux in the trace metal buffered BESAW medium. Cell preconditioned in Fe-deficient BESAW (Fe^{3+} = $10^{-20.2}$ M) were inoculated in BESAW medium at various free Fe(III) concentrations (Fe^{3+} = $10^{-20.2}$ M, $10^{-19.2}$ M, $10^{-18.6}$ M, $10^{-18.2}$ M, $10^{-18.0}$ M, $10^{-17.2}$ M, $10^{-15.9}$ M). Bioluminescence was measured following 12 h of incubation and normalized to the number of bioreporter cells mL^{-1}. Values reported represent the average value obtained for 3 experiments performed in identical conditions. Error bars represent the standard deviations of 3 experiments (n = 9). Regression analysis of the linear range (pFe 15.9 (Fe^{3+} = $10^{-15.9}$ M) to pFe 18.6 (Fe^{3+} = $10^{-18.6}$ M) is shown (R^2 = 0.935; *slope* = 0.070 and *y intercept* = - 0.684).

surface waters throughout the study period (Fig. IV.3). Fe concentration did not display a nutrient-like (scavenged) profile in the surface layer, suggesting that our survey was preceded by a mixing event or a period of intense regeneration. The initial concentration profile (February 5, 2003) showed a minimum of 43 pM at 32 m depth. A slight transient increase of ~ 29 pM in dissolved Fe concentration was observed at this depth on February 10, 2003.

Macronutrients exhibited a classic nutrient-type profile with a nutricline between 40 and 50m depth coinciding with the well-developed seasonal pycnocline (Fig. IV.4). Vertical profiles illustrated a strong seasonal nutrient depletion within the summer-warmed surface mixed layer at the FeCycle site (Fig. IV.4). These conditions are characteristic of subantarctic waters in late summer as a result of biological and physicochemical feature changes within the upper water column (Boyd, 2002; Ellwood, 2004). Phosphate concentrations ranged between 0.55 and 0.64 μM within the mixed layer. Silicic acid remained at submicromolar level ([DSi] = 0.47 – 0.60 μM) throughout the mixed layer and one order of magnitude lower than nitrate concentrations (5.43 – 6.57 μM). Nitrate (NO_3) concentrations were *ca*. 50% of those previously reported for this region (Hutchins et al., 2001b). Over the course of the experiment, NO_3 concentrations displayed a slight decrease (ΔNO_3 ~ -1 μM at 30 – 40 m depths) concurrent with the increase observed in Fe concentrations. Ammonium (NH_4) concentrations were relatively high in the mixed layer, ranging from 0.22 μM to 0.59 μM and increased with depth. These observations suggest that either a mixing or a lateral advection episode altered transient Fe and NO_3 levels within the mixed layer during the course of the experiment.

Silicic acid to dissolved nitrogen (DSi:DIN) ratios in surface waters averaged 0.081 ± 0.009, The dissolved DIN:P ratios were lower than the corresponding Redfield ratio throughout the upper water column, averaging 11.58 ± 1.06. The dissolved Si:P ratios were ~ 0.94 ± 0.14 over the same depth range. These observations suggest that phosphate concentrations were not limiting.

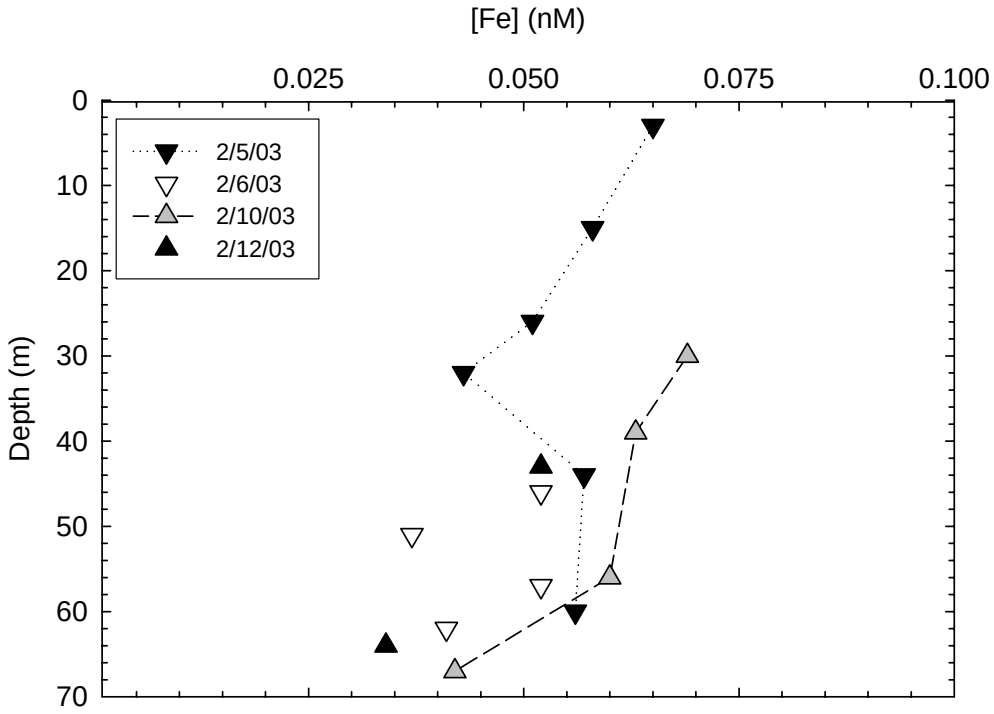


Fig. IV.3. Depth profiles of total dissolved Fe (< 0.4 μm) concentrations determined at four stations located at the center of the SF_6-labeled patch.

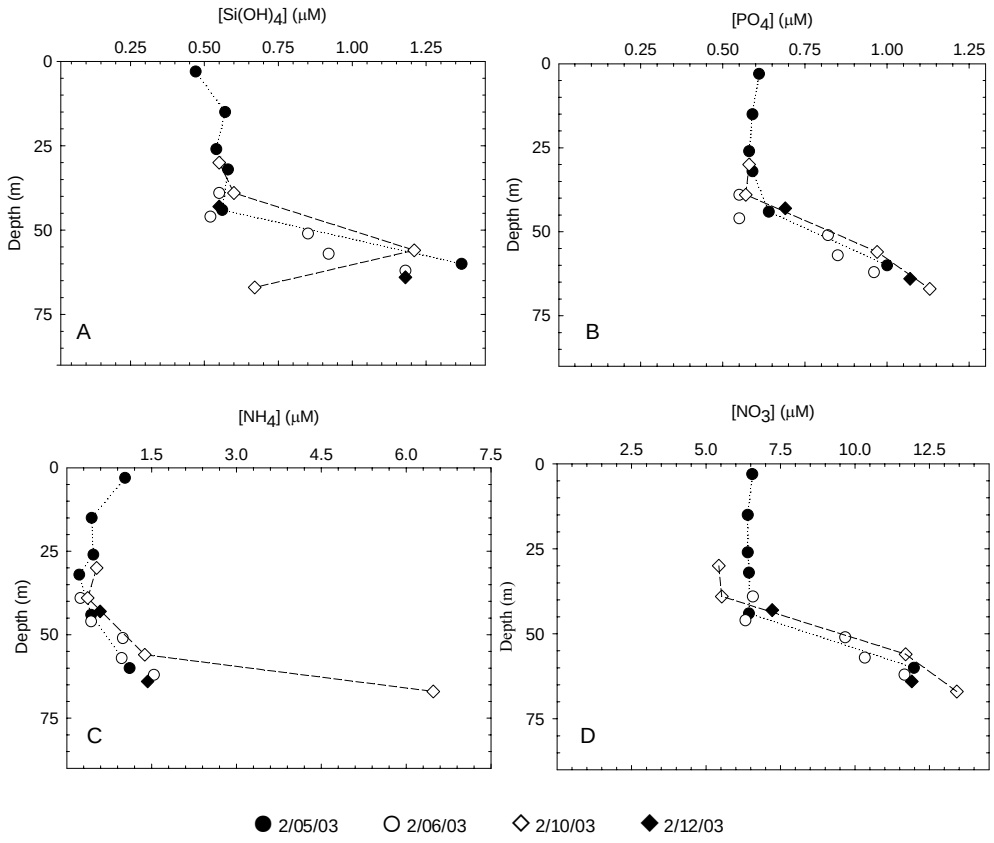


Fig. IV.4. Macronutrient profiles determined at four time points located within the SF_6-labeled patch during the late summer FeCycle voyage. A. Orthosilicic acid ($Si(OH)_4$); B. Phosphate (PO_4); C. Nitrate (NO_3); D. Ammonia (NH_4).

Evolution of dissolved Fe and macronutrients within the SF_6-labelled patch during FeCycle – Figure IV.5 shows the evolution of macronutrients concentrations in mixed-layer waters during the study period. Initial dissolved Fe concentrations were very low (30 pM; Fig. IV.5A). Although total dissolved Fe concentration remained low throughout the course of the experiment, a transient increase was observed. Fe concentration increased by a factor 2.3 between February 2 and February 8, reaching a maximum level of 69 pM. Elevated Fe concentration remained stable until February 10. On February 12, Fe level had dropped back to the initial level of 30 pM. Although DSi levels remained lower than 1 μM in the surface waters of the patch, a reduction of a factor ~ 2 was observed during the study period, with initial concentration of ~ 0.50 μM and a minimum of 0.27 μM reached on February 12 (Fig. IV.5B). However, such a decrease in DSi concentration did not occur until after February 6. Similarly, NO_3 concentrations remained steady until February 5 (Fig. IV.5C). Subsequently, NO_3 level decreased of about 1.40 μM (*i.e.* 20%) between February 6 and February 12, which is lower than what would be expected given the Redfield/Brzezinski $Si(OH)_4$:NO_3 molar depletion ratio of 0.9:1 (Brzezinski et al., 2003). Such a lag time was not observed in the variations of NH_4 concentrations. NH_4 concentrations decreased linearly by a factor 4 from February 3 to February 8 (Fig. IV.5C). In contrast to dissolved Fe levels which returned to initial concentration at the end of the study period, both DSi and NH_4 levels exhibited a greater depletion at the last time-point as compared to the initial time point (by factor 1.8 and 2.8 respectively), reaching subnanomolar concentrations at the end of the study period. It is also noticeable that both DSi and NH_4 exhibited a spike in concentration on February 10. Such peaks of concentration may reflect a mixing event with water mass richer in these two macronutrients. It has effectively reported that the SF_6 patch started to break up by the end of the FeCycle voyage, suggesting instability of the water mass (Law et al., in prep.). These observations suggest once again that mixing episodes may have altered the chemical features of the surface waters during the study period. Dissolved phosphate (PO_4) levels remained globally constant over the study period, averaging 0.58 ± 0.02 μM (Fig. IV.5D). This observation suggests that PO_4 were not utilized or that the supply rate and the utilization rate were in equilibrium.

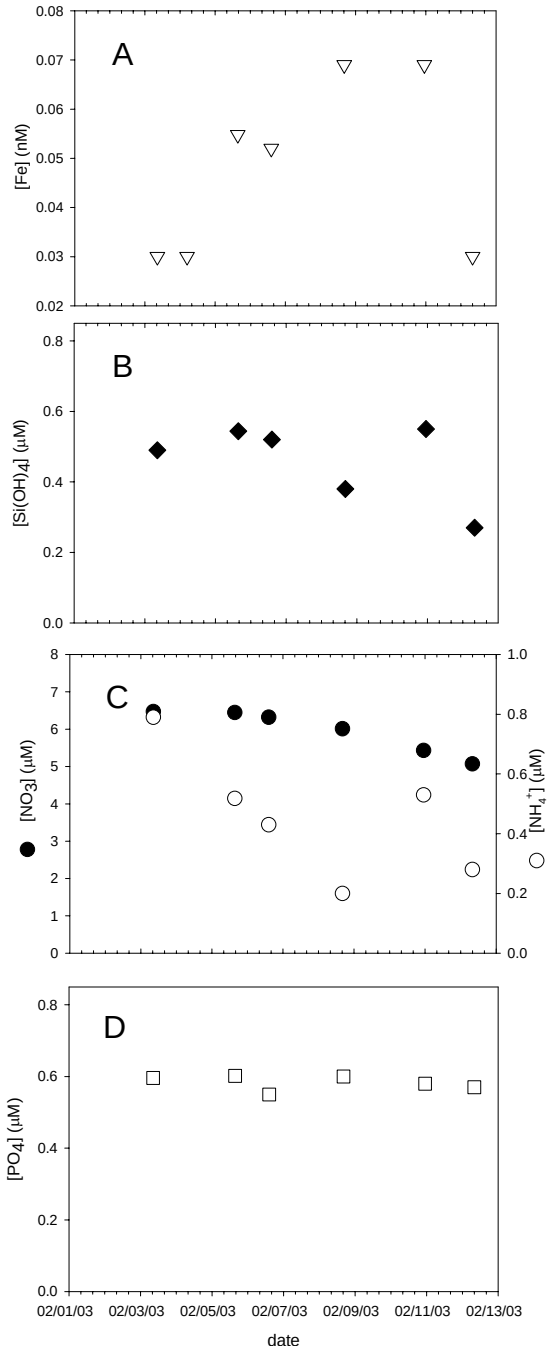


Fig. IV.5. Fe (nM) and major nutrients (µM) concentrations within the mixed layer throughout the course of the FeCycle experiment. A. Dissolved Fe ($[Fe]_D < 0.4$ µm), B. Nitrate and ammonia, C. Silicic acid, D. Phosphate.

The NO_3 to NH_4 molar ratios increased by a factor 3.7 between February 3 (ΔNO_3:NH_4 = 8.19) and February 8 (ΔNO_3:NH_4 = 30.05), indicating a faster utilization of NH_4 relative to NO_3 while total dissolved Fe levels increased. The descriptive parameter for ecosystem's nitrogen signature NH_4Av (Goeyens et al., 1995), remained high ([NH_4^+] > 5% total dissolved inorganic nitrogen) however throughout the study period. NO_3 to PO_4 molar ratios remained relatively constant during the study period, averaging 10.22 ± 0.98 μM. This trend confirms the low consumption of these two macronutrients. By contrast, NH_4 to PO_4 ratios were more variable, they decreased of a factor 4 between February 3 (ΔNH_4:PO_4 = 1.33) and February 8 (ΔNH_4:PO_4 = 0.33) and shifted between 0.91 and 0.49 at the last two time-points of the study period. Such variations were driven by variations in NH_4 levels and suggest therefore a decoupling between NH_4 and PO_4 utilizations and supplies within the surface waters at the sampling site.

3.3. Biological response

Total chlorophyll a – Surface nutrient depletion coincided with low chl *a* concentrations (< 0.40 μg L^{-1}) in the mixed layer at the beginning of the study period (Fig. IV.6B). No accumulation was observed in the upper water during the two first days of the study. Concomitant to the transient increase in dissolved Fe level, total community chl *a* concentrations increased (~ 30%) between February 4 and February 6, stabilizing at 0.55 ± 0.02 μg L^{-1} for the remaining of the study period. This observation suggests that the transient increase in dissolved Fe concentration (*ca*. ~ 40 pM) was sufficient to induce a small total chl *a* accumulation. Unfortunately, since no size-fractionated chl *a* information is available, it is not known if the biological response to the transient Fe increase was size-class specific.

Phytoplankton photosynthetic efficiencies (Fv/Fm) – Phytoplankton photosynthetic efficiencies (F_v/F_m), measured in the upper water column of the patch, are represented in Figure IV.6C. Resident F_v/F_m values were well below the theoretical maximum that would be expected for nutrient replete growth (~ 0.65, Kolber and Falkowski, 1993). Initial F_v/F_m (February 4, 2003) in surface seawater were low (~ 0.25)

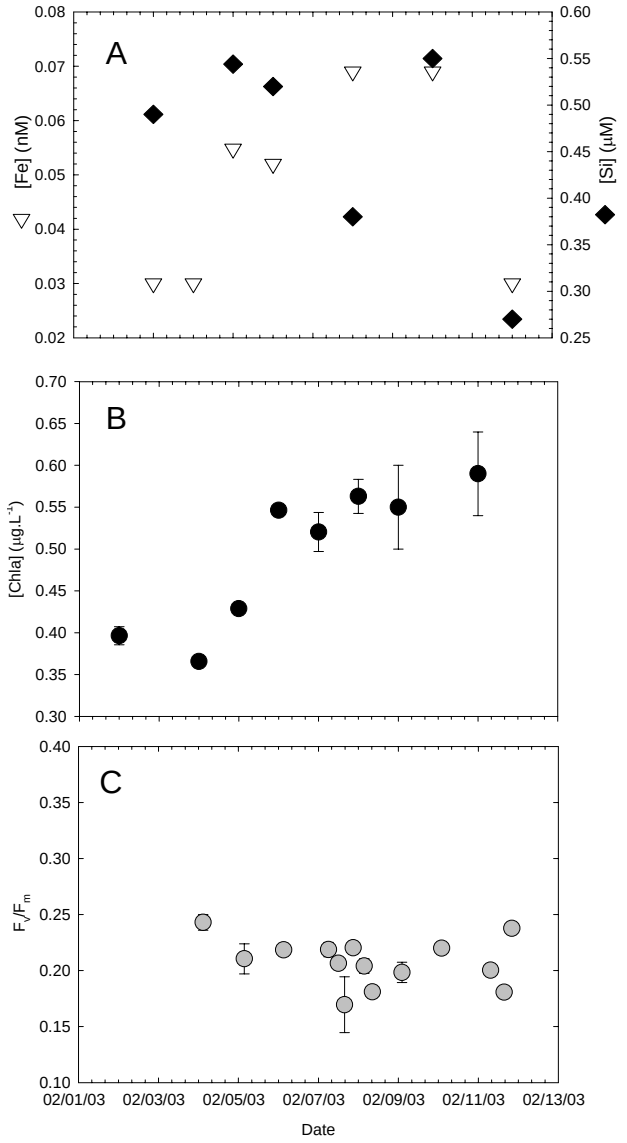


Fig. IV.6. Change in biological and chemical environmental factors within the mixed layer of SF_6-labelled waters over the course of the FeCycle voyage. A. Dissolved Fe and $Si(OH)_4$ concentrations; B. Chl *a* (> 0.2 µm) concentration; C. Temporal trend in the index of photochemical conversion efficiency, computed as variable over maximum fluorescence yield of photosynthesis (F_v/F_m) and determined by fast repetition rate fluorometry in the upper water column over the course of the FeCycle voyage.

suggesting that ambient phytoplankton photosynthetic activity was impaired. Values of F_v/F_m remain stable over the study period, averaging 0.21 ± 0.01. Comparable F_v/F_m values have previously been reported for Fe-Si co-limited phytoplankton community in subantarctic surface waters (Hutchins et al., 2001b) and for phytoplankton communities from other HNLC regime (Southern Ocean and Equatorial Pacific) dominated by picoplanktonic organisms (Boyd, 2002, Sosik and Olson, 2002). These observations suggest that the resident phytoplankton community was Fe-limited and that the transient increase in Fe concentration was insufficient to alleviate physiological stress.

Incubation experiment – Initial characteristics of the seawater sample used for on-deck incubation experiments and variations in nutrient concentrations upon the 72 h incubation period are summarized in Appendix IV.3. No significant decrease in DSi or PO_4 relative was observed in any treatment at the end of the 72 h incubation experiment ($p > 0.05$). There was no significant difference in the DSi and PO_4 concentrations between Fe- or DFB-amended treatments at the end of this study period ($p > 0.05$). By contrast, NO_3 levels decreased markedly in the Fe-amended treatment relative to the control treatment ($p < 0.05$). However, NO_3 levels in +DFB treatments did not significantly differ from that of the control at the end of the experiment ($p > 0.05$). NH_4 concentrations were much noisier than that of NO_3, perhaps due to grazing or cell leakage. Surprisingly, NH_4 levels decreased in significantly less extent in the +1.5 nM Fe and + 3 nM Fe treatments than in the control treatment ($p < 0.05$). These observations suggest that Fe addition to the enclosed assemblage stimulated significantly NO_3 utilization, while it did not impact the utilization of the two other macronutrients.

Biological responses observed during incubation experiment are presented in Figure IV.7. Small but significant increase in total chl *a* concentration (> 0.2 μm) relative to control was observed for the Fe-amended treatments ($p < 0.05$) while addition of DFB did not significantly alter the chl *a* level as compared to the control ($p > 0.05$, Fig. IV.7A). Fe addition also resulted in a slight but statistically significant increase of photochemical energy conversion efficiency (F_v/F_m) relative to control ($p < 0.05$,

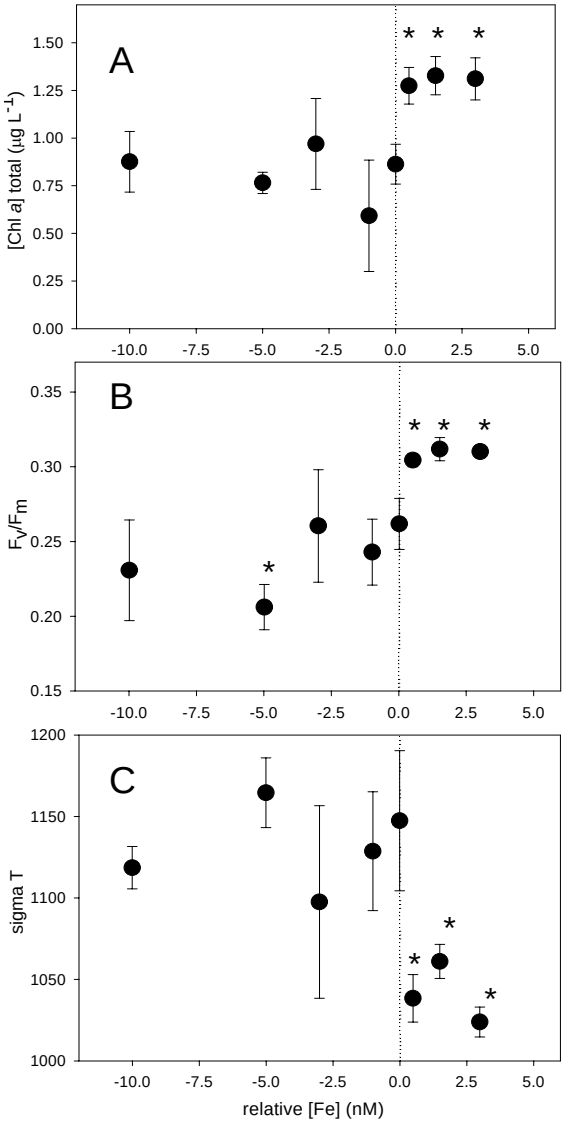


Fig. IV.7. Shipboard enrichment incubations conducted during FeCycle cruise. Seawater samples were supplemented with increasing amount of DFB (1 – 10 nM, represented as "- relative Fe") or $FeCl_3$ (1 – 3 nM). The control treatment (0 nM) was neither amended with DFB nor $FeCl_3$. Chl *a* and F_v/F_m data were collected upon 72-hour incubation period. A. Total chlorophyll *a* (chl *a* > 0.2 µm; µg L^{-1}), B. Variable over maximum fluorescence yield of photosynthesis (F_v/F_m) determined by fast repetition rate fluorometry, C. Photosystem II functional absorption cross-section (sigma T, σ), derived from the rate of increase from initial level of fluorescence (F_0) to F_m. Error bars represent the standard deviation of the means (n = 3). Samples significantly different from the control (*Dunnett's*-test, $p < 0.05$) are denoted by an asterisk.

Fig.IV.7B). Interestingly, chl *a* and F_v/F_m values plateau at low level of Fe addition (*ca*. concentration = 0.5 nM), as no significant differences with the amount of Fe added was observed in bulk F_v/F_m measured between +Fe treatments ($p > 0.05$). Moreover, even the highest F_v/F_m ratios observed for the +Fe treatments remained below the theoretical maximum F_m/F_v expected for nutrient replete phytoplankton (*ca*. 0.65). These data indicate that addition of Fe at subnanomolar level (0.5 nM) was sufficient to relieve partially the physiological stress of the bottled phytolanktonic assemblage but that other factors may be involved. The photosystem II functional cross-section (sigma T) displayed a slight but significant decrease in +Fe treatments as compared to the control ($p < 0.05$; Fig. IV.7C). Sigma T values measured in DFB treatments were not significantly different than that of control treatment ($p > 0.05$). These data suggest that the number of functional reaction centers increased in cells from +Fe treatments relative to cells growing in control and +DFB treatments. Furthermore, the lack of significant difference between DFB and control treatments for all the diagnostic indexes tested indicates that cells could not be further stressed. Taken together, these observations suggest that Fe was the principal factor limiting the enclosed phytoplankton community but that nanomolar Fe addition was insufficient to repair fully the PSII function in 72 hours.

Size fractionated chl *a* data set indicates that the response to Fe addition was size-classe specific (Fig. IV.8). Initial chl *a* was dominated by the picoplankton size class (0.2 – 2 μm). As compared to the control treatment, chl *a* levels represented by this picoplanktonic size fraction increased significantly upon Fe addition ($p < 0.05$). Slight but statistically significant chl *a* accumulation was also observed in the 5 – 20 μm size class ($p < 0.05$) and >20 μm ($p < 0.05$) upon Fe addition as compared to controls. However, there was no statistically significant difference in chl *a* level between +Fe and control treatments in the 2-5 μm size-class ($p > 0.05$). DFB addition did not result in statistically significant change relative to control in all size-fractions ($p > 0.05$). Our results indicate that picophytoplankton (0.2 – 2 μm) and nanophytoplankton (5 – 20 μm) were the most responsive to Fe addition. It is noticeable that Fe additions did not alter the relative hierarchy between size-classes in term of contribution to chl *a* biomass as

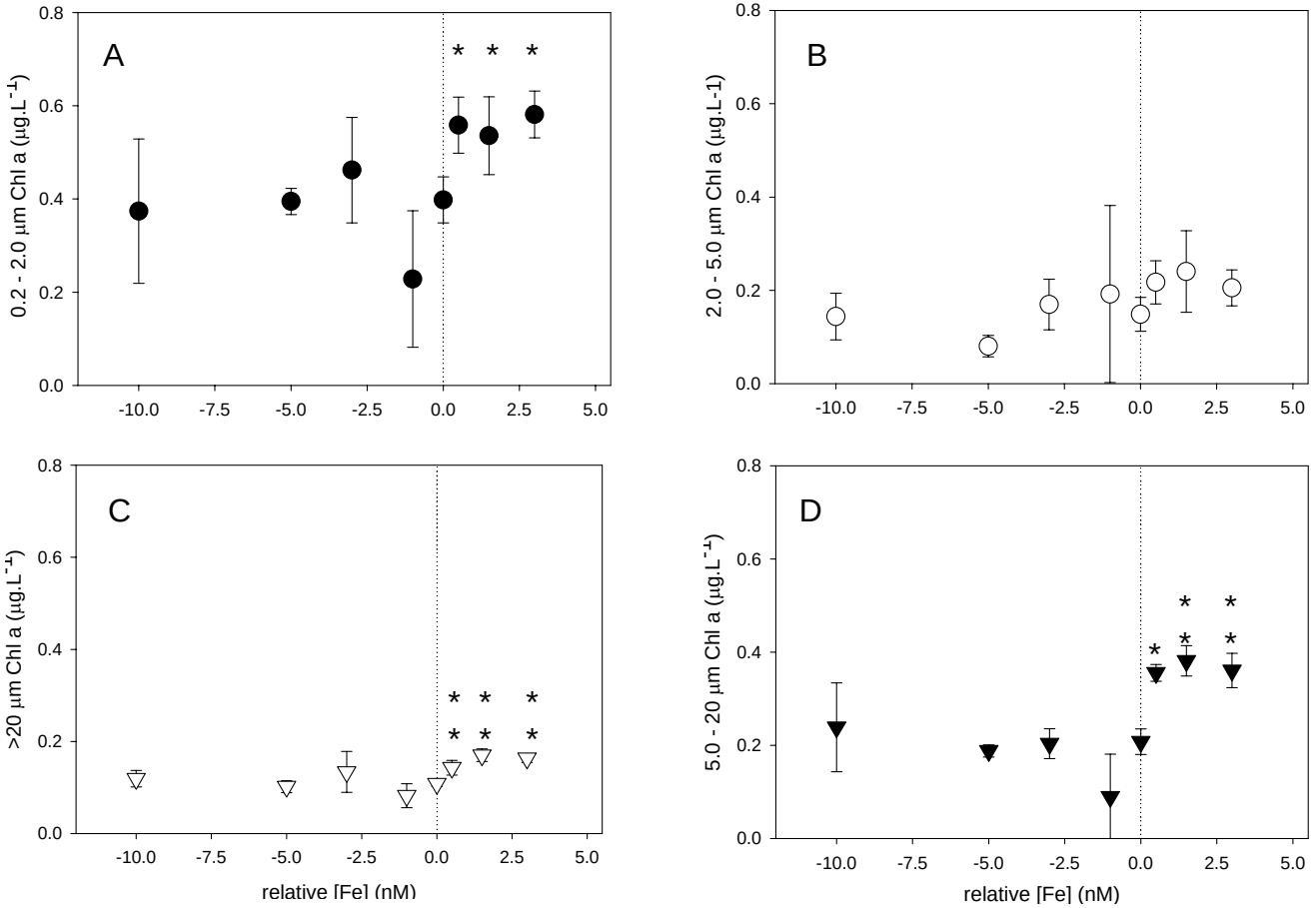


Fig. IV.8. Size-fractioned chl *a* in shipboard amendments: A. 0.2 – 2 µm size class, B. 2 – 5 µm size class, C. 5 – 20 µm size class, and D. > 20 µm size class. Error bars represent the standard deviation of the means ($n = 3$). Samples significantly different from the control (*Dunnett's*-test, $p < 0.05$) are denoted by an asterisk.

compared to the control. This result indicates that during the short period of incubation, no major shift occurred within the phytoplankton assemblage. Indeed, undesirable bottle effects appear limited and our results reflect the responses of the different size-classes of the phytoplankton community.

3.4. Assessment of Fe availability over the course of the Fe cycle voyage

Bioavailable Fe level (estimated as pFe = -log[Fe^{3+}]) in dissolved fraction (< 0.2 μm) of surface seawater predicted from *P. putida* FeLux bioluminescent reporter cells analyses are represented on Figure IV.9. Initial bioavailable Fe (February 2, 2004) was lower than $10^{-18.6}$ M Fe' as bioreporters produced light at the saturation level of the calibration curve, suggesting that high affinity Fe transport systems of *P. putida* FeLux cells were fully derepressed. Based on bioluminescent reporter analyses, the evolution of Fe bioavailability over the study period can be divided in two main phases. Between February 2 and February 6, as dissolved Fe in surface water increased, inferred pFe decreased significantly ($p < 0.05$) and in a log-linear fashion ($R^2 = 0.99$), dropping to a minimal pFe value of 16.46 ± 0.85. This observation suggests that the transient increase of dissolved Fe in the surface water was sufficient to be sensed by heterotrophic bacteria such as *P. putida* FeLux and to allow for the repression of high-affinity Fe transports. During this period, inferred pFe appeared to follow an evolution parallel to ammonia concentrations in surface waters (Fig. IV.5C), suggesting that the increase in Fe bioavailability stimulated the uptake of this macronutrient within the patch. From February 6 to February 8, when dissolved Fe concentrations in surface waters peaked (0.069 nM, Fig. IV.9A), inferred pFe varied around a mean pFe value of 16.57 ± 0.94. The variability in inferred pFe during this period coincides with variability in other parameters (DSi and NH_4) suggesting that instability in the water column may have affected the bioavailability of Fe as well. It is noticeable that chl *a* and Fe concentrations mirror in less extent the variation of inferred pFe at this sampling time.

Addition of 1.5 nM of Fe to the same seawater samples, resulted in lower luminescence level characteristics of Fe-replete cells and inferred pFes lower than pFe

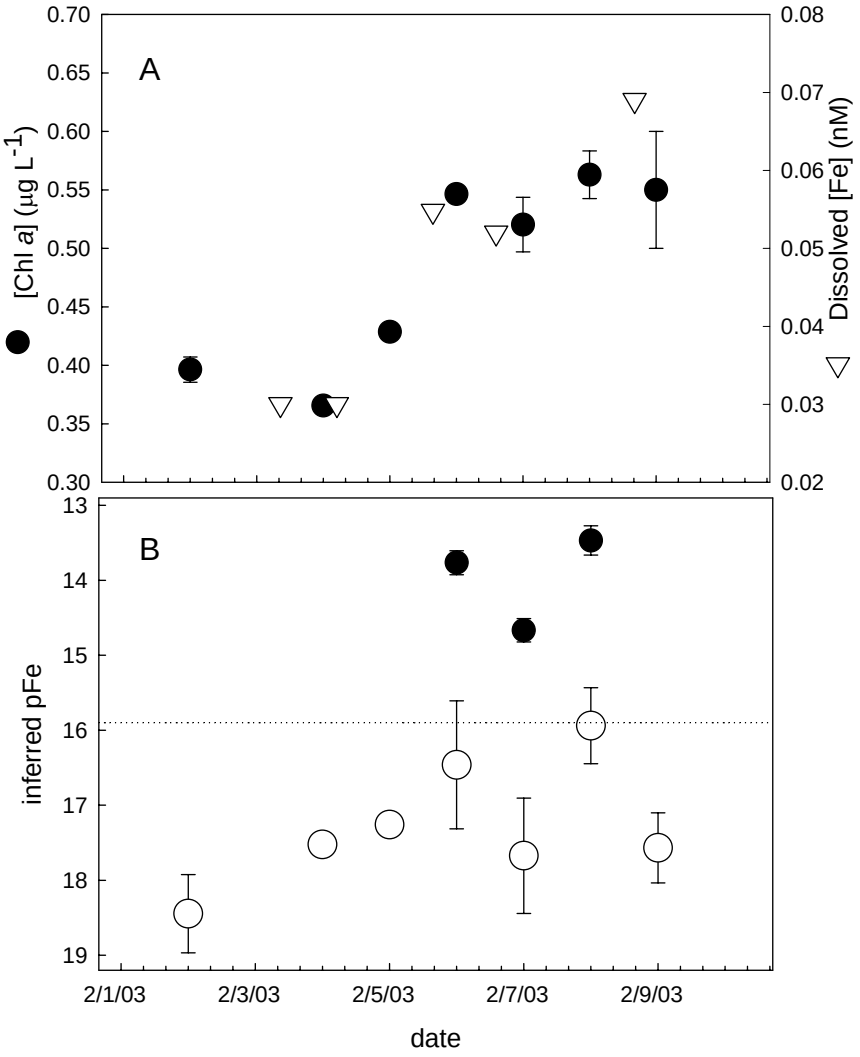


Fig. IV.9. Comparison of changes in dissolved Fe concentration, Chl *a* and Fe bioavailability within the mixed layer of SF_6-labelled waters. A. Dissolved Fe and Chl *a* concentrations within the FeCycle patch, B. Bioavailable Fe level (estimated as pFe = -log[Fe^{3+}]) predicted from *P. putida* FeLux bioreporter. Cells preconditioned in Fe-deficient BESAW medium were used to inoculate seawater samples amended with 1.5 nM $FeCl_3$ (●) or not (○). Luminescence values normalized by cell density (cell mL^{-1}) were used to estimate the corresponding pFe based on the calibration curve obtained in the trace metal buffered BESAW medium. Error bars represent the standard deviations between replicates (*n=3*). The dash horizontal line represents the lower pFe limit from the calibration curve (pFe 15.9), at this pFe level, bioreporter cells are Fe-replete. Data from Fe-amended treatment were analyzed by extrapolating the calibration curve equation to pFe lower than 15.9, assuming a linear relationship between bioluminescence and Fe bioavailability within this range.

15.9 (Fig. IV.9B). This result suggests that, although bioreporter cells sensed an increase in bioavailable Fe in the surface seawater, their high-affinity transport systems were never completely repressed. On February 9, Fe bioavailability dropped back to pFe level comparable to that estimated on February 4. Unfortunately, dissolved Fe concentrations were not determined at this time point, but the total dissolved Fe level and chl *a* concentration determined at the next time-point suggest that both parameters had dropped back to concentrations as low as that observed initially on February 2 (Fig. IV.6A). Taken together, these results suggest a strong correlation between the total Fe pool and the bioavailable Fe pool.

4. Discussion

4.1. Information provided by heterotrophic bioreporter

To the best of our knowledge, this article is the first to describe the use of bioluminescent reporter in seawater to directly estimate Fe bioavailability. The use of *P.putida* FeLux strain as a diagnostic tool of Fe bioavailability presents several advantages. First, the regulation of Fe metabolism in response to Fe availability *via* the ferric-uptake regulator protein (FUR) is widespread among heterotrophic bacterial strains (Escolar et al., 1999; Andrews et al., 2003). Therefore, bioreporters based on the Fur-regulated *fepA-fes* promoter fusion are suitable to estimate the Fe-status of the bacterial component in complex environments such as seawater. Second, owing to the halotolerance of *P. putida* FeLux, this strain can be used to detect Fe bioavailability in a wide range of environments (*e.g*. along a salinity gradient). Previously, this bioreporter had been applied to determine Fe availability in freshwater field samples and to elucidate the impact of various Fe chelators in freshwater culture medium (Mioni et al., 2003). In this study, we have demonstrated the applicability of this bioreporter to seawater systems. Using this bioreporter strain and an incubation time of 12 hours in a synthetic seawater medium, the bioluminescent reporter cells responded to increasing bioavailable Fe level in a dose dependent way within the range of pFe values of 18.6 to 15.9. Although these

pFe values may appear relatively high, results from water samples collected within the FeCycle patch fell within the linear portion of our calibration curve, allowing us to estimate corresponding bioavailable Fe levels. Furthermore, our results indicate that our bacterial reporter was sensitive enough to sense subnanomolar changes of bioavailable Fe in this environment. Effectively, a transient picomolar increase in Fe (*ca*. 40 pM) was sufficient to increase Fe bioavailability to the heterotrophic bacteria *P. putida* FeLux. Moreover, our data suggest that this strain can provide insight regarding coupling/decoupling between bioavailable Fe and total dissolved Fe. Effectively, during the transient increase in total dissolved Fe (February 2 – February 6), if organic Fe-ligands were released from indigenous microorganisms, they were at least partially available to *P. putida* FeLux since bioavailable Fe level inferred from our bioreporter analyses evolved in parallel to the total dissolved Fe in the surface waters. Although, analyses using a single strain of bacteria should be cautiously extrapolated to the natural populations (as Fe requirements no doubt vary between species), the data suggest that the transient increase in dissolved Fe may have been high sufficient to stimulate the prokaryotic component of the microbial community.

4.2. What controlled the phytoplankton within the FeCycle patch?

In contrast to the bioreporter, the response of the ambient phytoplankton assemblage to the increase in dissolved Fe level was not as noticeable. Although chl *a* level increased slightly in response to elevated Fe concentration, photosynthetic efficiencies remained low ($F_v/F_m \sim 0.2$) over the course of the study period suggesting that a strong physiological limitation prevailed in the resident community during this transient enrichment in Fe. Indeed, this observation suggests that Fe speciation may have been unfavorable for growth or that the transient increase in dissolved Fe level may have not been sufficient to stimulate eukaryotic phytoplankton while it may have stimulated only prokaryotes (although enough to repress their high-affinity Fe transport systems).

Several lines of evidence suggest that conditions were not favorable to eukaryotic phytoplankton growth within the FeCycle SF_6-labelled patch. Previous studies in various

HNLC areas reported a threshold Fe concentration for marked increase in F_v/F_m of 0.1 nM – 0.2 nM (Appendix IV.4). Firme et al. (2003) have also reported that phytoplankton show signs of Fe deprivation for Fe concentrations of ~ 0.1 – 0.6 nM. Such Fe concentrations were never reached within the surface waters of the FeCycle patch, the highest dissolved Fe concentrations were two-time lower than these threshold concentrations (*ca*. 0.069 nM on February 8 and February 10). Therefore, total dissolved Fe concentrations within the patch may not have increased sufficiently to induce a substantial response from the resident phytoplankton community. This hypothesis is confirmed by the results from on-deck experiments. Fe additions in nanomolar range were high enough to improve the physiological status of the enclosed phytoplankton community (as shown by the increased in photochemical energy conversion and photosystem II functional absorption cross-section) and to stimulate a significant chl *a* accumulation within a 72 h incubation period. By contrast, addition of DFB could not push the physiological stress further, indicating that the ambient phytoplankton assemblage was globally Fe-starved (Eldridge et al., 2004). Previous lab experiments also suggest that cell division, of both cyanobacteria (Geider and Laroche, 1994) and eukaryotic phytoplankton (Greene et al., 1992, 1994) is delayed until photosynthetic apparatus is almost fully recovered. Therefore, the transient Fe enrichment of 40 pM may not have been sufficient for full recovery of the photosynthetic apparatus of the resident phytoplankton cells. Indeed, variable fluorescence parameters from our incubation experiments indicate that nanomolar Fe additions were not sufficient to result in a full recovery of the photosynthetic apparatus over a 72-h long study period. Although the physiological status of the phytoplankton assemblage improved in Fe amended treatments, F_v/F_m values remained low (~ 0.31) as compared to the theoretical maximal F_v/F_m value for replete growth (0.65; Kolber and Falkowski, 1993) for all concentrations of Fe tested. Additionally, no significant dose-response in chl *a* concentration was observed between Fe-amended treatments. These results suggest that factors other than Fe may have contributed to the physiological stress of the phytoplankton and may have been secondarily limiting for the accumulation of chl *a* biomass. Conversely, the results can also be explained by the suggestion that our frame of reference (72 h) was too short,

and that to observe recovery of populations in this region from Fe-stress may require a longer period of observation.

Another factor that could have impacted the phytoplankton response is the low availability of silicic acid. Based on results from other incubation experiments performed during the FeCycle voyage, it has been suggested that silicic acid was the primary limiting factor to diatoms (LeBlanc et al., 2004) or that Fe and silicic acid were co-limiting within the SF_6 patch (Ellwood, 2004). Although no +Fe treatment was performed to fully demonstrate this assumption, Ellwood did observe a significant increase in chl *a*, nutrient draw-dawn and F_v/F_m value relative to control for the silicate + Fe treatments while addition of $Si(OH)_4$ alone did not result in significant change relative to control. Previous results reported for incubation experiments performed in subantarctic waters at this period of the year also support this assumption. Hutchins et al. (2001b) observed a significant increase in total chl *a* (> 0.2 μm) in the silicate + Fe treatment as compared to the +Fe treatment in comparable conditions ($[Si(OH)_4]$ = 0.66 – 0.80 μM). Moreover, DSi to dissolved Nitrogen (DIN) ratios in surface waters of the SF_6-labeled patch averaged 0.081 ± 0.009, nowhere near the minimal utilization molar ratio DSi:DIN of 1:1 for diatoms (Brzezinski, 1985). Furthermore, $Si(OH)_4$ levels were lower than most half saturation constants (0.2 – 7.7 μM) documented in the literature for DSi uptake by marine diatoms (Martin-Jézéquel et al., 2000; Frank et al., 2003; Leynaert et al., 2004). In this study, incubation data suggest that picoplankton (0.2 – 2 μm) and nanoplankton (5 – 20 μm) class-sizes were the most responsive to Fe amendment. Therefore, only lightly-silicified and non siliceous phytoplankton may have been able to take advantage of the elevated Fe availability. These results are consistent with incubation data collected in this region at this period of the year. Indeed, it has been shown previously that nanoplanktonic diatoms and other lightly silicified picoeukaryotes (*e.g.* silicoflagellates) residing in subantarctic waters have silicic acid requirements low enough to take advantage of an increase in Fe bioavailability (Hutchins et al., 2001b; Boyd et al., 2004). Therefore, silicic acid concentrations within the FeCycle patch may have been too low to support frustule formation, and hence cell division of large silicified phytoplankton

(Martin-Jézéquel et al., 2000). Moreover, it has been recently demonstrated that Fe enrichment, spanning between 0.008 and 0.12 nmol Fe' L^{-1}, results in an increase of both K_{Si} and V_{max} of the marine diatom *Cylindrotheca fusiformis* (Leynaert et al., 2004). Indeed, a subnanomolar increase in Fe availability would result in lowering large diatoms affinity for $Si(OH)_4$, which would be disadvantageous in such a HNLSiLC environment. In summary, our data suggest that while Fe was acting as the agent for primary production in the region (*i.e.,* for the whole community), silicic acid concentrations may have played a significant role in regulating the community structure (by limiting siliceous phytoplankton) in the system..

The other nutrient limitation known to be linked to low F_v/F_m is nitrogen limitation (Greene et al., 1994). Although NO_3 concentrations within the patch were well above what would nominally be considered growth-limiting, the low ambient Fe availability may have impaired NO_3 utilization (Price et al., 1991) and NO_3 uptake kinetics (Franck et al., 2003). It is well known that Fe-limited phytoplankton have reduced nitrate-reductase and nitrite-reductase activities (Timmermans et al., 1994; Milligan and Harrison, 2000) and it has been previously demonstrated that, under Fe limitation, the nitrogen metabolism is affected to a greater extent relative to the silica metabolism (Franck et al., 2000; Firme et al., 2003). Such assumption would explain the greater draw-down of DSi relative to NO_3 observed in the surface waters of the patch. Moreover, our incubation experiments show a significantly higher draw-down in NO_3 in Fe-amended treatments as compared to control, suggesting an increase in NO_3 assimilation. However, our data also suggest a rapid and preferential use of NH_4 relative to NO_3 within the FeCycle patch concomitant to the transient increase in Fe bioavailability. The relatively high NH_4 concentrations observed within the FeCycle patch may have also contributed to a partial, inhibition of NO_3 uptake by resident phytoplankton. Indeed, NH_4 levels in FeCycle waters were higher than published values of K_i (*i.e.* half saturation for NH_4 inhibition of NO_3 uptake) (Price et al., 1994; Harrison et al., 1996; Elskens et al., 1999). Within the patch, NH_4 concentrations remained higher than 200 nM over the course of the study period and a slight decrease in NO_3 concentration was only perceptible when NH_4 levels dropped below 400 nM.

Furthermore, the abundance of small-sized cells and the high values of the NH_4Av index (*ca.* > 5 % ammonium in total dissolved inorganic nitrogen stock) observed within the surface waters of the FeCycle patch, illustrate a "NH_4-based community", generally characterized by preferential ammonium uptake and by the predominance of non-siliceous species in a regenerated production regime (Goeyens et al., 1995). Taken together, these observations suggest that the plankton assemblage was dominated by components of the microbial loop.

The predominance of smaller size-classes of algae may also explain the low F_v/F_m value observed within the patch and the incubation treatments. Recent investigations in Southern Ocean led to the conclusion that the relationship between Fe limitation and and F_v/F_m may be species or size- dependent (Sosik and Olson, 2002). Compiled literature evidence suggest that nutrient-replete cyanobacteria growing at optimal growth rate often display F_v/F_m values no greater than 0.5 (Berges et al., 1996; Raateoja et al., 2004). Differences between cyanobacteria and eukaryotic phytoplankton light-harvesting machinery have been shown to impact the measurement and interpretation of variable fluorescence parameters : prokaryotic algae generally display high F_0 relative to F_m, resulting in low F_v/F_m values as compared to that of eukaryotic algae (Raateoja et al., 2004). Indeed, the F_v/F_m signature may be impacted by the relative contribution of the prokaryotic component of the microbial community. In agreement with this assumption, F_v/F_m values reported for on-deck experiments in which cyanobacteria or small phytoplankton cells in Fe-amended treatment outnumbered eukaryotic algae typically remain lower than 0.5 (Boyd et al., 1998, Hutchins et al., 2001b; Eldridge et al., 2004). Flow-cytometry analyses performed during FeCycle voyage suggested that *Synechococcus* cell density was initially one order of magnitude higher than that of eukaryotic cells in the surface waters of the patch (Ellwood, 2004). During our incubation experiments, *Synechococcus* abundance also remained one order of magnitude higher than the eukaryotic phytoplankton (data not shown). It is therefore plausible that the F_v/F_m values measured both within the patch and in the incubation treatments may be impacted by the taxonomic composition of the phytoplankton assemblage.

The abundance of cyanobacteria and heterotrophic prokaryotes (Higgins et al., in prep.) may have contributed to the maintenance of Fe-limitation of eukaryotic phytoplankton by partitioning the dissolved Fe pool through the release of Fe-ligands (Hutchins et al., 1999b). Indeed, Rue and Bruland (1997) have suggested that the increase in *Synechococcus* activity during IRONEX II was responsible for an increase in Fe- and that the ligands may have delayed the algal bloom in response to Fe infusion. However, increased siderophore production disagrees with our bioreporter analyses, as we observed that high-affinity systems appear to be rapidly repressed by the Fe enrichment in the ambient environment. Recent works suggests that prokaryotic virus-mediated lysis may explain such a concurrent increase in Fe availability and Fe-ligands production (Poorvin et al., 2004; Higgins et al., in prep.). Since most grazers (but not viruses) were removed by filtration during our incubation experiment, such a virus pressure may explain why *Synechococcus* abundance remained steadily high and why the 2.0 – 5.0 μm size class did not respond significantly to Fe addition. Indeed, combined with our bioreporter analyses, our results suggest that Fe-ligands complexes present in the surface water of the patch (derived from viral-mediated lysis and/or siderophore production) were available to our bioreporter but may not have been available to eukaryotic phytoplankton.

4.3. Significance at the ecosystem level

The current tenets of oceanography assume that picoplankton dominate HNLC areas because they are less physiologically limited by Fe availability than large phytoplankton species such as diatoms (Sunda et al., 1991; Greene et al., 1994) or, in many cases, because they maintain high-affinity transport systems (Wilhelm and Trick, 1994). However, diagnostic indexes currently available failed to demonstrate this hypothesis and suggested a global physiological limitation of the resident picoplankton-dominated community (Greene et al., 1994). Most diagnostic markers or signals of environmentally imposed stress are generally optimized for or specific to the photosynthetic, and mostly the eukaryotic, component of HNLC assemblages (Beardall et al., 2001, Firme et al., 2003). At this time, no other diagnostic marker specific to

heterotrophic bacteria is available. The heterotrophic bioluminescent reporter represents the first diagnostic tool specifically optimized to estimate the degree of Fe-limitation of the marine prokaryotic component. The data presented here suggest that heterotrophic bacterioplankton are more sensitive than eukaryotic phytoplankton to natural variations in Fe bioavailability. Although both heterotrophic bacterial cells (*ca*. bioreporter) and phytoplankton (diagnosed with F_v/F_m diagnostic index) were Fe-limited at the beginning of the survey, only bacterial cells demonstrated a relaxation of their Fe-limited state during the transient elevation in Fe availability. Moreover, our bioluminescent reporter was showing signs of Fe-repletion upon Fe amendments of the seawater samples in the nanomolar range while phytoplankton cells still demonstrated signs of nutrient stress (as reflected by F_v/F_m values below the theoretical maximum of ~ 0.65).

Taken together, our data suggest that the factors controlling microbial productivity are tightly interconnected in a very complex pattern, resulting in unfavorable conditions for eukaryotic phytoplankton growth. Although previous studies in Southern ocean support the importance of Fe in controlling community structure and function (Boyd et al., 2000, Gervais et al., 2002), some of them suggest that Fe may not be the ultimate control (Hutchins et al., 2001b; Boyd et al., 2001; Ellwood et al., 2004; Coale et al., 2004). The Fe- and silicic acid-depleted subantarctic waters represent more than the half (65%) of the Southern Ocean, and accumulated evidence suggest that during summer, these conditions affect primary productivity and community structure in this zone (Boyd, 2002; Coale et al., 2004). While Fe input induces the growth of large diatom in the southern silicic acid-replete region of the Southern ocean (Boyd et al., 2000, Gervais et al., 2002, Coale et al., 2004), it stimulates the growth of non-siliceous flagellated phytoplankton and small pennate diatom with low $Si(OH)_4$ and Fe requirements (Coale et al., 2004). Hutchins et al. (2001b) suggested that such HNLSiLC regimes may have prevailed in the glacial Southern Ocean. Although SOFeX mesoscale experiment results suggest a higher than expected biological C pump (*ca*. biomass build-up and export in the HNLSiLC subantarctic waters) (Bishop et al., 2004, Coale et al., 2004), the $Si(OH)_4$ concentrations were one order of magnitude higher than that observed in the FeCycle patch. Thus, the initial SOFeX community may not have shifted toward

nonsiliceous picoplankton and nanoplankton as the FeCycle patch did during natural Fe spike. This would explain why Fe addition in our incubation treatment may have stimulated picoplanktonic species. Based on our observation, heterotrophic bacteria and picophytoplanktonic species would be the most responsive to Fe addition in severely DSi-depleted water. Although small scale experimental results need to be interpreted with caution, one may hypothesize that such a picoplankton-dominated community would export carbon with little efficiency and would not result in the depletion of the excess macronutrients. Owing to these uncertainties, the long term consequences of a large-scale fertilization of the oceans with Fe are difficult to predict. In the long run, mass fertilization of the ocean with Fe may not help in transferring excess CO_2 from the atmosphere to the ocean floor if Fe is the proximate but not the ultimate control (Chisholm et al., 2001).

5. Conclusion

This work validates the use of bioreporters as a novel biotechnology tool to estimate the bioavailability of Fe in complex environments such as seawater. At this time, no other diagnostic marker specific to the heterotrophic bacteria component is available. Other indexes used to estimate the degree of Fe limitation are generally specific to the photosynthetic component of the microbial community. Coupled with other traditional methods, heterotrophic bioluminescent bioreporter can provide insight with regard to the microbial community response to minute Fe variations.

Acknowledgements

The authors thank Russell Frew, Julie Hall, Clint Hare, Julie Higgins, David Hutchins, Cliff Law, Stuart Pickmere, and the captain and crew of R/V *Tangaroa* for assistance in sample collection. The authors also thank Melanie Eldridge, Gary Sayler, Paul Tréguer and the reviewers for valuable comments during the preparation of the manuscript. Support was provided by NSF OCE – 0002968 and NSF INT- 0240092 to SWW, as well

as by a University of Tennessee Center for Environmental Biotechnology Graduate Fellowship, a Ryan Kelley Memorial scholarship – International Women's Fishing Association 2003, and a Daniel Jouvance Award "Recherches sur le plancton marin 2003" to CEM.

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APPENDIX (PART IV)

Appendix IV.1 –

Composition of BESAW medium as modified from Berges et *al*. (2001). Nutrients and salts were prepared as 2 time concentrated stocks solutions, Glycerol was added from a separately Chelex 100 treated stock solution (35% v/v, in BESAW salts), trace metals and vitamins were added from a ×1,000 and a ×10,000 stock solutions respectively,

Substance	Final concentration (M)
Anhydrous salts solution	
NaCl	3.63×10^{-1}
Na_2SO_4	2.50×10^{-2}
KCl	8.04×10^{-3}
$NaHCO_3$	2.07×10^{-3}
KBr	7.25×10^{-4}
H_3BO_3	3.72×10^{-4}
NaF	6.57×10^{-5}
Hydrated salts and buffer solution	
$MgCl_2 \cdot 6H_2O$	4.12×10^{-2}
$CaCl_2 \cdot 2H_2O$	9.14×10^{-3}
$SrCl_2 \cdot 6H_2O$	8.20×10^{-6}
Hepes Buffer	5.00×10^{-2}
Nutrients	
$NaNO_3$	5.49×10^{-4}
NH_4SO_4	2.00×10^{-2}
NaH_2PO_4	2.10×10^{-5}
Carbon solution stock	
Glycerol	1.44×10^{-1}
Trace Metals stock	
$ZnSO_4 \cdot 7H_2O$	2.54×10^{-7}
$CoSO_4 \cdot 7H_2O$	5.69×10^{-9}
$MnSO_4 \cdot 4H_2O$	2.42×10^{-6}
$Na_2MoO_4 \cdot 2H_2O$	6.10×10^{-9}
Na_2SeO_3	1.00×10^{-9}
$NiCl_2 \cdot 6H_2O$	6.30×10^{-9}
Iron stock	
$FeCl_3 \cdot 6H_2O$	$5.00 \times 10^{-9} – 5.00 \times 10^{-5}$
$Na_2EDTA \cdot 2H_2O$	1.00×10^{-4}
Vitamine stock	
Thiamine-HCl	2.97×10^{-7}
Biotin	4.09×10^{-9}
B12	1.47×10^{-9}

Appendix IV.2 –

Total concentrations of trace metals added to BESAW medium (pH 7.8) with an EDTA concentration of 100 μM and correspondence to free metal ion concentrations for each treatment as computed using the chemical equilibrium program MINEQL.

[Fe] (M)	**EDTA** (M)	**[Fe^{3+}]** (M)	**pFe**	**pZn**	**pCo**	**pMn**	**pNi**
5.0×10^{-9}	10^{-4}	$10^{-20.2}$	20.2	9.38	11.2	6.12	13.1
5.0×10^{-8}	10^{-4}	$10^{-19.2}$	19.2	9.38	11.2	6.19	13.1
2.0×10^{-7}	10^{-4}	$10^{-18.6}$	18.6	9.38	11.2	6.18	13.1
5.0×10^{-7}	10^{-4}	$10^{-18.2}$	18.2	9.38	11.2	6.18	13.1
7.5×10^{-7}	10^{-4}	10^{-18}	18.0	9.38	11.2	6.18	13.1
5.0×10^{-6}	10^{-4}	$10^{-17.2}$	17.2	9.36	11.2	6.17	13.1
5.0×10^{-5}	10^{-4}	$10^{-15.9}$	15.9	9.09	10.9	6.02	12.8

Appendix IV.3 –

Seawater sample characteristics for on-deck incubation experiment measured in the initial collected water and on day 3 (72 h). Nutrients concentrations are expressed in μM. Ratio (δ) represent the variation of each nutrient concentration relative to initial concentration for each treatment (in μM μM^{-1}). Errors are standard deviations to the mean (n = 3). Treatments in which in nutrient drawdown differed significantly from the control values upon the 3 days of incubation are denoted with an asterisk (*: t-test, $p < 0.05$; **: t-test, $p < 0.01$))

Nutrients concentrations		Control	Fe treatments			DFB treatments			
			0.5nM	1.5nM	3nM	1nM	3nM	5nM	10nM
NO_3	Initial	6.53 *(0.01)*	–	–	–	–	–	–	–
	72 h	5.30 *(0.02)*	4.71 *(0.17)*	4.70 *(0.18)*	4.70 *(0.18)*	5.24 *(0.20)*	5.01 *(0.57)*	5.18 *(0.05)*	5.18 *(0.41)*
	δ NO_3	**0.81** ***(0.00)***	**0.72**** ***(0.03)***	**0.72**** ***(0.03)***	**0.72**** ***(0.03)***	**0.80** ***(0.03)***	**0.77** ***(0.09)***	**0.79*** ***(0.01)***	**0.79** ***(0.06)***
NH_4	Initial	0.88 *(0.07)*	–	–	–	–	–	–	–
	72 h	0.27 *(0.23)*	0.44 *(0.15)*	0.75 *(0.21)*	1.18 *(0.43)*	0.47 *(0.66)*	0.19 *(0.66)*	0.53 *(0.37)*	1.06 *(0.71)*
	δ NH_4	**0.29** ***(0.25)***	**0.50** ***(0.15)***	**0.84*** ***(0.19)***	**1.32*** ***(0.40)***	**0.52** ***(0.71)***	**1.32** ***(0.66)***	**0.63** ***(0.50)***	**1.17** ***(0.74)***
DSi	Initial	0.52 *(0.04)*	–	–	–	–	–	–	–
	72h	0.48 *(0.02)*	0.46 *(0.09)*	0.44 *(0.03)*	0.45 *(0.03)*	0.47 *(0.03)*	0.52 *(0.05)*	0.48 *(0.03)*	0.68 *(0.27)*
	δ DSi	**0.93** ***(0.10)***	**0.90** ***(0.17)***	**0.85** ***(0.06)***	**0.87** ***(0.07)***	**0.91** ***(0.07)***	**1.01** ***(0.11)***	**0.93** ***(0.07)***	**1.36** ***(0.67)***
PO_4	Initial	0.54 *(0.02)*	–	–	–	–	–	–	–
	72 h	0.49 *(0.02)*	0.51 *(0.06)*	0.47 *(0.02)*	0.49 *(0.06)*	0.49 *(0.04)*	0.53 *(0.01)*	0.51 *(0.02)*	0.53 *(0.06)*
	δ PO_4	**0.90** ***(0.04)***	**0.94** ***(0.14)***	**0.87** ***(0.05)***	**0.91** ***(0.14)***	**0.90** ***(0.05)***	**0.97** ***(0.02)***	**0.95** ***(0.05)***	**0.98** ***(0.14)***

Appendix IV.4 –

Recorded literature data of dissolved Fe threshold concentrations to stimulate an F_v/F_m increase.

Region	Fe threshold for F_v/F_m increase	Reference
Equatorial Pacific	0.15 – 0.2 nM	Coale et al., 1996
Polar Front (S.O.)	0.15 nM	Olson et al., 2000
Polar Front (S.O.)	0.20 nM	Boyd and Abraham, 2001
Polar Front (S.O.)	0.10 nM	Sosik and Olson, 2002
Subarctic Pacific (Station KNOT)	0.05 – 0.17 nM	Liu et al., 2002
Subarctic Pacific	< 0.28 nM[1]	Suzuki et al., 2002
Peruvian Upwelling	0.06 nM[2]	Hare et al., 2004

[1] estimated control contamination
[2] threshold concentration of labile iron (Fe')

Part V

ASSESSMENT OF IRON BIOAVAILABILITY IN THE SUBARCTIC PACIFIC OCEAN DURING THE SUMMER OF 2003

Part V

ASSESSMENT OF IRON BIOAVAILABILITY IN THE SUBARCTIC PACIFIC OCEAN DURING SUMMER 2003

1. Introduction

The subarctic Pacific is one of the three large HNLC regions of the World's ocean, together with the eastern equatorial Pacific and the Southern Ocean. The first field trials aiming to test the "Fe hypothesis" were performed in the northeastern subarctic Pacific basin (Martin and Fitzwater, 1988; Martin et al., 1989). Although controversial at the time, it is now established that HNLC conditions in the subarctic Pacific results from both Fe limitation (Tsuda et al., 2003; Boyd et al., 2004) and microzooplankton grazing (Frost 1991; Strom et al., 2000). However, the subarctic Pacific is far from being representative of all the known HNLC systems, as it differs from the other HNLC region in terms of the endemic biology (Harrison et al., 1999; Tsuda et al., 2003) and by a marked longitudinal gradient in dust deposition (Duce and Tindale, 1991).

The subarctic Pacific extends over *ca*. 10,000 Km in an east-west direction and spans from the Aleutian Islands to the subarctic boundary (~ 40°N) in a north-south direction (Fig. V.1, Banse and English, 1999; Harrison et al., 2004). It is commonly divided in two main hydrographic domains associated to two large cyclonic (anti-clockwise) gyres: the Alaskan Gyre (AG) to the east, and the Western subartic Gyre (WSG) to the west (Banse and English, 1999; Harrison et al., 2004). These two domains differ from each other with regard to their biology, physicochemical environment and Fe supplies. The AG displays the typical characteristics of an HNLC region, *i.e*. low phytoplankton standing stock (Chl*a* < 1 μg L^{-1}) and excess of macronutrients in surface

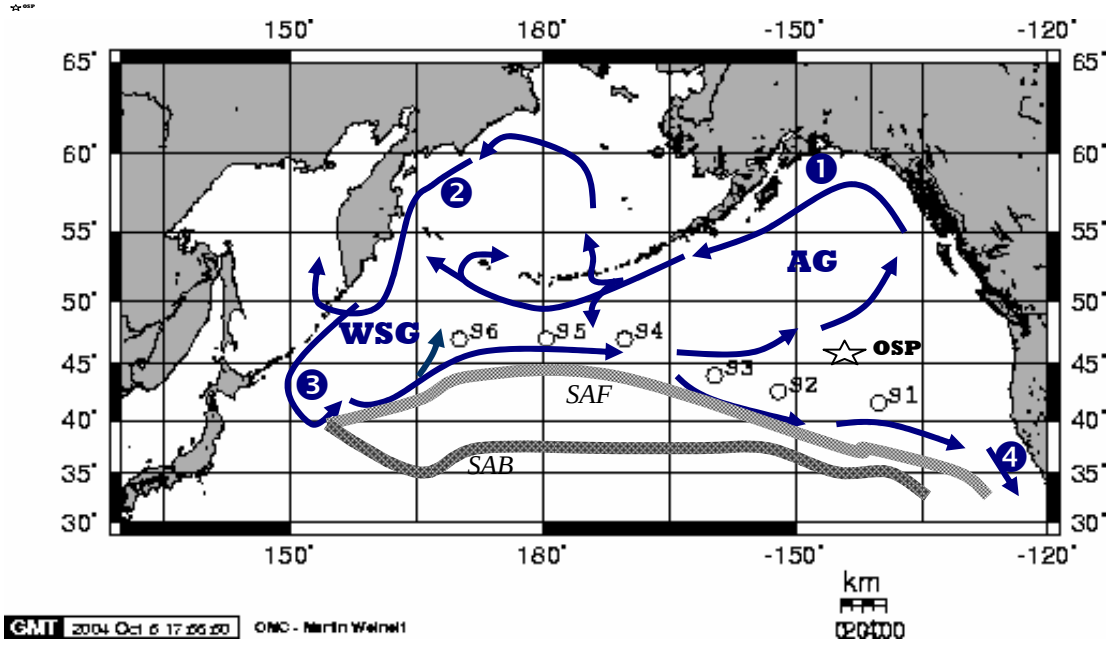

Fig.V.I. General circulation in the Subarctic North Pacific and sampling stations. Following the hydrographic domains of Banse and English (1999): S1-S4 = central subarctic domain, Alaskan Gyre (AG); S5 = boundary of Alaskan and Western Subarctic Gyres (WSG); S6 = Western Subarctic Gyre. The subarctic Boundary (*SAB*) separes the subarctic region to the north from the subtropical Pacific region to the south.❶: Alaskan stream, ❷: East Kamchatka Current, ❸: Oyashio, ❹:California Current. *SAF*: Subarctic front. The Ocean Weather Station Papa (OSP) is shown.

throughout the year (Banse and English, 1999; Harrison et al. 1999). The phytoplankton community is dominated by picoplankton species which are less sensitive than larger species (*e.g.* diatoms) to Fe limitation but are also more accessible to microzooplankton grazing (Boyd et al., 1996; Harrison et al., 1999; Strom et al., 2000). The WSG also displays HNLC traits but is characterized by higher macronutrient supplies and a shallower mixed layer in summer as well as phytoplankton biomass and nutrient utilization that tend to be higher than in the AG (Harrison et al., 1999, 2004). Recent investigations further suggest that phytoplankton are physiologically less severely Fe-limited in the WSG as compared to the AG (Suzuki et al., 2002). Indeed, depending on locations, Fe concentrations can be higher in the surface mixed layer of the WSG than in the AG (Appendix V.1; Suzuki et al., 2002; Nishioka et al., 2001, 2003; Johnson et al., 2003). The main external Fe source to the WSG comes from Fe enriched coastal waters transported by the Kamchatka current and the Oyashio (Fig. V.1; Banse and English, 1999; Suzuki et al., 2002; Harrison et al., 2004). Aeolian dust originating from the arid regions of Asia (*e.g.* Gobi desert) and Kamchatka vulcanoes, and spread eastward over the SWG of the North Pacific by favorable westerly winds, also constitutes a subsequent source of Fe to the WSG (Banse and English, 1999). In contrast, the AG is located further from atmospheric Fe sources and relies mainly on Fe inputs from the Fe-impoverished Alaskan stream and subarctic current (Harrison et al., 2004). Sporadically, volcanic ashes from the Aleutian peninsula can enrich the AG surface waters as well (Boyd et al., 1998). Nevertheless, picoplankton and nanoplankton also dominate the phytoplankton community in the western subarctic Pacific (except in coastal areas) but are also kept in check by microzooplankton grazing (Liu et al., 2004). Therefore, Fe bioavailability controls phytoplankton community structure and production in these two gyres, especially during summer (Tsuda et al., 2003; Boyd et al., 2004; Harrison et al., 2004; Liu et al., 2004).

Increasing evidence suggests that heterotrophic bacterioplankton play an important role in element cycling, including Fe, in the subarctic Pacific Ocean. Heterotrophic prokaryotes account for up to the half of the plankton carbon biomass (Kirchman et al., 1993; Tortell et al., 1996) and an equivalent amount of biogenic Fe

(Tortell et al., 1996). Experiments performed in the northeastern Pacific have demonstrated that bacteria size class (0.2 μm – 1 μm) contributes for up to 70% of the total Fe assimilation by the plankton community; this group is also characterized by higher Fe:C ratios than that of phytoplankton (Tortell et al., 1996; Maldonado and Price, 1999; Schmidt and Hutchins, 1999), implying that heterotrophic bacteria compete with phytoplankton for this limiting nutrient. Moreover, analytical assessments of Fe speciation in the North Pacific (Rue and Bruland, 1995; Takata et al., 2004) have shown that organic ligands bind more than 99% of the dissolved Fe. Although the source of these ligands is uncertain, they display similar stability constants than that of siderophores which are generally produced by bacteria under Fe limitation (Rue and Bruland, 1995). Furthermore, it has been reported that heterotrophic bacteria distribution in the upper 100 m of the water column is very similar to that of Fe(III) solubility (Takata et al., 2004), suggesting that they could be responsible for the high solubility of Fe(III) in seawater through the release of Fe-binding ligands such as siderophores or by mediating the chemical transformation of existing organic matter (Powell and Donat, 2001; Tani et al., 2003; Takata et al., 2004). Nevertheless, the concept of direct limitation of bacterioplankton by Fe, and thereby siderophore production, is still hotly debated. Indeed, subarctic Pacific bacterioplankton display low growth and production rates as compared to other open ocean regions, including other HNLC systems (Sherry et al., 2002; Liu et al., 2002), however, doubt remain with regard to the factor(s) controlling their development. Although experiments performed in the AG suggest that heterotrophic bacteria may be physiologically Fe-limited (Tortell et al., 1996) and that Fe availability controls bacterial community composition (Hutchins et al., 2001), other experiments suggest that bacterioplankton growth is primarily limited by carbon (Kirchman et al. 1990; 1993).

Gaining insight with regard to the factors driving bacterial processes in the Ocean is necessary to better understand the biogeochemistry of key elements such as Fe and carbon, and by extension to gain insights into Global change. Indeed, bacterioplankton control the carbon pump by remineralizing a significant fraction of the exported POC (Boyd et al., 2004) in the subarctic Pacific, and therefore act as a sink in place of a link

when Fe limitation is relieved. Here we provide a first estimate of Fe bioavailability using a heterotrophic bacterial bioreporter along a E-W transect crossing the subartic Pacific during summer 2003. Our results suggest that, in this region of the Pacific Ocean, variations in Fe bioavailability to heterotrophic bacteria are directly correlated to phytoplankton biomass in the mixed layer (in term of Chl *a*). Moreover, a significant fraction of bioavailable Fe is colloidal (0.02 – 0.45 μm) in nature.

2. Material and methods

2.1. Study area and sample collection

This study was conducted during R/V *Kilo Moana* cruise KM0311, which crossed the subarctic Pacific along an east-west transect in the early summer (June 24 to July 15) of 2003 (Fig. V.1). Six stations were investigated along the transect (Table V.I.). Following the hydrographic domains of Banse and English (1999), these stations can be defined as follow: stations S1 – S4 (140ºW- 170.5ºW) as the Alaskan Gyre (AG) stations of the central subarctic domain, station S5 (180ºW) as the boundary station between the Alaskan and the Western Subarctic Gyres (WSG) and station S6 (170ºE) as the Western Subarctic Gyre station. Station S6 was located at the vicinity of the SEEDS site (Tsuda et al., 2003).

Table V.1

Sampling dates and local time, locations, sampling depth and ambient parameters (Fluo : Fluorescence, T; surface temperature).

Station	Date	Local Time	Location	Depth (m)	T (°C)	S (‰)	Fluo
S1GF1	25-Jun-03	18:00	41.6°N, 140.1°W	15	14.4	32.95	0.44
S2GF3	2-Jul-03	11:00	42.6° N, 152.0°W	20	11.1	33.10	0.30
S3GF1	4-Jul-03	09:00	44.0° N, 159.6°W	20	10.4	32.90	0.51
S4GF1	7-Jul-03	09:00	47.0° N, 170.3°W	13	07.9	32.82	0.33
S4GF1	7-Jul-03	09:00	47.0° N, 170.3°W	26	07.3	32.88	2.60
S5GF2	12-Jul-03	09:00	47.0° N, 179.6°W	13	08.6	32.87	2.34
S6GF1	15-Jul-03	07:00	47.0° N, 170.1°E	20	08.5	32.88	1.51

At these stations a CTD rosette loaded with Niskin bottles was cast for Chl *a* and macronutrients. The Chl a concentrations were determined by HPLC analyses and were kindly provided by Dr. Brian Popp. Hydrographic data were collected using the ships seabird CTD. Seawater samples for bioreporter analyses were collected at 6 stations within the surface layer of the water column using acid-washed GoFlo bottles mounted on Kevlar line. Waters were homogenized in acid-cleaned container. Filtered (0.45 or 0.02 μm) mixed-layer seawater samples for bioreporter assay experiments were collected in acid-cleaned polycarbonate flasks and stored frozen (*ca.* -20º C) until use.

2.2. Estimation of Fe bioavailability to P. putida FeLux

Pseudomonas putida FeLux (Mioni et al., 2003) stock cultures were maintained on Pseudomonas Isolation Agar (PIA) supplemented with 50 μg mL^{-1} of tetracycline (Tc) at 30°C. Prior to the experiment, one colony was selected based on its response to Fe availability as described in Part IV. Cultures from the selected colony were maintained in microwave-sterilized (Keller et al., 1988) marine broth 2216 (Difco; 50 μg mL^{-1} Tc) at 25°C with shaking. Bioreporter cells were preacclimated in Fe-deficient BESAW medium (pFe 20.2) 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 filled with 18-mL aliquot of trace metal buffered BESAW medium (calibration treatments) or 18-mL aliquot of macronutrient-amended seawater. All treatments were performed in triplicate. The optical density at 600 nm (OD_{600}) and light production were measured every 2 hours over an experimental period of 12 hours using a spectrophotometer (Biomate 5, Thermospectronic corp.) and luminometer (FB-15, Zylux corp.). Cell number were estimated from OD_{600} using a previously empirically determined linear function (Mioni, Poorvin and Wilhelm, 2004). For all replicate, bioluminescence (light production) was normalized to cell abundance. Subsequently, concentrations of bioavailable Fe present in seawater samples, expressed as pFe, were estimated from linear regression of light production per cell on pFe determined from calibration treatments as described in Part IV. These Fe concentrations will be referred as

predicted pFe or inferred pFe in the text. Values reported here are the means of triplicate cultures from the t = 12 h time-point.

2.3. Statistical analyses

Statistical analyses for all the data presented here were performed using SPSS (ver. 12) software. Analyses of variance and independent *t*-tests (two-tailed) were performed assuming equal variance on mean values. The homogeneity of variance was tested in each analysis using the Levene test. For incubation experiments, Analysis of variance was used to establish the statistical significances of variation among different treatments. In parallel, multiple comparison tests were performed to determine where the differences lied. Dunnett's test was used to analyze the significance of the variations of the means of a set of amended treatments relative to the control treatment mean (Corston and Colman, 2003). The Tukey's honestly significant difference test (*Tukey-HSD* test) was used to establish the statistical significance of variations among a set of treatments means (Corston and Colman, 2003). For all analyses, a 95 % confidence interval was used. Results were considered significant at $p < 0.05$ unless otherwise stated.

3. Results

3.1. Laboratory characterization

To characterize the response of the bioreporter to Fe availability, preconditioned *P. putida* FeLux cells were incubated for 12 h in the trace-metal buffered synthetic seawater medium BESAW at various levels of Fe complexed to 100 μM EDTA ($[Fe^{3+}]$ = $10^{-20.2}$ M, $10^{-19.2}$ M, $10^{-18.6}$ M, $10^{-18.2}$ M, $10^{-18.0}$ M, $10^{-17.2}$ M, $10^{-15.9}$ M) (Figure V.2). As observed in Part IV, bioluminescence per cell increased linearly by a factor ~ 2.4 between treatment pFe 15.9 ($[Fe^{3+}] = 10^{-15.9}$ M) and treatment pFe 18.6 ($[Fe^{3+}] = 10^{-18.6}$ M). This increase in light production was highly significant (*ANOVA*, $p < 0.001$). The dose-

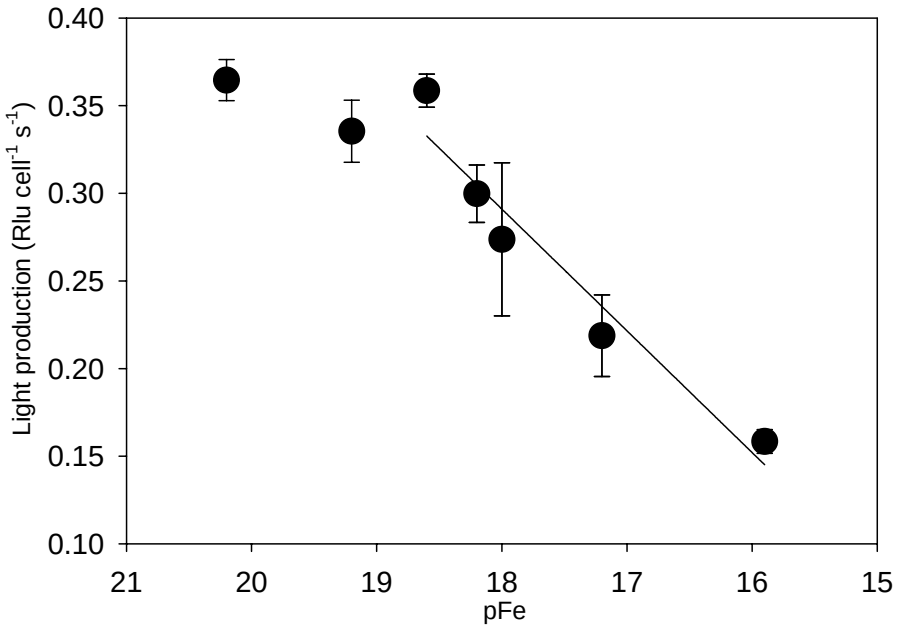

Fig. V.2. Dose-response characterization of the Fe bioreporter *P. putida* FeLux in trace metal buffered BESAW medium. Cell preconditioned in Fe-deficient BESAW ($Fe^{3+} = 10^{-20.2}$ M) were inoculated in BESAW medium at various iron levels complexed to 100 μM. Regression analysis extending from pFe 15.9 and pFe 18.6 is represented (R^2 = 0.996; *slope* = 0.0695 and *y*-intercept = -0.9593).

response signal saturated for treatments of Fe (III) concentrations below $10^{-18.6}$ M. All environmental samples tested were within the linear range of the calibration curve. A linear regression performed using the regression curve fitting of the Sigma Plot software (ver 9.0, SPSS corporation) in the linear region of this calibration curve provided the following equation (R^2 = 0.996):

$$y = 0.0695x - 0.9593.$$

Although the light production per cell was about two times lower than that observed in part IV, the slope of the calibration curve was similar to that obtained in Part IV (*slope*: 0.0701). This observation suggests that, despite differences in light production between experimental sets due to variability between bioreporter clones and variability in physiological status of the cells, there is a good reproducibility of the results. It highlights as well the importance of performing calibration treatments in parallel to the unknown

treatments for each experimental set to allow for a comparison of data sets. Although this may sounds constraining, traditional chemical analyses (*e.g.* colorimetric reaction-based analyses) require the same standards.

3.2. Study site description

Salinities – Salinities were similar between stations along the transect, averaging *ca*. 33.0‰ (Table V.1; Fig. V.3). Station S2 exhibited slightly higher salinities (*ca.* 33.0 – 33.3‰). This observation implies that we crossed several water masses along the transect.

Temperatures – The surface seawater temperatures decreased gradually by a factor ~ 2 from station S1 (14.4°C) to station S4 (7.9°C) (Table V.1; Fig.V.4). Surface temperatures and vertical profiles were nearly equal for the stations S4, S5, and S6. Surface summer mixed layer was shallower at the western stations (~ 20 – 50 m) of the transect than in the eastern stations (~ 75 m). These results are similar to previously

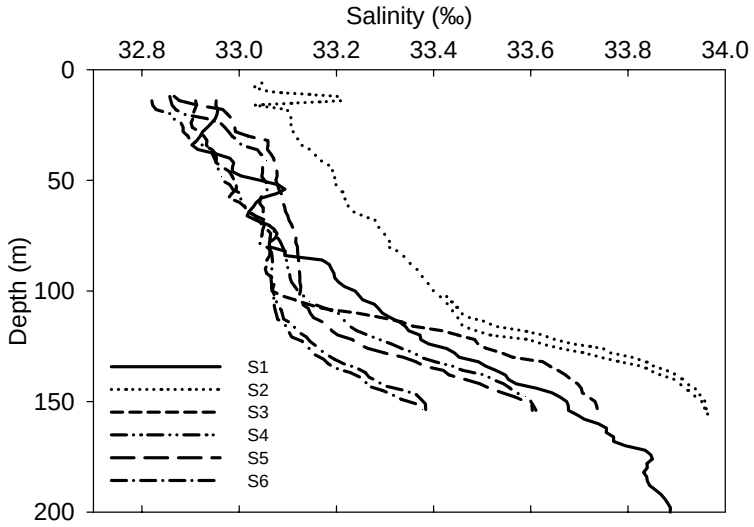

Fig. V.3. Salinities at six stations along a E-W transect across the subarctic Pacific during summer 2003.

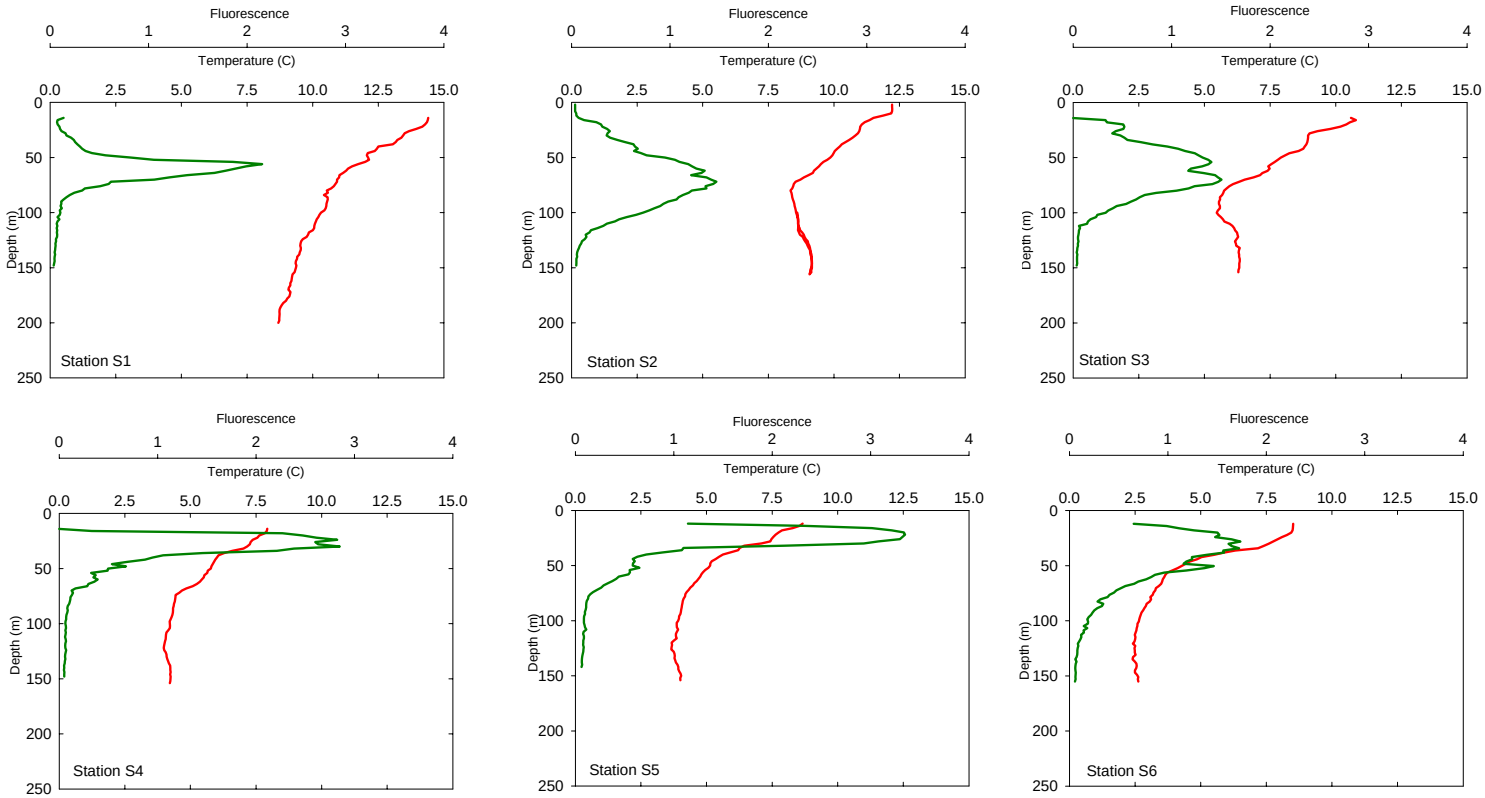

Fig.V.4. Phytoplankton biomass (in term of fluorescence, green profiles) and seawater temperature (red profiles) in the surface layer (200 m) of the water column of six stations along a NW transect in the subarctic Pacific Ocean during summer 2003.

reported range of temperatures during summer (Harrison et al., 1999 and ref. therein).

Fluorescence – As expected, maximal phytoplankton biomass (in term of fluorescence values was higher within the mixed layer of each station (Fig.V.4). Phytoplankton biomass was almost nill below 100 m depth at each station. Although fluorescence values have to be extrapolated with precaution, especially in Fe-limited regions, the mean fluorescence in the mixed layer of the three eastern stations (S1, S2, and S3) and the westernmost station (S6) were two times lower than that of the central stations (S4 and S5). This observation implies that phytoplankton biomass was the highest in the central part of the transect.

Chlorophyll a – Expectedly, Chl *a* concentration in the mixed layers of sampling station follows globally the same evolution than fluorescence (Fig.V.5A). Chl *a* concentrations mostly ranged from *ca.* 0.14 (Stations S1 – S2) to ~1 μg L^{-1} (Stations S4 – S5) except station S3 and S6 which displayed intermediate concentrations of 0.37 μg L^{-1} and 0.49 μg L^{-1} respectively. This range is similar to earlier measurements made in summer (Harrison et al., 1999; Banse and English, 1999).

3.3. Assessment of Fe availability along a E-W transect in the subarctic Pacific during summer 2003

Bioavailable Fe level in the dissolved fraction (< 0.45 μm) of summer surface mixed layer of the 6 stations investigated by using *P. putida* FeLux luminescent bioreporter are represented in Figure V.5A. Fe bioavailability increased significantly (*ANOVA*, $p < 0.001$) between station S1 and station S3, from pFe ~ 17.75 to pFe value at the lower limit of the calibration curve (*ca*. pFe ~15.9). No significant differences were observed between the predicted pFe means of stations S3, S4 and S5 (*Tukey's-HSD* test, $p > 0.05$). This observation implies that, although the high affinity Fe transport systems of *P. putida* FeLux cells were never fully repressed, conditions were close to repletion at these three intermediate stations. Fe bioavailability at the westernmost station (pFe 17.84) was not significantly different than that predicted at station S1 (*Tukey's-HSD*, $p > 0.05$).

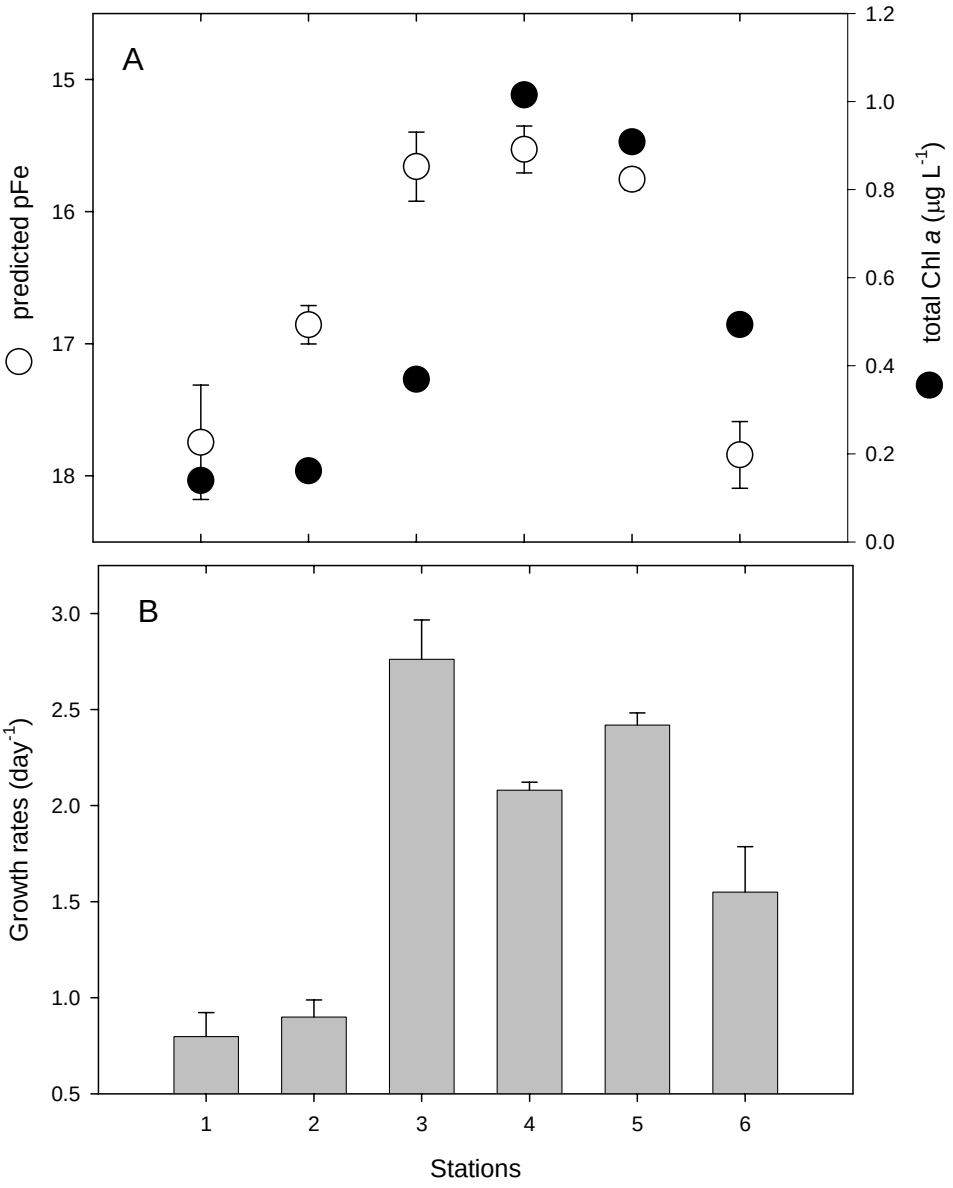

Fig. V.5. A: Total Chl *a* concentrations (●) and quantitative assessment of bioavailable Fe level (expressed as pFe, ○) using bioreporter strain *P. putida* FeLux. Cells preacclimated in BESAW (pFe 20.2) were used to inoculate 0.45 µm filtered seawater samples. Each assay was conducted in triplicate. Values reported here were determined from bioreporter luminescent signal upon 12 h of incubation using the calibration curve regression analysis (Fig. V.2). **B: growth rates of the bioreporter in each treatment.** Values are means ± S.D., n = 3 replicates per treatment (n = 6 for S1).

Taken together, these results suggest that the increase in Fe bioavailability in the surface water was sufficient to be sensed by heterotrophic bacteria such as *P. putida* FeLux and to allow for the repression of high-affinity Fe transports. Changes in Fe bioavailability is also reflected by variations in bioreporter cell growth rates (Fig.V.5B). Bioreporter cells divided two times faster when they grew in the surface mixed layer samples of the intermediate stations (S3-S5; $\mu = 2.1 - 2.7\ d^{-1}$) than when they were growing in the two easternmost stations (S1-S2, $\mu = 0.79 - 0.89\ d^{-1}$). Interestingly, bioreporter grown in station S6 seawater samples displayed intermediate growth rates (*ca.* $1.5 \pm 0.2\ d^{-1}$), although Fe bioavailability was similar to that at station S1 and significantly lower to that at station S2 (*Tukey's HSD* test, $p < 0.001$).

Along the transect inferred pFe appeared to follow an evolution mostly parallel to chl *a* concentrations in surface waters (Fig. V.5A), suggesting that the increase in Fe was also perceived by resident phytoplankton. However, the increase in chl *a* concentration at stations S2 and S3 appears low when compared to what would be expected based on bioavailable Fe levels predicted by *P. putida* FeLux.

3.4. Quantitative assessment of bioavailable Fe concentrations in the dissolved (< 0.45 μm) and soluble (< 0.02 μm) fractions of Station S4

To refine our assessment of Fe bioavailability, we compared the level of bioavailable Fe between two size fractions at the most productive station (station S4): the dissolved Fe fraction (< 0.45 μm) and the soluble Fe fraction (< 0.02 μm) (as defined by Nishioka et al., 2001). Indeed, the dissolved Fe fraction in this study comprises the soluble Fe fraction as well as the large labile particulate (> 0.1 μm) and the small colloidal (0.03 – 0.1 μm) Fe fractions (Nishioka et al., 2001). Two depths were tested within the surface mixed layer: 13 m depth, located above the chl *a* maximum (0.718 μg L^{-1}), and 26 m depth which corresponded to the chl *a* maximum depth (1.015 μg L^{-1}). Results are presented in Figure V.6. Fe bioavailability decreased significantly (*ANOVA, p* < 0.001) in the 0.02 μm filtered treatments as compared to the 0.45 μm filtered treatment for the two depths tested. Fe bioavailability was significantly lower (*Tukey's-*

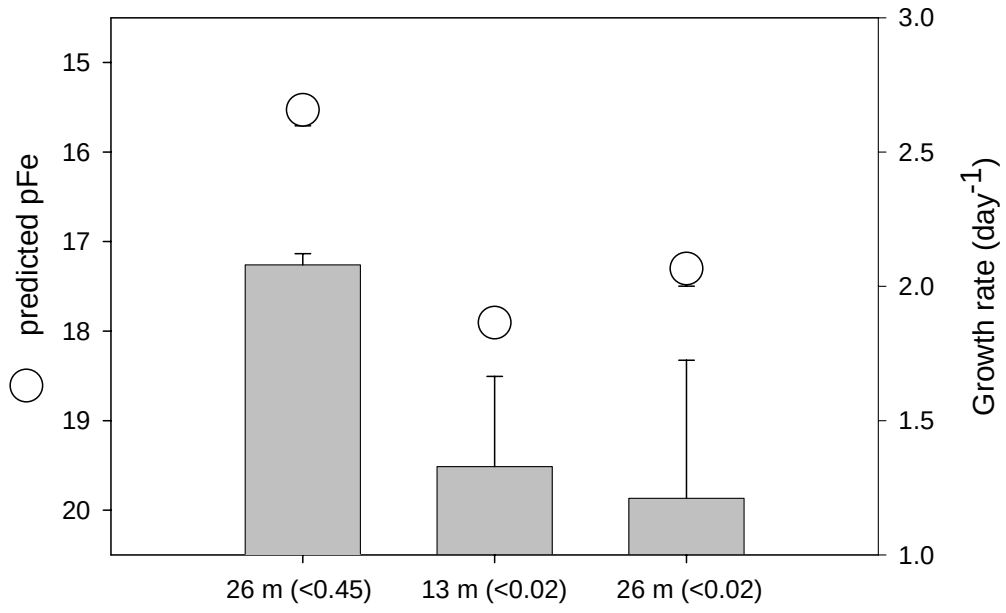

Fig. V.6. Growth rates (grey bars) and quantitative assessment of bioavailable Fe concentrations (○) in the dissolved (< 0.45 μm) and soluble (< 0.02 μm) fraction of station S4 using bioreporter strain *P. putida* FeLux. Cells were preacclimated in BESAW (pFe 20.2) were used to inoculate filtered seawater samples (0.45 μm or 0.02 μm). Each assay was conducted in triplicate. Values reported here were determined from bioreporter luminescent signal upon 12 h of incubation using the calibration curve regression analysis presented in Fig. V.2. Values are means ± S.D., $n = 3$ replicates per treatment.

HSD test, $p < 0.001$) in the soluble fraction at 13 m depth (pFe = 17.91 ± 0.03) as compared to that predicted for 26 m depth (pFe = 17.30 ± 0.19). Growth rates decreased by *ca.* 30% in the 0.02 μm filtered samples as compared to the 0.45 μm filtered treatment. The large standard deviations for the growth rate means in the 0.02 μm filtered treatment may reflect the physiological stress of the bioreporters. Such variations were not observed however for the light production, suggesting that the luciferase reaction was not impacted by Fe deficiency. Taken together, these results suggest that a substantial fraction of bioavailable Fe was present in the 0.02 – 0.45 μm size-class, including the large labile particulate Fe and the small colloidal Fe.

4. Discussion

Several conclusions can be drawn from this study. Our results further confirm that Fe-dependent bioreporters such as *P. putida* Felux can be used in complex oceanic systems to efficiently assess variations of Fe bioavailability at environmentally relevant Fe concentrations ranges. Inferred pFe values reported in this study fell in the same range than that reported for the HNLSiLC region of the Subantarctic Pacific (Part IV). Although we currently lack Fe concentrations data for this study (which does not allow us to go further in the comparison of the two regions), the similarity of these results suggests there is both reliability and reproducibility of the data acquired by our bioreporter analyses. Indeed, a heterotrophic bacterial luminescent bioreporter can be valuable tool to complement the traditional analytical techniques to improve our understanding of Fe biogeochemistry in oceanic systems. Moreover, our results demonstrate that Fe bioavailability in the WSG and AG is low enough to derepress the high-affinity transport systems of heterotrophic bacteria such as *P. putida* FeLux, and therefore to induce siderophore production. In contrast, increased Fe bioavailability in the central subarctic Pacific was sufficient to repress, at least partially, the expression of these high-affinity uptake systems. These results may have important implications for our understanding of Fe speciation in marine systems. Lastly, bioreporter analyses suggest that the 0.02 – 0.45 μm size class, including colloidal and small particulate Fe, contribute for a substantial

fraction of bioavailable Fe. To the best of our knowledge, this is the first study that directly demonstrated that Fe bound to natural colloidal-small particulate Fe fraction is bioavailable to heterotrophic bacteria. These different points will be discussed below.

4.1. Fe bioavailability in the subarctic Pacific

Grow out experiments (Boyd et al., 1996; Boyd and Harrison, 1999) and mesoscale Fe fertilization studies in the two gyres (Tsuda et al., 2003; Boyd et al., 2004) have now demonstrated that Fe controls phytoplankton growth in the subarctic ocean. In contrast, although it is now recognized that heterotrophic bacteria growth rates are lower in the subarctic Pacific than in most open ocean systems including HNLC regions (Sherry et al., 2002; Liu et al., 2002), the concept of direct Fe limitation of the heterotrophic bacterial community remains a source of debate. Only two studies performed in the Southern Ocean suggests that the bacterioplankton are directly limited by Fe (Pakulski et al., 1996; Arrieta et al., 2004) and another, also performed in the same HNLC region, suggests a co-limitation by both carbon and Fe (Church et al., 2000). Moreover, bacteria activity has been shown to be enhanced by Fe fertilization in the AG, leading to an increase in remineralization rates from particles and an important increase (~35%) in DIC concentrations in the surface waters of the mixed layer (Boyd et al., 2004). Another report in which the Fe quota of an Fe-deficient laboratory culture were compared to that of natural bacterioplankton community in the northeastern subarctic Pacific, suggests that resident bacterial assemblage may be physiologically limited by this micronutrient (Tortell et al., 1996). Other investigations from the AG have also reported that, although resident bacterioplankton biomass accumulates Fe at a faster rate than phytoplankton biomass, surface specific uptake rates in the bacteria size-class is more than 30 times slower than that of phytoplankton (Maldonado and Price, 1999). Recent laboratory experiments further suggest that nitrogen utilization is impaired under Fe-depleted condition (Kirchman et al., 2003). On the other hand, field studies in the subarctic Pacific suggest that bacterial growth rates and ammonium utilization are primarily regulated by the supply of organic carbon and *in situ* temperature in the Pacific north-east (Kirchman et al., 1990, 1993). However, none of these studies directly examined Fe-limitation, and

conclusions were based on correlations between phytoplankton (the main source of organic substrate) and bacterial distributions.

Although we cannot discard the hypothesis of carbon limitation, our results clearly indicates that Fe availability was low enough in at least three stations (S1, S6, and in less extent S2) to derepress the high affinity transport systems of *P. putida* FeLux. Stations S1 and S2 coincided with the lowest chl *a* concentrations observed along the transect, thereby carbon may have been as well co-limiting at these stations. However, increases in Fe bioavailability and in phytoplankton biomass appear decoupled at station S2. Also, although phytoplankton biomass (in term of chl *a*) was more than two times higher at the station S6 relative to both stations S1 and S2, bioavailable Fe concentrations predicted from the bioreporter cells were not significantly different than that of station S1. Indeed, carbon and Fe availabilities appeared decoupled in an inverse fashion at these two stations (S2 and S6), suggesting that the phytoplankton biomass may have been controlled by other factors in addition to Fe bioavailability (*e.g.* temperature, macronutrient concentrations or community structure). The high salinity at station S2 may suggest that subtropical oligotrophic waters may have intruded in this frontal region, but the physical environment at station S6 was similar to the adjacent stations. Nevertheless, our results imply that the bacterioplankton may be at least co-limited by Fe and carbon at these stations. In contrast, Fe bioavailability in the central subarctic Pacific stations (S3, S4, and S5) was high enough to partially repress the high affinity transport system of the bacterial bioreporter. Bioreporters growth rates were also significantly higher at these intermediate stations and were close to optimal growth rates observed for this bacterial strain in Fe-replete laboratory cultures (Mioni et al., 2003). This increase in bioavailability was also positively correlated to an increase in phytoplankton biomass. Indeed, the surface waters may have been enriched in Fe locally, either through dust deposition from Aleutian volcanoes or, more realistically, from the Asian arid lands (Boyd et al., 1998), or through advection of Fe-rich coastal water transported by the Alaskan stream (Banse and English, 1999; Harrison et al., 2004). These observations may have important implications with regard to our understanding of Fe and carbon flows in oceanic systems. Indeed, if bacterioplankton is Fe limited in some areas of the WSG and

AG, as our results suggest, they may indeed produce siderophores which will globally increase Fe bioavailability in the surface mixed layer by increasing Fe solubility, but will also partition the bioavailable Fe pool (Hutchins et al., 1999; Weaver et al., 2003). As a result, bacterioplankton may directly compete with the phytoplankton for the limiting resource as suggested previously (Maldonado and Price, 1999).

4.2. Bioavailability of the colloidal and small particulate phase

Because Fe bioavailability is influenced both by its physical and chemical speciation (Gobler et al., 2002), we investigated the differential bioavailability of the dissolved Fe size-class and the soluble Fe size class at station S4. A commonly used definition of dissolved Fe fraction (< 0.45 μm) includes both colloidal and chelated Fe, and eventually the biota itself. Gaining a better understanding with regard to the bioavailability of the Fe in colloidal and small particulate fractions is of interest as they can constitute a substantial fraction of the dissolved Fe fraction in seawater (Nishioka et al., 2001). Indeed, Fe tends naturally to form colloids at seawater pH (Martin et al., 1995) and chemical analyses performed in the Central North Pacific and other open ocean regions suggested that more than 99% of dissolved Fe was chelated to organic ligands (Rue and Bruland, 1995).

It is generally assumed that colloids are not bioavailable to phytoplankton and that the low molecular weight size class is the only fraction bioavailable to planktonic organisms (Rich and Morel, 1990; Sunda, 2001; Wells, 2003). Although, recent Fe assimilation experiments suggest that natural oceanic and coastal colloidal Fe can be efficiently used by cyanobacteria (Wang and Dei, 2003) or diatoms (Chen et al., 2003), these results were acquired through the introduction of high amount of exogenous inorganic Fe (2 – 7nM!) to the seawater sample and therefore does not reflect truly the bioavailability of natural Fe colloids to planktonic organisms. Moreover, surface washing steps (*e.g.*,Ti-citrate-EDTA) necessary to remove superficially associated extracellular Fe (Hudson and Morel, 1989) was omitted in one of these two studies (Wang and Dei, 2003), and may have altered their interpretations. By contrast to phytoplankton, it is presently assumed that heterotrophic bacteria can efficiently solubilize particulate Fe (*e.g.*

Fe hydroxides) through their siderophore transport systems (Sunda, 2001). Since siderophores are themselves included in the low molecular weight colloid size-class (0.3-1.0 KDa) (Macrellis et al., 2001), they cannot enter the cell through porins or simple diffusion (Andrews et al., 2003). Two major transport systems are thought to mediate the acquisition of Fe chelated to such organic binding ligands: the siderophore uptake systems, which involves ligand-specific outer membrane transport systems, and the ferrous or ferric ion membrane transporters that bind to the free ion after photoreduction of the ligand-Fe(III) complex (Sunda, 2001). Owing to the high energetic cost necessary for the production and maintenance of siderophore uptake systems, they are only induced under Fe-stress and therefore are not likely to contribute to Fe acquisition in Fe-replete environment (Andrews et al., 2003).

At station S4, one may assume that siderophores were not likely to be abundant in the surface waters of the mixed layer. Indeed, bioreporter cells were showing reduced expression of high affinity transport systems and optimal growth rates, implying that the resident heterotrophic bacteria assemblage was close to Fe repletion. Regeneration through grazing and/or viral mediated lysis is therefore most likely to have driven the recycling of organically bound Fe in surface at station S4. Laboratory evidences suggest that viral-mediated lysis releases a large fraction of Fe in the soluble size-class, *ca.* < 200 kDa or < 0.03 μm (Nishioka et al., 2001), but also in the soluble-colloidal (30 kDa – 0.2 μm) size-class (Poorvin et al., 2004). Unfortunately, the cut offs used for the fractionation of Fe in these experiments does not allow to distinguish for the relative contribution of the colloidal fraction and the rest of the soluble fraction (30KDa – 200KDa) in the Fe pool and may have lead these authors to overestimate the bioavailability of the truly soluble Fe. Other studies performed on other model organisms suggest that viral-mediated lysis release only 5% of Fe in the dissolved fraction (Gobler et al., 1997), implying that the physical speciation of Fe released through viral lysis depend on the identity of the organism being lysed.

Results presented here confirm that soluble Fe (< 0.02 μm) in the surface waters of the mixed layer constitutes a non-negligible pool of bioavailable Fe to heterotrophic bacteria. Nevertheless, removal of the small colloidal and large labile particulate Fe

resulted in a significant decrease of Fe bioavailability to *P. putida* FeLux, implying that heterotrophic bacteria may rely on at least a fraction of these larger size-class. A recent study performed during summer in the subarctic Pacific suggests that concentrations of the small colloidal Fe are generally lower (0.01 – 0.06 nM) than that of the truly soluble Fe (0.05 – 0.07 nM) in the mixed layer of the HNLC waters of the AG (OSP station; Nishioka et al., 2003). Moreover, low concentrations of small colloidal Fe correlated to areas that are not productive, such as summer mixed layer of the station OSP (Nishioka et al., 2003). However, limitation in the analytical method may have led these authors to underestimate a significant fraction of the organic ligands present in the small organic/inorganic colloidal phase. Indeed, their method only detects Fe weakly bound with organic ligands. Strong organic ligands, such as the L1-class ligands which are mostly distributed in surface of the mixed layer in the Central North Pacific (Rue and Bruland, 1995), or Fe-organic complexes where the Fe is covalently bound (*e.g.*, Rieske Fe-S centers from dead cells) may have therefore been unnoticed by Nishioka et al. (2003).

As discussed earlier, viral- and grazing- mediated lysis of ambient phytoplankton may result in an increase of Fe in different size fractions, including intracellular organically bound-Fe (Poorvin et al., 2004). Microzooplankton grazing is known to be one of the factors keeping in check phytoplankton biomass in the subarctic Pacific, therefore, one may assume that zooplankton grazers contribute to a substantial fraction of the Fe recycling in the mixed layer. Also, in contrast to bacterial siderophore concept, it has been demonstrated that the cultures of the ubiquitous phytoplankton species *Emiliana huxleyi* can release Fe organic ligands with stability constant comparable to that of DFB (Log K_{FeL} = 21.5 ~ 21.6) when Fe is added (Boye and van den Berg, 2000). Moreover, at least two mesoscale Fe fertilization experiments performed in different HNLC regions (IronEX II: equatorial Pacific; SOIREE: Southern Ocean) resulted in an increase of organic ligands associated with the increase in phytoplankton biomass (Rue and Bruland, 1997; Boyd, 2002). It is therefore possible that organic ligands were released either through lysis of plankton cells or through release of organic ligands similar to those produced by *Emiliana huxleyi* cells. Such ligands may have been present in the small

colloidal Fe size-class of surface layer of Station 4 and may have been available to *P. putida* FeLux. Our results suggest that the soluble Fe fraction (*i.e*. the Fe species thought to be the most available to phytoplankton) was slightly but significantly higher at the chlorophyll maximum depth (26 m) as compared to 13 m depth. This result appears paradoxical as a greater depletion of soluble Fe would be expected in proximity to higher phytoplankton biomass. Since viral-mediated lysis results in the release of a significant portion of Fe (*ca*. > 50%) in this size-class, it may have contributed to the observed increase of Fe in the soluble size-class at this depth. However, analytical analyses would be required to support this hypothesis.

5. Conclusion

In summary, Fe bioavailability in the subarctic Pacific varies greatly between regions as determined by bioreporter analyses. Although we cannot rule out a carbon-Fe colimitation, our results demonstrate that the bacterioplankton can be directly limited by Fe in at least three of the stations investigated. Our results further confirm the tight relationship between Fe bioavailability and Fe speciation. Although kinetics considerations suggest that colloidal and small particulate Fe is not bioavailable to phytoplankton, it appears that this size fraction constitutes a non-negligible reservoir of bioavailable Fe to bacteria.

Acknowledgements

We would like to thank Debora Iglesias-Rodriguez, Darren Hepworth, Gary Fones, Jim Moffett, Seth John and others, as well as the captain Chris Best and all the crew members of R/V *Kilo Moana* for assistance in, among many other things, sample collection. C.E.M would like to thank especially Gabe Foreman, the "wizard of the electronic" who repaired the luminometer in the middle of the Bering Sea, eventually allowing her to finish this Ph.D. work. And she would like to thank as well Lawson Worrell IV for his loving support all over this cruise. Support was provided by NSF OCE – 0002968 and NSF INT-0240092 to SWW, as well as by a University of Tennessee Center for Environmental

Biotechnology Graduate Fellowship, a Ryan Kelley Memorial scholarship – International Women's Fishing Association 2003, and a Daniel Jouvance Award "Recherches sur le plancton marin 2003" to CEM.

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APPENDIX (PART V)

Appendix V.1 –

Iron in the surface waters of the subarctic Pacific Ocean (adapted from deBaar and de Jong, 2001; their table A2). Stations of the KM0311 cruise are indicated between brackets for corresponding published sampling sites.

°Lat., °Long.	Depth (m)	Date	Fe range (nM)	Mean [Fe] (nM)	Reference
Alaskan Gyre					
50°00'N, 145°00'W	20 – 50	Aug 1987	0.05 – 0.06	0.055	Martin et al. (1989)
50°00'N, 145°00'W		May, Sep 1995	0.10 – 0.60	0.10 - 0.60	La Roche et al. (1996)
50°00'N, 145°00'W	0 – 35	May 1995	N.A.	<0.29	Boyd et al. (1998)
50°00'N, 145°00'W	0 – 100	Sep 1997	0.06 – 0.12	0.12	Nishioka et al. (2001)
50°00'N, 145°00'W	0 – 100	Jun 1998	0.07 – 0.11	0.09	Nishioka et al. (2001)
50°00'N, 145°00'W	0 – 100	Sep 1998	0.11 – 0.16	0.13	Nishioka et al. (2001)
50°00'N, 145°00'W	0 – 800	Jun 1998	N.A.	0.03	Johnson et al. (2003)
49°59'N, 145°08'W	0 – 200	Jul 1999	N.A.	<0.28	Suzuki et al. (2002)
49°53'N, 144°54'W	0 – 40	Sep 1997	N.A.	< 0.28	Fujishima et al. (2001)
45°00'N, 142°87'W (S1)	20 – 100	Aug 1987	0.06 – 0.42	0.08	Martin et al. (1989)
52°65'N, 145°W	0 – 100	Jun 1998	N.A.	0.04	Johnson et al. (2003)
49°57'N, 138.67°W	0 – 800	Jun 1998	N.A.	0.02	Johnson et al. (2003)
49°28'N, 134.67°W	0 – 600	Jun 1998	N.A.	0.02	Johnson et al. (2003)
48°97'N, 130.67°W	0 – 35	May 1995	N.A.	~1.96	Boyd et al. (1998)
48°97'N, 130.67°W	0 – 800	Jun 1998	N.A.	0.05	Jonhson et al. (2003)
48°65'N, 126.67°W	0 – 35	May 1995	N.A.	>2.94	Boyd et al. (1998)
48°65'N, 126.67°W	0 – 600	Jun 1998	N.A.	0.12	Johnson et al. (2003)
59°N, 148°W	20 – 80	Aug 1987	0.08 – 0.12	~0.10	Martin et al. (1989)

Appendix V.1 – Continued

°Lat., °Long.	Depth (m)	Date	Fe range (nM)	Mean [Fe] (nM)	Reference
Western subarctic Gyre					
43°00-47°30'N, 175°30'E	0 – 200	Jul 1994	0.25 – 1.04	~0.57	Kuma et al (1998)
45°00'N, 165°00'E	0 – 40	Sep 1997	N.A.	<0.28	Fujishima et al. (2001)
~48°N, 168°E (S6)	0 – 200	Jul 1999	0.02 – 0.04	~0.03	Suzuki et al. (2002)
40°00'N, 155°00'E	5 – 125	Jun 2002	0.00 – 0.18	~0.06	Takata et al. (2004)
41°30'N, 145°57'E	5 – 75	Jun 2002	0.13 – 0.57	~0.3	Takata et al. (2004)
41°00'N, 155°00'E	5 – 50	Jun 2002	0.03 – 0.2	0.1	Takata et al. (2004)
44°00'N, 155°00'E	5 – 75	Jun 2002	0.10 – 0.28	~0.15	Takata et al. (2004)

Part VI

VIRUS AND SIDEROPHORE-MEDIATED TRANSFER OF AVAILABLE FE BETWEEN HETEROTROPHIC BACTERIA: CHARACTERIZATION USING AN FE-SPECIFIC BIOREPORTER

Part VI

VIRUS AND SIDEROPHORE-MEDIATED TRANSFER OF AVAILABLE FE BETWEEN HETEROTROPHIC BACTERIA: CHARACTERIZATION USING AN FE-SPECIFIC BIOREPORTER

This chapter is a revised version of a paper by the same name to be submitted to the journal *Aquatic Microbial Ecology* in 2004 by Cécile E. Mioni[*], Leo Poorvin[*], and Steven W. Wilhelm.

[*] These authors contributed equally to this study.

My use of "we" in this chapter refers to my co-authors and myself. My primary contributions to this paper include (1) experimental design and realization of the experiments involving the heterotrophic bioluminescent reporter *Pseudomonas putida* FeLux (except Fe uptake experiment, collective work with Leo Poorvin), (2) extraction and characterization of *Pseudomonas putida* FeLux siderophores (collective work with Leo Poorvin), (3) data analyses, (4) most of the gathering and interpretation of literature, (7) pulling the various contributions into a single paper, and (8) most of the writing.

1. 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., 2002). Due to a low solubility in oxic marine surface waters, rapid removal rates via export and particulate scavenging, and low external input from atmospheric dust deposition (Martin and Gordon, 1988; Duce and Tindale, 1991) and/or deep water upwelling (Coale et al., 1996), Fe levels in oceanic surface waters are globally low, averaging 0.07 nmol kg^{-1} (Johnson et al., 1997). This growth limiting concentration of Fe has been suggested in

many studies to control phytoplankton production (Boyd, 2004). Moreover, most (> 99%) of the dissolved Ferric Fe is complexed by strong organic ligands, leaving less than 0.1 pmol kg^{-1} as free ferric species (Rue and Bruland, 1995; Wu and Luther, 1995). During the past decade, several studies have demonstrated how organic complexation can control Fe concentration and Fe speciation and thus its bioavailability to marine plankton (Gledhill and van den Berg, 1994; Wu and Luther, 1995; Boye et al., 2001). Although uncertainties remain regarding the source(s) and chemical structure of these organic ligands, a compilation of data from the literature suggests that they fall into two distinct ligand classes, denoted L1 and L2. It has been proposed by some researchers that the strong ligand class (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 (Rue and Bruland, 1995; Butler, 1998; Tortell et al., 1999; Powell and Wilson-Finelli, 2003). More recently we have proposed that a significant component of these Fe-ligand complexes are the byproducts of cellular mortality (Poorvin et al., 2004). And while little is known with regard to the relative bioavailability and the fate of these organic ligands in natural systems, recent lab experiments suggest that the chemical nature of these Fe complexes may influence competition between prokaryotes and eukaryotes for this limited micronutrient (Hutchins et al., 1999a).

Although an important role for heterotrophic bacteria in the pelagic C cycle has been recognized for many years (Pomeroy, 1974; Azam et al., 1983), their role in Fe biogeochemistry has only recently been considered. The similarities between the stability constants (Rue and Bruland, 1995; Lewis et al., 1995) and structural groups (Martinez et al., 2003; Gledhill et al., 2004) of the natural organic Fe ligands and those for naturally produced siderophores has lead to a surge of interest with regard to the microbial component of the marine plankton assemblage, in spite of results that demonstrate dissociation constants of Fe-ligand complexes which preclude significant concentrations of siderophores (Witter et al., 2000).

It has been demonstrated that marine heterotrophic bacteria account for as much as 50% of the biomass (and thus biogenic Fe) in oceanic systems (Whitman et al., 1998). Furthermore, bacteria contain significantly more Fe per biomass unit than phytoplankton

and are responsible for significant Fe uptake in Fe-depleted seawaters (Maldonado and Price, 1999). As well, although it bacterial productivity is primarily limited by carbon (Kirchman et al., 2000), evidence suggests that heterotrophic bacterial productivity may be Fe-limited at low, *in situ* concentrations (Pakulski et al., 1996). Finally, conflicting reports suggest that Fe may (Eldridge, 2004) or may not (Hutchins et al., 2001; Arrieta et al., 2004) influence bacterial diversity. However, the question of how resources (bottom-up) and predator factors (top-down) control and limit the development of bacterial populations is not fully resolved yet (Flaten et al., 2003).

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 and Colwell, 2000), resulting in a global population of *ca.* 3.5×10^{29} viruses in the world's oceans (Wilhelm and Suttle, 2000). Moreover, findings suggest that prokaryotic viruses dominate aquatic systems (Fuhrman, 1999; Breitbart et al., 2002). As such, several researchers have postulated that viruses play and important role in the regeneration of organically-complexed nutrients in aquatic systems (Gobler et al., 1997; Fuhrman, 1999; Wilhelm and Suttle, 1999). Very little is known with regard to the chemical nature, molecular size and bioavailability of nutrients such as Fe that are released during the phage-mediated lysis of natural populations of prokaryotes. Recent laboratory experiments, using model planktonic organisms, have shown that such a viral lysis resulted in the release of a range of dissolved to particulate Fe, mostly in the low molecular weight size-class (< 3 kDa) 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, virus control of 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 diversifying organically-bound Fe sources that are, at least in part, readily available to the prokaryotic component of the oceanic food web. To test this hypothesis, we compared the bioavailability of Fe released from the lysate of the heterotrophic bacterium *Vibrio natriegens* PWH3a to that of Fe complexed to a model synthetic Fe chelator (EDTA) using a heterotrophic bioluminescent reporter for Fe availability (*Pseudomonas putida* FeLux). Combined with estimates of Fe assimilation from ^{55}Fe-labeled lysates by a bioluminescent bacterial reporter, our data suggest that organic Fe complexes released during viral lysis are both highly available and efficiently assimilated by bacterial cells. To contrast this to classic high-affinity (siderophore-mediated) transport by prokaryotes, 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. The results support our working hypothesis that viral activity plays a crucial role in the recycling of biologically available Fe complexes in surface seawater.

2. Material and methods

2.1. Production of virus-mediated bacterial lysates

To reduce residual 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 used in this study were soaked in dilute HCl and rinsed with Chelex-100 treated Milli-Q water prior use. All manipulations were performed under a class-100 conditions to maintain both 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 (enriched seawater, artificial water; Berges et al., 2001) supplemented with glycerol and ^{55}Fe (3.33×10^{-9} M total Fe concentration) at 25°C for 4 days. Cells were collected by centrifugation and washed with

Ti(III)-citrate-EDTA (Hudson and Morel, 1989) to remove surface associated Fe. Cells were then resuspended in carbon-free ESAW medium supplemented with non-radioactive Fe (1×10^{-9} M) and the lytic bacteriophage PWH3a-P1. Bacterial cells and virus particles were incubated for 24 hours to allow for virus infection and subsequent cell lysis. The dissolved fraction of the lysate was collected after filtration (0.22 μm). The final Fe concentration in the lysate was estimated at 4.33×10^{-9} mol L^{-1} (3.33×10^{-9} M ^{55}Fe and 1×10^{-9} M Fe).

2.2. Estimation of Fe bioavailability to P. putida FeLux

Pseudomonas putida FeLux (Mioni et al., 2003) stock cultures were maintained on Pseudomonas Isolation Agar (Remel) supplemented with 50 μg mL^{-1} of tetracycline (Tc) at 30°C. For each experiment an individual colony was selected and amplified to a stock culture for use with all treatments. Cultures from the selected colony were maintained in microwave-sterilized (Keller et al., 1988) marine broth 2216 (Difco) supplemented (50 μg mL^{-1} Tc) at 25°C with shaking. Bioreporter cells were acclimated in Fe-deficient BESAW medium (pFe 20.2) 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 hours over an experimental period of 12 hours 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 complexes were prepared as described previously (Part IV). Sterile aliquots of Fe-free BESAW (18 mL) were dispensed into acid washed and microwave sterilized Oakridge tubes. The concentration of $FeCl_3 \cdot 6H_2O$ was altered to create an increasing range of free Fe^{3+} spanning from pFe 20.2 to pFe 15.9 (pFe = -log [Fe^{3+}]. The final concentration of EDTA was maintained constant in all treatments (100 μM).

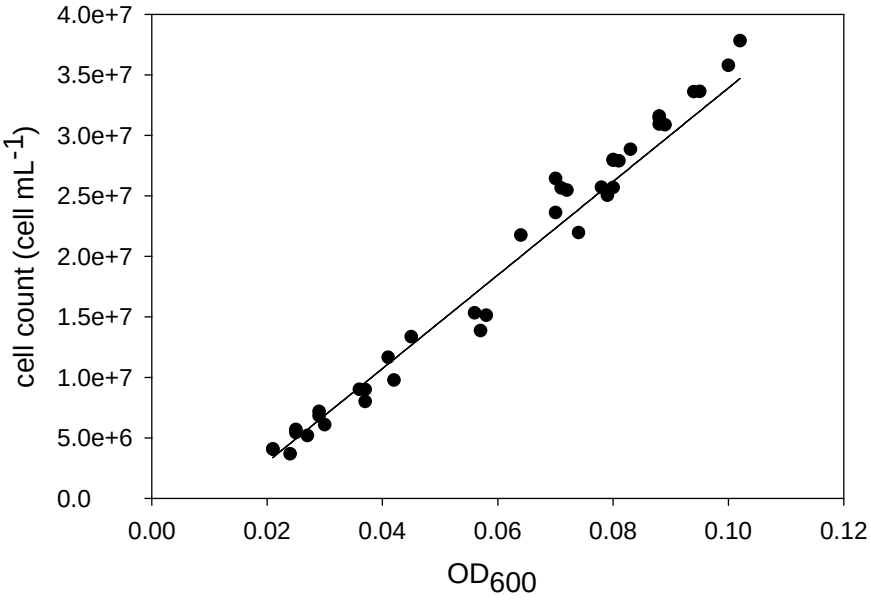

Fig. VI.1. Laboratory characterization of *P. putida* FeLux as a bioreporter in the trace metal buffered BESAW medium. Typical calibration curve of OD_{600} and cell density in BESAW medium with resulting linearization ($R^2 = 0.97$; *slope* = 3.866×10^8, *y*-intercept = $- 4.743 \times 10^6$).

Light production per OD_{600} values were converted to light production per reporter cells mL^{-1} using the empirically determined linear function (Fig. VI.1):

$$y = 3.459 \times 10^8 - 3.611 \times 10^6 x$$

where y is the number of bioreporter cell per milliliter and x the corresponding OD_{600} value. Values are the means of triplicate cultures from the time-point for which the signal was the strongest (t = 12 h).

Treatments amended with ^{55}Fe-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 diluted in sterile Fe-free BESAW medium (total volume = 18 mL) to obtain the following final total Fe concentrations: 43.3×10^{-12} mol L^{-1}, 110×10^{-12} mol L^{-1}, 160×10^{-12} mol L^{-1}, 210×10^{-12} mol L^{-1}, 540×10^{-12} mol L^{-1}, and 4.33×10^{-9} mol L^{-1}. The last was treatment being composed fully of lysate (100% v/v). Chelex-100 treated glycerol was supplied

directly to replicate tubes to obtain a carbon level similar to that in the other treatments. For all lysate-amended treatments, in parallel to bioluminescence and OD_{600} monitoring, 1 mL of culture was fixed with glutharaldehyde (2.50 % v/v) at t = 12 hours and t = 24 hours and enumerated by epifluorescence microscopy after treatment with acridine orange (Hobbie et al., 1977) on a Leica DMRXA epifluorescence microscope.

2.3. Lysate uptake studies

Fe-uptake was assessed for the lysate-amended treatments as previously described (Poorvin et al., 2004). At time t = 12 hours and t = 24 hours, 2 mL of each lysate-amended treatment were collected by filtration through 47 mm diameter, 0.2 μm polycarbonate filters. Extracellular Fe was removed by washing the filters with Ti(III)-citrate-EDTA (Hudson and Morel, 1989). Filters were placed into 4-mL scintillation vials and dissolved for 1 hour with 500 μL of ethyl acetate. Scintillation fluid was added prior to analyses for ^{55}Fe. Reported values are the mean uptake rates of triplicate cultures normalized to cellular carbon content (23.3 fg $cell^{-1}$, Poorvin et al., 2004 and ref. therein). Preliminary tests demonstrated that residual bioluminescence does not interfere with the results from the Wallac scintillation counter (data not shown).

2.4. 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 ($FeCl_3$) 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 and 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. The sterile medium was supplemented with a filter-sterilized enriched vitamin solution

(Trick and Wilhelm, 1995). 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 filtrate (< 0.22 μm) of the bacterial cultures as described previously (Wilhelm and 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.

2.5. Characterization of siderophores

Extracts were assayed for Fe-binding compounds by thin layer chromatography (TLC) on cellulose plates (Merck) using methanol:H_2O (70:30, v/v) as a solvent. Fe binding compounds were resolved by spraying the dried TLC plate with 1.0% $FeCl_3$ in ethanol (Wilhelm and Trick, 1994).

To estimate the total concentration of Fe-binding compounds, we performed the Chrome Azurol S (CAS) assay (Schwyn and Neilands, 1987) Standard curves for the CAS assay were performed 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).

2.6. 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 to 18 mL of BESAW medium supplemented with 15 nM of $FeCl_3$ and 5 nM or 15 nM of the siderophore extract. For comparison, the following two treatments were

also tested: inorganic Fe (15 nM $FeCl_3$) 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 assimilated by the *P. putida* FeLux bioreporter (Mioni et al., 2003). Light production and bacterial density were determined as described above. Treatments for calibration of the bioreporter bioluminescence were prepared and analyzed as described earlier in the text. All treatments were repeated in triplicate.

To demonstrate that the bioluminescent response to the fungal siderophore DFB was due to an inability of the reporter cells to acquire Fe from this source, we evaluated the impact of equimolar Fe:DFB complex additions on the bioreporter in the presence of sufficient total Fe (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-time 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 $FeCl_3$ plus the Fe:DFB premix to the final concentrations of 0 nM (control), 5 nM, 10 nM, 15 nM, and 20 nM DFB. EDTA was omitted from the recipe. All treatments were triplicated. Assuming that the added DFB bound to Fe(III) in 1:1 ligand stochiometry (Rue and Bruland 1995; Liu and Hider, 2002), the total inorganic Fe concentration was 15 nM between treatments.

2.7. 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 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 and 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 and Colman 2003). Unless stated, results were considered significant at $p < 0.05$.

3. Results

3.1. Bioavailability of Fe released from virus-mediated lysate

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 18.6 ($Fe^{3+} = 10^{-18.6}$ M) to pFe 15.9 ($Fe^{3+} = 10^{-15.5}$ M) (Fig.VI. 2A). Linear regression analysis was performed over this linear region using Sigma-Plot software (ver. 9, SPSS Inc.) yielding the equation $y = 0.027x - 0.079$ ($R^2 = 0.995$). The luminescent signal was saturated for pFe values higher than 18.6 as no significant differences were observed between the treatments pFe 20.2, pFe 19.2 and pFe 18.6 (*ANOVA*, $p > 0.05$). The results suggest that that all high-affinity transport systems were derepressed at free Fe^{3+} concentrations lower than $10^{-18.6}$ M (*ca.* 200 nM of EDTA-buffered total Fe).

The bioluminescent response of *P. putida* FeLux in treatments supplemented with increasing levels of Fe derived from the *V. natriegens* PWH3a lysate followed also a sigmoidal function ($R^2 = 0.997$; Fig.VI.2B). Light production reached a maximal level of 0.037 ± 0.013 Rlu s^{-1} $cell^{-1}$ in treatments supplemented with 43.3 pM to 0.16 nM of 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 ≥ 0.21 nM in lysate treatments, relative to total Fe concentrations > 200 nM (*i.e.* pFe 18.6) when Fe was complexed to EDTA. 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 the trace-metal buffered Fe in BESAW medium. The dose-response elicited by the addition of Fe in form of bacterial lysate was dramatically greater than when Fe was provided as Fe-EDTA complex (Fig.VI.2C). Light production

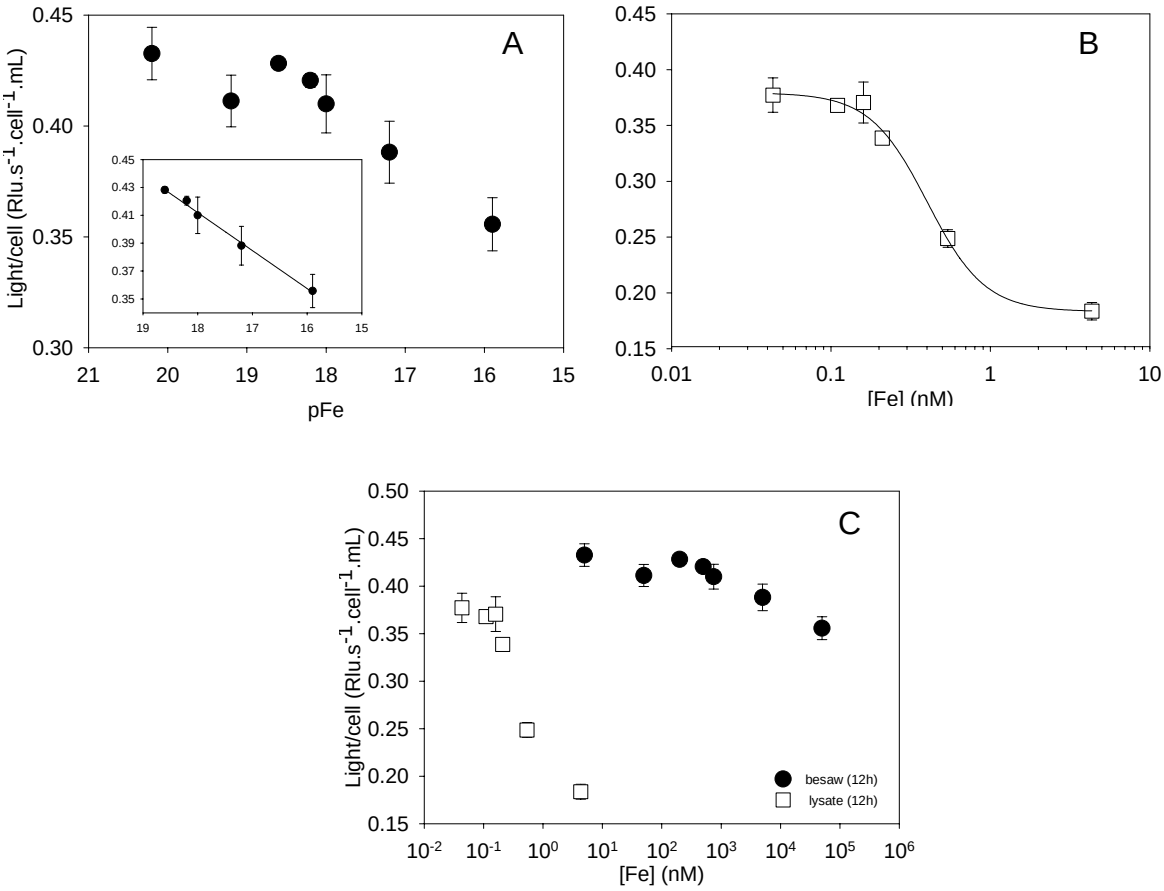

Fig. VI.2. Dose-response characterization of the Fe bioreporter *P. putida* FeLux in BESAW medium. A: Dose-response in the trace-metal buffered BESAW medium. Cells preconditioned in Fe-deficient BESAW (pFe 20.2) were inoculated into BESAW medium at various Fe concentrations with 100 μM EDTA (pFe 20.2, pFe 19.2, pFe 18.6, pFe 18.2, pFe 18.0, pFe 17.2, pFe 15.9). Regression analysis extending from pFe 15.9 and pFe 18.6 is shown (R^2 = 0.995; *slope* = 0.027 and *y-intercept* = - 0.079). B: Bioavailable Fe level (represented here as total Fe concentrations) predicted from *P. putida* FeLux cells luminescence analyses 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. C: Dose response of *P. putida* FeLux in both Fe-EDTA- and lysate-supplemented BESAW medium. Fe concentrations are reported as total concentration 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).

decreased by a factor 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 of only 20% in between pFe 18.6 and pFe 15.9 treatments.

3.2. Impact of lysate on Fe uptake rates

The rate of Fe assimilation by the bioreporter cells from *V. natriegens* PWH3a lysate was measured to investigate the impact of the Fe-lysate mixture on Fe acquisition and the relationship between acquired Fe and light production (Fig. VI.3). Short term Fe uptake rates did not exhibit saturation characteristics of facilitated or active transport due to limitations in the Fe concentration of cell lysates. Over the range of experimental concentrations tested, short-term ^{55}Fe uptake rates increased linearly with respect to Fe concentrations in the lysate amended treatments, following the equation: $y = 1.218 \times 10^{-23} x + 6.534 \times 10^{-21}$ ($R^2 = 1$; Fig. VI.3A). This result suggests that Fe levels assimilated by bioreporter cells were directly correlated to external concentrations of Fe within the range of concentrations tested, and thus that maximal velocity of transport was not reached.

A comparison between uptake kinetics and bioreporter bioluminescent response (*i.e.* high-affinity transport systems expression) is presented in Figure VI.3B. Over the range of concentrations tested, the bioluminescent signal produced by *P. putida* FeLux reporter grown in BESAW medium amended with bacterial lysate decreased as ^{55}Fe uptake rates increased, following a four-parameter logistic function ($R^2 = 0.999$). This result suggests a strong indirect and non-linear correlation between bioluminescent signal and Fe bioavailability, and therefore 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 1.02×10^{-21} ($\pm$ 2.41×10^{-23}) 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.

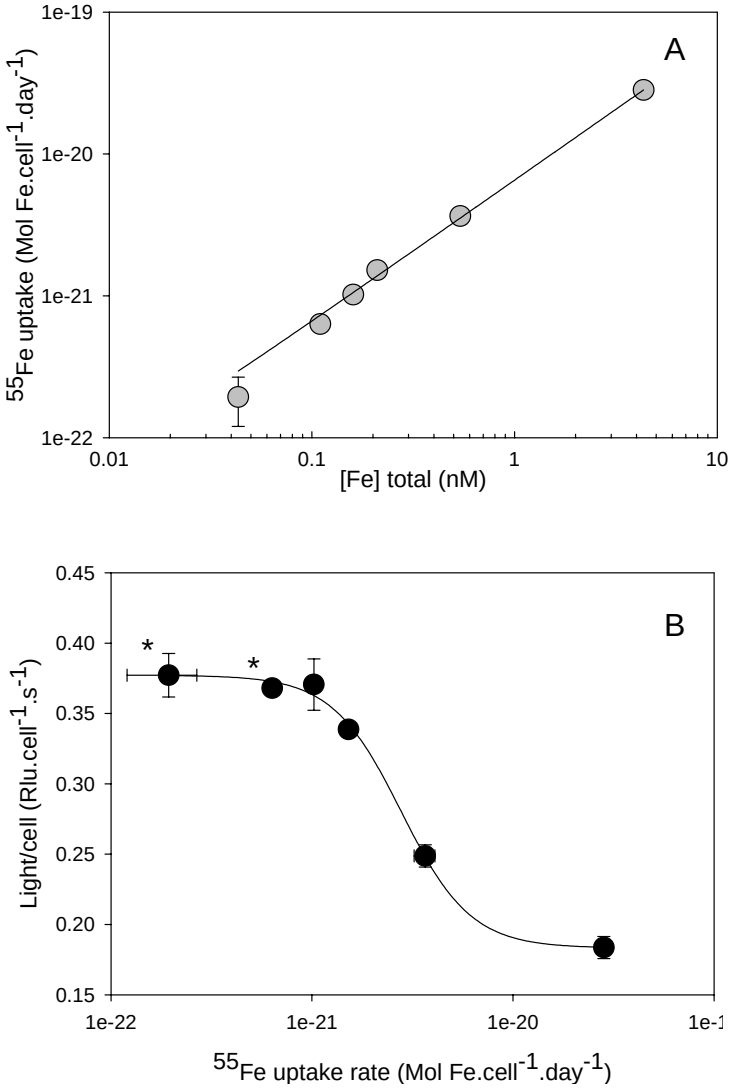

Fig. VI.3. Uptake of Fe from *V. natriegens* PWH3a lysate by *P. putida* FeLux. A: Fe assimilation rates as a function of total Fe concentrations in lysate-supplemented BESAW. Regression analysis for the whole data set is represented (R^2 = 1, *slope* = 1.218×10^{-23} and *y-intercept* = 6.534×10^{-21}). Error bars represent the standard deviations between replicates ($n = 3$). B: Comparison of Fe bioavailability with Fe uptake. The four-parameter logistic function (linear; R^2 = 0.999) characterizing the relationship between these two parameters as determined by using Sigmaplot 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 ($p < 0.05$).

Table VI.1

Concentrations of the Fe ligands isolated from the supernatants of *V. natriegens* and *P. putida* FeLux. The dash lines indicate that the concentration was below the detection limit of the assay (*i.e.* 10 nM for the Csaky test).

Strain	**CAS assay** (μM ligands)	**Csaky test** (μM hydroxamate)	**Rioux Assay** (μM catechols)
V. natriegens (PWH3a)	9.25	_	> 50
P. putida FeLux	12.00	_	40.9

3.3. Characterization of siderophores

Extracellular Fe-ligands isolated from the supernatant of the both bacterial strains studied here were detected with CAS assay (table VI.1). 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, cultures of *V. natriegens* sp. PWH3a and *P. putida* FeLux yielded total Fe-complexing concentrations of 9.25 μM and 12.00 μM respectively. These siderophores will be referred as "V.n.$_{Sid}$" and "P.p. $_{Sid}$". Both siderophore extracts provide results below our detection limits with 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 P.p. $_{Sid}$, indicating that this extract may be dominated by a siderophore(s) that are tricatecholate. V.n.$_{Sid}$ also reacted positively with the Rioux assay but the concentration of catechols detected was above the range of the calibration curve of this assay. Such a result may indicate that in addition to the siderophores, other compounds (*e.g.* phenolics) that reduce Fe at low pH may have been present in the supernatant of *V. natriegens* cultures (Granger and Price, 1999).

3.4. Assessment of siderophore bioavailability

The bioavailability of the siderophores to the bioreporter *P. putida* FeLux was examined in a separate set of experiment and compared to the bioavailability of inorganic Fe and Fe-DFB complex (Fig. VI.4).

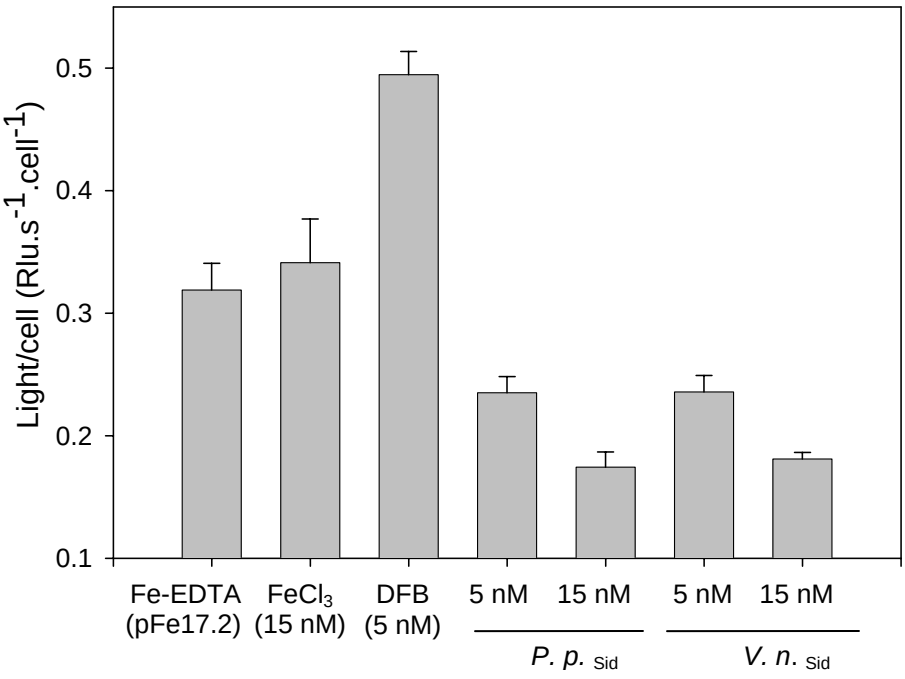

Fig. VI.4. Comparison of the bioavailability of Fe in presence of various ligands, including the siderophores produced by *V. natriengens* and *P. putida* FeLux. Error bars represent the standard deviations between replicates (*n = 3*).

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 17.2 (*t*-test, $p > 0.05$).

DFB addition resulted in a significant increase in 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 > 18.6) 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 $FeCl_3$ (Fig. VI.5).

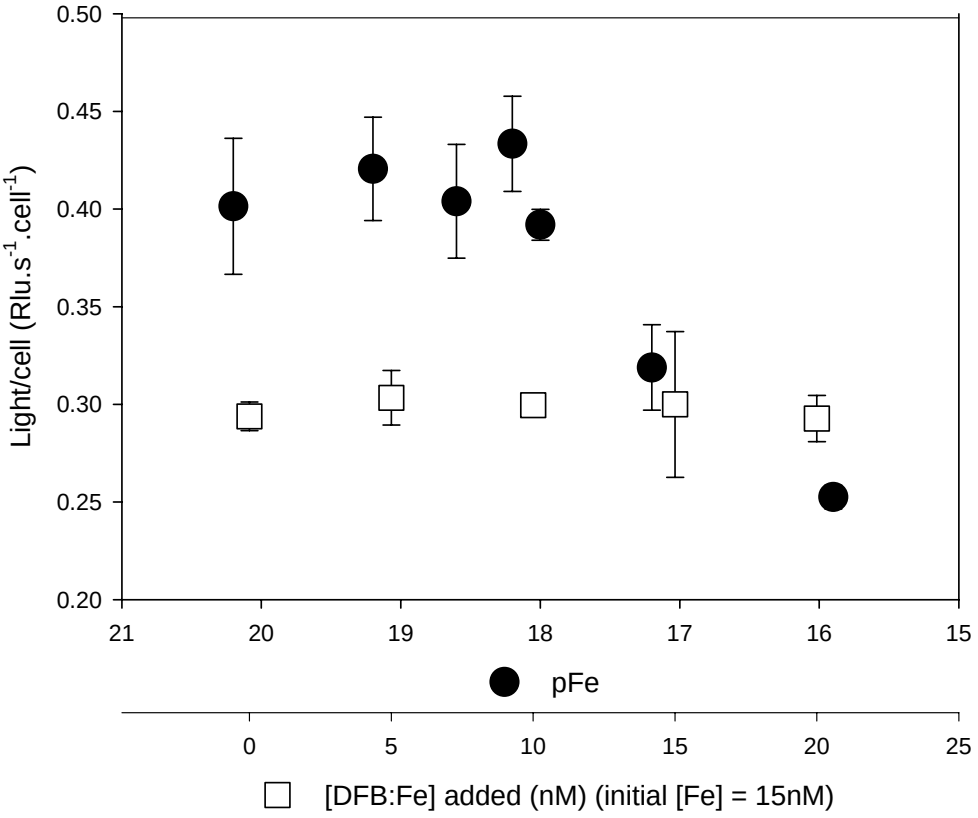

Fig. VI.5. Determination of the impact of DFB on bioavailable Fe pool. ●: Dose-response characterization of the Fe bioreporter *P. putida* FeLux in the trace-metal buffered BESAW medium. Cells were prepared as described in Fig.VI.2. □: Bioavailable Fe level predicted from *P. putida* FeLux bioreporter cells luminescence analyses in BESAW medium supplemented with equimolar Fe:DFB complex. Cells preconditioned in Fe-deficient BESAW medium (pFe 20.2) were used to inoculate BESAW medium ([$FeCl_3$] = 15 nM, EDTA omitted) supplemented with increasing amounts of Fe:DFB complex (Fe:DFB complex = 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 VI.2.

Table VI. 2

Evaluation of the impact of DFB addition on the bioluminescent signal of *P. putida* FeLux. pFe values reported here represent the mean value (± 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 VI.5, treatments are designated as the total concentration of $FeCl_3$ and DFB added.

Treatments	**Total Fe** (nM)	**DFB** (nM)	**Inferred pFe**
0	15 nM	0 nM	17.03 (± 0.05)
5	20 nM	5 nM	17.10 (± 0.09)
10	25 nM	10 nM	17.08 (± 0.09)
15	30 nM	15 nM	16.97 (± 0.42)
20	35 nM	20 nM	17.02 (± 0.10)

No significant variation in the bioluminescent responses (*ANOVA*, $p > 0.05$; *Dunnett's* test, $p > 0.05$) were observed between Fe(III):DFB amended treatments as compared to the unamended control. For all treatments, bioluminescent signals fell into the linear range of the calibration curve, enabling us to determine the concentrations of bioavailable Fe (expressed as pFe = -log $[Fe^{3+}]$) for these treatments (Table VI.2). In all treatments, inferred bioavailable concentrations were not significantly different than that of the pFe 17.2 treatment (*ANOVA*, $p > 0.05$). These data demonstrate that DFB effects on Fe assimilation and light production by these reporter cells are a direct response to perceived bioavailable Fe in the extracellular environment (*i.e*. Fe complexation by DFB when DFB is in excess). Moreover, the consistency of the results (*i.e.* 15 nM $FeCl_3 \sim 10^{-17.2}$ M Fe^{3+}) highlight 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 ~ 15.9). No significant difference was observed between treatments amended with similar level of V.n $_{Sid}$ and P.p. $_{Sid}$ (*Tukey-HSD* test, $p > 0.05$ for both 5 nM and 15 nM addition). This suggests that *V. natriegens* siderophores were available to *P. putida* FeLux. Slight but significant decreases in light production cell^{-1} were observed upon addition of 15 nM siderophore as compared to the treatments amended with 5 nM of siderophore (*t*-test, $p = 0.003$ [V.n. $_{Sid}$ 5nM – V.n. $_{Sid}$ 15 nM], $p = 0.004$ [P.p.$_{Sid}$ 5nM – P.p.$_{Sid}$ 15 nM]). However, light production was never completely "switched off", suggesting that a basal level of activity of the high-affinity transport systems remained activated.

4. Discussion

Two main conclusions can be drawn from this study. First, independent lines of evidence are presented which further validate the use of the *P. putida* FeLux bioreporter as a quantitative tool to estimate the biological availability of Fe to heterotrophic bacteria. 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 decreases as uptake rates are enhanced 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 between the degrees of bioavailability of various Fe species. Using the bioluminescent response of *P. putida* FeLux, we were able to rank the Fe sources tested here in a decreasing order of bioavailability order: Fe-P.p $_{Sid}$ = Fe-V.n. $_{Sid}$ > inorganic Fe = Fe:EDTA (pFe 17.2) > Fe:DFB. Last, 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 environment of the cells. Taken together, our results support therefore the conclusion that the bioreporter *P. putida* FeLux could be a valuable tool to estimate variation in

bioavailability of naturally occurring Fe-ligand complexes, such as lysis products and ferrisiderophores, in marine systems.

As suggested in Poorvin et al. (2004), this study also confirms that viruses infecting prokaryotes may play a crucial role in mediating Fe transfer between planktonic organisms. Both the bioluminescent reporter signal and Fe uptake rates of the bioreporter cells provided a dose-dependent response to increasing Fe from the dissolved (< 0.2 μm) fraction of *V. natriegens* lysate. As well, these results suggest that Fe from the lysis products enters the bioreporter cells via multiple transporters, as the high-affinity system 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, multiples 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 validate the hypothesis that Fe in lysates are 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).

4.1. Determination of Fe bioavailability from lysate using P. putida FeLux

Results from both the bioreporter and ^{55}Fe assimilation assays demonstrated that Fe present in the dissolved phase of *V. natriegens* was significantly (*ANOVA*, $p > 0.05$) more available to *P. putida* FeLux than Fe:EDTA complexes. 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 by *P. putida* FeLux Fe in the lysate-amended treatments while repressing light production indicates that Fe complexes

released from virus-mediated lysis enter the cells through more than one transport systems, *i.e*. both non-specific (low-affinity system) and specific routes (high-affinity systems) (Winkelmann, 1990). Indeed, it has been previously 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 linearily ($R^2 = 1$). 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 μm) 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 and Bruland, 1995; Wu and 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, as they suggest that Fe-uptake rates were directly proportional to the extracellular Fe concentrations over the range of concentrations tested. Therefore, although the high-affinity Fe transport systems were fully derepressed, they were not sufficient to maintain a steady Fe uptake 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 maximal transport rates were only influenced by the density of 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 (Ferric uptake regulator)-regulated transport was repressed. This highlights the

difficulty of characterizing Fe transport in systems where multiple transporters (and variant Fe-complexes) persist. Taken together, these results suggest that Fe permeated the cells through pathways other than that of FUR-regulated outer membrane receptors.

Previously reported fractionation analyses have shown that virus-mediated lysis of *V. natriegens* cells releases Fe predominantly in the < 3 kD size-fraction (Poorvin et al., 2004). Furthermore, previous studies with cyanobacteria (Wang and Dei, 2003) have demonstrated that Fe present in the < 1 kDa size fraction of natural seawater samples was assimilated *ca.* 1.7 times faster than Fe-complexes of higher molecular weight (1 kDa – 0.2 μm). It is therefore possible that a fraction of the organic Fe-complexes released from virus-mediated lysis are small enough to pass through porins (size limit ~ 0.6 kDa, Andrews et al., 2003) and constitute a non-negligible source of bioavailable Fe to prokaryotic cells. The available literature data documenting the "low-affinity" Fe transport systems are scarce, and mainly focuses on the two potential pathogen bacteria *Escherichia coli* and *Helicobacter pylori* (Velayudhan et al., 2000; Andrews et al., 2003). Several transport pathways, from outer-membrane receptors specific to other metals or cations to non biologically-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 bacteria *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 present a different mode of regulation than that involved the acquisition of Fe from cognate siderophores (Venturi et al., 1995; Ratledge and Dover, 2000). 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 and 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 heterologous organic ligands. Therefore, Fe acquisition by heterotrophic bacterial cells may rely on several transport pathways to maintain growth.

In summary, these results suggest that Fe sources present in virus-mediated lysates are highly available to heterotrophic bacteria and may be internalized into the 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 bioreporter 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 with regard to 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 and Bruland, 1995; Wu and Luther, 1995), we speculate that microorganisms may rely on multiple Fe acquisition systems in oceanic environments. As such our data support the general conclusion that virus-mediated Fe recycling in the surface waters of aquatic environments may play a crucial role in regenerating the bioavailable Fe to resident plankton organisms, and thereby on Fe cycling (Poorvin et al., 2004).

4.2. Specificity of Fe-siderophore acquisition and cross-utilization

Under the experimental conditions, the bioluminescent signal was higher when Fe was provided as inorganic Fe ($FeCl_3$) than when Fe supplied as a Fe(III)-siderophore complex. 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 (Zimmermann et al., 1989), inorganic Fe may be internalized into cells as Fe(III)-siderophore complexes through outer-membrane receptor (van der Halm, 1998) or through a broader Fe(III) exchange mechanism requiring also 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 theproduction of siderophores (Granger and Price 1999, Guan et al. 2001). Our results further suggest that inorganic Fe was utilized more efficiently than Fe:EDTA complexes. Previous studies have effectively shown that EDTA is not available to *Pseudomonas putida* (Meyer and Hohnabel, 1992). Indeed our results confirm that the bioluminescence

signal is directly related to the estimated concentration of Fe (III) in the environment of the bioreporter and not to the total Fe concentration.

At comparable concentrations, Fe released from *V. natriegens* lysates appeared to more efficient at satiating the bioreporter cells as compared to the Fe(III)-siderophore complexes. This result comforts our hypothesis that Fe released from virus-mediated lysis enters cells through other transport systems than the specific outer-membrane receptors. In the case of siderophores, high-affinity systems needs to remain turned on to maintain production of cognate outer-membrane receptors (Andrews et al., 2003). Effectively, Fe-siderophores complexes are too large to be internalized through 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 (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; van der Halm, 1998). The lack of significant difference observed in the bioluminescent signal between the P.p. $_{Sid}$- and V.n. $_{Sid.}$- amended treatments suggest 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 lysates-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 de-repression between genes involved in Fe-acquisition at identical Fe concentrations. Ferric-enterobactin uptake genes (such as the *fep*A acquisition system used in our bioreporter) were among the most weakly de-repressed indicating that such a system is more strongly controlled by the cell Fe status than the acquisition apparatus itself. Thus, we speculate that the bioluminescent signal background 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 systems expression, even under suboptimal Fe conditions may also reflect a compromise of maintaining uptake rates and reducing transporter proteins with also act as targets for bacteriophage and antibiotics (Andrews et al., 2003).

In contract to the P.P. $_{Sid}$ and the exogenous V.n. $_{Sid}$, the fungal siderophore DFB sequestered Fe from the bioreporter cells. Although it has been shown that DFB has toxic effect on eukaryotic cells (Fukuchi et al. 1994; Leardi et al. 1998),we have demonstrated that this it is not the case for our heterotrophic bioreporter. Therefore, our results 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) necessary to transport Fe into the cells. 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 to the planktonic community (Mioni et al., 2003 and ref. therein; Weaver et al, 2003; Eldridge et al., 2004; Poorvin et al., 2004) and that the transport kinetics of Fe:DFB complexes would not be favorable to a sustainable growth (Hutchins et al., 1999b). However, Fe acquisition from Fe:DFB complexes is still source of debate as it has been also suggested that phytoplankton communities from natural assemblage could access to at least a fraction of Fe chelated to DFB (Maldonado and 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 and 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 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 tri-hydroxamate siderophore would be needlessly expensive for natural bacterioplankton and would explain why DFB addition results in uniformly Fe stress in oceanic biological communities.

Although the cross-availability of various siderophores to diverse aquatic organisms has been documented for laboratory culture or enclosed-seawater samples (Trick, 1989; Granger and Price, 1999; Hutchins et al., 1999a; Guan et al., 2001; Weaver et al. 2003), the artificial conditions of such incubation experiment might have been source of bias. Indeed, it has been argued that relying on siderophore as a sole Fe source may be inefficient and energetically expensive in an aquatic environment due to the strong probability of a rapid diffusion of the produced siderophores away from the cell that could subsequently limit the probability of recapturing Fe(III)-loaded siderophores (Hutchins et al., 1991; Völker and Wolf-Gladrow, 1999), especially if other distantly related microbes could exploit this newly formed complexes (Griffin et al., 2004). The cost of siderophore excretion by an isolated heterotrophic cell would be metabolically expensive, especially where organic carbon supplies may also be limiting (Kirchman et al., 2000). Model predicts that Fe acquisition through freely diffusible siderophores may be an efficient strategy in oceanic environment at the sole condition that the concentration of organic ligands in the surrounding environment is high enough to allow the bacteria cell to use siderophores (cognate and xeno- siderophores) excreted by other cells (Völker and Wolf-Gladrow, 1999).

One way to counter this problem is to produce compounds with reduced diffusive potential in aquatic systems. Wilhelm and Trick (1994) proposed such a model, where production of catechol-type siderophores (like those discussed in this study) allows for cells to maintain a surface concentration of active chelates (since catecholates are generally hydrophobic in nature). More recently Martinez et al. (2003) 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.

5. Conclusion

Results from this study reinforce that Fe released from virus-mediated lysis would be an important source of highly bioavailable Fe to the bacterioplankton (Poorvin et al., 2004). Through this virus-mediated Fe transfer, cells would acquire Fe at a minimal cost as compared to the siderophores-mediated Fe uptake. Increasing pieces of evidence suggest that viruses play a pivotal role in Fe transfer in aquatic environments (Gobler et al., 1997; Wilhelm and Suttle, 2000; Poorvin et al., 2004; Higgins et al., 2004). In the case of heterotrophic bacteria, the high bioavailability of Fe might be related to the high diversity of organic Fe species released through virus lysis. Considering the importance of viral lysis in nutrient regeneration in natural ecosystems, we therefore 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 (Fig. VI.6). Such processes might explain why marine prokaryotes are able to sustain higher Fe assimilation rates and cells Fe:C quotas relative to large eukaryotic phytoplankton (Tortell et al., 1999).

Acknowledgements

This work was supported by NSF OCE – 0002968 and NSF OCE-9977040 to SWW, as well as by a University of Tennessee Center for Environmental Biotechnology Graduate Fellowship, a Ryan Kelley Memorial scholarship – International Women's Fishing Association 2003, and a Daniel Jouvance Award "Recherches sur le plancton marin 2003" to CEM.

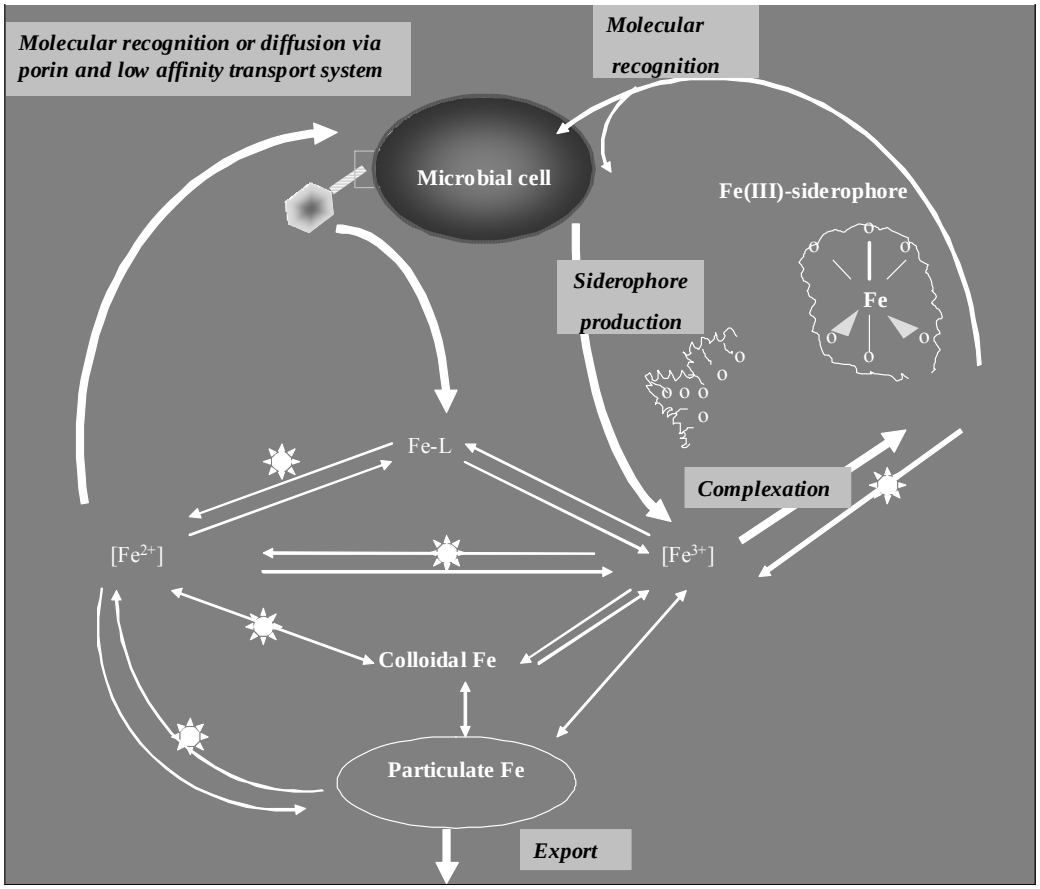

Fig. VI.6. Schematic representation of Fe acquisition by microbial cells from their environment, involving the siderophore-mediated and the virus-mediated acquisition pathways. Virus-mediated lysis of bacterial cells releases Fe under diverse forms of Fe(II)- or Fe(III)- organic complexes (Fe-L), mainly in the < 3KDa size-fraction (Poorvin et al., 2004). At least a portion of these Fe sources released from virus-mediated lysis is internalized into the cell through unspecific transport systems (*e.g.* diffusion through porines, low affinity systems) which do not require molecular recognition. Siderophore- mediated acquisition involves siderophore production and release by the cell, recognition of Fe(III) ions and complexation, diffusion to the cell surface and internalization upon molecular recognition of the Fe(III)-siderophore complex by outer membrane receptors. It has been argued that relying on siderophore as a sole Fe source may be inefficient and energetically expensive in an aquatic environment due to the strong probability of a rapid diffusion. Diffusion may drive the produced siderophores away from the cell and may subsequently limit the probability of recapturing Fe(III)-loaded siderophores (Hutchins et al., 1991; Völker and Wolf-Gladrow, 1999).Through virus-mediated Fe transfer, cells would acquire Fe at a minimal cost as compared to the siderophores-mediated Fe uptake. Photoreduction driven by sunlight is also represented (☼) (adapted from Boukhalfa and Crumbliss, 2002; and Gerringa et al., 2000).

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Part VII

INTRODUCTION OF A CONSTITUTIVE *GFP* MARKER FOR THE *PSEUDOMONAS PUTIDA* FELUX BIOREPORTER

Part VII

INTRODUCTION OF A CONSTITUTIVE *GFP* MARKER FOR THE *PSEUDOMONAS PUTIDA* FELUX BIOREPORTER

1. Introduction

As discussed in the previous chapters, the heterotrophic bacterial bioreporter *P. putida* FeLux appears to be a powerful tool for the assessment of Fe bioavailability in aquatic environments. However, the identification and the quantification of the bioreporter cells, required for the standardization of our results, is complicated in complex environments where resident bacterial cells can reach cellular densities as high as 10^7 cells mL^{-1} (Whitman et al., 1998). Indeed, traditional tools to determine microbial cells density are generally lacking the specificity required to easily monitor a bioreporter in environmental samples (Jansson, 1995; Jansson and Prosser, 1997). During the past decade, biotechnological tools, such as genetic tagging, have been developed to overcome such problems (Jansson and Prosser, 1997; Jansson, 2003). Previous studies have demonstrated that such tools are very powerful in detecting and quantifying a specific population of microbial cells in complex environments such in soils (Unge et al., 1999), plant leaves (Gau et al., 2002; Sabaratnam and Beattie, 2003), biofilms (Rice et al., 2003) and aquatic systems (Leff and Leff, 1996; Arana et al., 2003).

One of the most popular genetic marker is the *gfp* gene, which encodes for the green fluorescent protein (GFP). This protein does not depend on any other external compounds (substrates, cofactors or ATP) to fluoresce (Chalfie et al., 1994) and can be expressed in a wide range of hosts (both prokaryotic and eukaryotic). GFP maturation requires only oxygen (Cody et al., 1993) and is independent from the cellular energy status (Unge et al., 1999). The expression of this protein does not appear to interfere with growth and can be detected in living cell at the single-cell level without disrupting the biosensor cell (Suarez et al., 1997; Tombolini et al., 1997).

One of the problems related to this marker is that, once formed, the wild type GFP protein is very stable (Andersen et al., 1998). Therefore, the utilization of an unstable *gfp* variant (i.e short half-life) may be preferable in biosensor system real-time studies (Andersen et al., 1998). The *gfp*-lva variant was previously shown to have the shortest half-life in *P. putida* strain (60 min for *gfp*-lva versus 190 min for other *gfp* variants; Andersen et al., 1998). Another issue to consider is the choice of the promoter that will regulate the expression of the *gfp* reporter gene. Most strong constitutive promoters are growth phase dependent, and as such the number of viable cells may be underestimated if the cells are not metabolically active. The P_L*tetO1* promoter has already been characterized and successfully used in *E. coli* (Lutz and Bujard, 1997; Elowitz and Leibler, 2000). This promoter has been shown to generate high levels of constitutive expression of another reporter gene (*lux*) in the absence of Tet repressor protein in the host organism (Lutz and Bujard, 1997).

Another criterion for the applicability of such a system to natural aquatic communities is the stable insertion of the genetic marker. Plasmid DNA constructs are not stable and generally require addition of a selective agent, such as an antibiotic, that may be a source of contamination or interfere with microbial interactions between natural and biosensors cells. Thus, it is preferable to integrate the construct into the chromosomal DNA of the preexisting biosensor cell. Although various transposon-based vectors are presently available to mediate the chromosomal insertion of genetic construction, their efficiency differs in function of the strains and the size of the insert to be mobilized. Recently, a series of modular mini-transposon derivatives based on transposon Tn5 function, which present broad host range and low target DNA sequence specificity, have been developed (Dennis and Zylstra, 1998). This vector system, named plasposon or pTn*Mod*, presents the advantage of cost efficiency relative to other commercially available minitransposon-based vectors (*e.g.*, pMOD, Invitrogen) since it does not require addition of transposase enzyme for the transformation of final host. Indeed, the transposase is encoded by the vector (outside the transposon element). This pTn*MoD* system has been tested in a wide range of microorganisms, including a *P. putida* strain.

The specific objective of the present research was to develop a better system for the standardization of the *P. putida* FeLux bioreporter results. As such, we needed a rapid and efficient way to enumerate cells in the background of the natural microbial population. To do so, we modified a *gfp* reporter gene to obtain the unstable variant *gfp*–lva (Andersen et al. 1998). Subsequently, we introduced a genetic construct, composed of this *gfp*–lva reporter gene fused downstream to the constitutive $P_L$tetO1 promoter (Elowitz and Leibler. 2000), to the preexisting bioreporter chromosome. The resulting bioreporter should enable us to determine the cell concentration in parallel to the measurement of the bioluminescent signal.

2. Material and methods

2.1. Bacterial strains, plasmids, and growth conditions

Strains and plasmids used in this study are listed in Table VII.1. The *E. coli* strain carrying the pTn*Mod*-OKm (accession number: AF061921) was kindly provided by Dr. Zylstra (Rutgers University, NJ) to the UT Center for Environmental Biotechnology. The pZE21-GFPasv plasmid carrying the constitutive promoter *$P_L$tetO1* fused upstream to an unstable variant of green fluorescent protein (*gfp*-asv; Andersen et al., 1998) was kindly provided to the UT Center for Environmental Biotechnology by Dr Michael Elowitz (Princeton University, NJ). The *E. coli* HB101 strain (Km^r) carrying the plasmid pRK201 (helper strain for tri-parental mating) was also provided by Dr Michael Allen (UT Center for Environmental Biotechnology – Oak Ridge National Laboratory), *E. coli* SV17 strain was kindly provided by Dr John Sanseverino (UT Center for Environmental Biotechnology). All strain were grown in Luria Bertani broth (Miller, pH 7.5) supplemented with the following antibiotics when appropriate: ampicillin (Amp, 150 μg mL^{-1}), kanamycin (Km, 50 μg mL^{-1}), and tetracycline (Tc, 50 μg mL^{-1}). *P. putida* FeLux strain was maintained on Pseudomonas Isolation agar (remel) supplemented with tetracycline. *E. coli* strains were grown at 37°C while *P. putida* were grown room temperature (unless otherwise specified).

Table VII.1

Bacteria and constructs used in this study.

	Relevant genotype/characteristics	Reference
Plasmids		
pCR2.1	3.9 kb cloning vector for PCR products with 3' A overhangs; Ap^R Km^R	Invitrogen, Carlsbad, CA
pTn*Mod*-OKm	5.098 kb Tn*Mod* variant with pMB1 *ori R*, mini-Tn5 Km^r	Dennis and Zylstra, 1998
pZE21-GFPasv	plasmid containing P_L*tetO1::gfp*-asv 867 bp DNA fragment, Km^R.	Elowitz and Leibler, 2000 [a]
pCR2.1< P_L*tetO1::gfp*-lva>	PCR2.1 containing P_L*tetO1::gfp*-lva 882 bp DNA fragment, flanked with *Not*1 and *Swa*1 restriction sites, Amp^rKm^r.	This study
pTn*Mod*-OKm < P_L*tetO1::gfp*-lva>	Delivery plasmid pTn*Mod*-OKm containing P_L*tetO1::gfp-lva* 882 bp DNA fragment, *E. coli* SV17, Km^r.	This study
Bacterial Strains		
E. coli DH5α F'	Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_K-,m_K+) *phoA supE44 thi-1 gyrA96 relA1* (Tn5 Km^R F' episome)	Invitrogen, Carlsbad, CA
E. coli SV17	λ *pir recA thi pro hsdR* M^+ RP4:2-Tc:Mu:Km Tn7Tp^RSm^R; mobilizing strain for pUT/mini-Tn5 derivatives	DeLorenzo et al., 1993
E. coli Gfp	*E. coli* SV17 containing pTnModGfp	This study
P. putida FeLux	*P. putida* containing a chromosomally inserted Tn5 *fepA-fes::luxCDABE*Tc^R Mini Tn5, Tc^R	Mioni et al., 2003
P. putida :: FeLux/Gfp	*P. putida* FeLux containing a chromosomally inserted Tn5 P_L*tetO1::gfp*-lva Km^R Mini Tn5, Tc^R Km^R	This study

[a] the plasmid reported here differs from the repressilator plasmid published in this article since it contains the gfp-asv variant in place of the gfp-aav variant.

2.2. Cloning procedures

Cloning procedure were performed as described by Sambrook et al. (2001). Plasmid DNA was isolated from *E. coli* or *P. putida* host cells with the Wizard® miniprep kit or the Wizard® plus maxiprep DNA purification system (Promega, Madison, WI). Genomic DNA was extracted using a phenol:chloroform extraction protocol (Sambrook et al. 2001). All PCR reactions were performed with pureTaq™ Ready-to-go™ PCR beads (Armersham Biosciences, Piscataway, NJ). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). For DNA ligation, we used the T4 DNA ligase from Promega (Madison, WI).

2.3. PCR Reactions

25 μL PCR reactions were prepared in PuRe Taq Ready-to-go™ PCR beads tubes (Amersham Biosciences) using ~10 ng of template DNA and 0.8 μM of primers. The following program was used: 94°C, 5 min; [94°C, 30 s; 56°C, 1 min; 72°C, 30 s]; 72°C, 15 min. The steps between brackets were repeated for 35 cycles.

2.4. Ligation Reaction

Several aliquots (*ca.* 2 μg of each plasmid DNA per 20 μL reaction) of the delivery plasmid vector (pTn*Mod*-OKm) and plasmid carrying the insert to be cloned (pCR2.1< P_L*tetO1::gfp-lva>)* were double-digested with the restriction endonucleases *Swa*I and *Not*I following the manufacturer's instructions with some modifications. Because the optimal temperatures for digestion reaction differ between these enzymes, both plasmid DNA were first incubated overnight (*ca.* 12 h) with *Swa*I (20 units) at 17°C in a rack filled with ice. This step reduces the activity of *Swa*I (optimal temperature = 25°C) and avoids over-digestion of the plasmid DNA. *Swa*I restriction enzyme was then heat inactivated (65°C, 20 min) and 30 units of *Not*I were added to each reaction. Reactions were incubated for *ca.* 4 h at 37°C. Linearized vector and insert DNA were purified from proteins and buffer salts using the Minielute Reaction Cleanup Kit – for

cleanup of enzymatic reactions® (Qiagen Inc., Valencia, CA). The linear vector was dephosphorylated using calf intestinal alkaline phosphatase from Promega (Madison, WI) according to the manufacturer's protocol. Both vector and insert DNA were gel purified from 1% agarose gels with the Qiaquick® extraction kit (Qiagen Inc., Valencia CA) according to the manufacturer's instructions. Extracted DNA was then quantified using a spectrophotometer (Biomate 5, Thermospectronic corp.). Spectrophotometry was used in place of fluorometry because it provides insight on protein contamination. Recovered DNA concentrations were estimated to *ca.* 10 ng mL^{-1} with a DNA ratio higher than 1.80 (insert: $\Delta = 1.85$, vector: $\Delta = 1.96$). The DNA ligation reaction was carried out with 20 μL of a 3:1 insert:vector molar ratio according to the manufacturer's recommendations. The reaction was incubated at 17°C overnight.

2.5. Transformations

PCR2.1 plasmid DNA was introduced into *E. coli* DH5α cells using the BTX Electroporator 600 (BTX, San Diego, CA) set at 2.45 kV, 125 Ω, and 50 μF.

Chemical transformation was used to transform *E. coli* SV17 with the minitransposon pTn*Mod*-OKm < P_L*tetO1::gfp*-lva>. Preparation of chemically competent cells and subsequent transformation was performed following a protocol modified from Sambrook and Russell (2001; Rakesh Gupta, pers. comm.).

Plasposons used for chromosomal integration of the P_L*tetO1::gfp*-lva genetic construct were transformed into *P. putida* FeLux cells by tri-parental mating (donor strain: *E.coli* SV17 carrying pTn*Mod*-OKm < P_L*tetO1::gfp*-lva>*)* as previously described (Dennis and Zylstra, 1998). Transformants were selected on PIA medium supplemented with kanamycin (50 μg mL^{-1}).

2.6. Sequencing analyses

All constructs were sequenced at the University of Tennessee Molecular Biology service facility using an Applied Biosystems 3100 Genetic Analyzer sequencer (Foster City, CA).

2.7. Direct visualization of fluorescent bioreporter cells

The strain *P. putida* FeLux-gfp harboring the miniTn5 - P_L*tetO1::gfp*-lva was grown at room temperature in LB medium supplemented with 50 μg mL^{-1} of tetracycline and kanamycin. At an optical density of *ca.* 0.1, culture samples (1 mL) were harvested, diluted in sterile LB and mounted of glass slide for immediate observation. Fluorescent cells were visualized on a Leica DMRXA epifluorescent microscope using a UV lamp for excitation. At this time, the specifc optical filter combination remains unavailable (microscopic or fluorometeric) and as such the present experiment should be considered as a "test experiment". We attempted to quantify both luminescent and fluorescent signals with proper wavelengths using the 1450 Microbeta Liquid Scintillation Counter but results were disappointing for both markers.

3. Results

3.1. Construction of a mini-Tn5 gfp cassette

A minitransposon was constructed for stable expression of the gfp gene into the chromosome of P. putida FeLux (Figure VII.1). The *pTet*::*gfp*-asv DNA fragment carried by the pZE21-GFPasv plasmid was genetically modified via PCR amplification in order to replace the *gfp*-asv variant by the *gfp*-lva. The GFP-lva variant was selected based on previous report which demonstrated that its in vivo half-life of this protein was of *ca.* 60 minutes in place of *ca.* 190 min for other GFP variants (Andersen et al., 1998). Indeed, since *P. putida* FeLux bioreporter cells are commonly harvested every 2 h for light production measurement, one of our selection criteria was that the half-life of the GFP protein be less than 2 hours. This modification was performed by altering the sequence of the reverse *gfp* primer in its 5' end in order to obtain the amino acids sequence leucine-valine-alanine in place of alanine-serine-valine. In the same time, two restriction sites were added in 5' and 3' end of the P_L*tetO1::gfg* fusion. *Swa*I was added at the 5' end of the P_L*tetO1* forward primer and NotI was added at the 5' end of the *gfp* reverse primer. The modifications listed above were introduced by using the following set of primers:

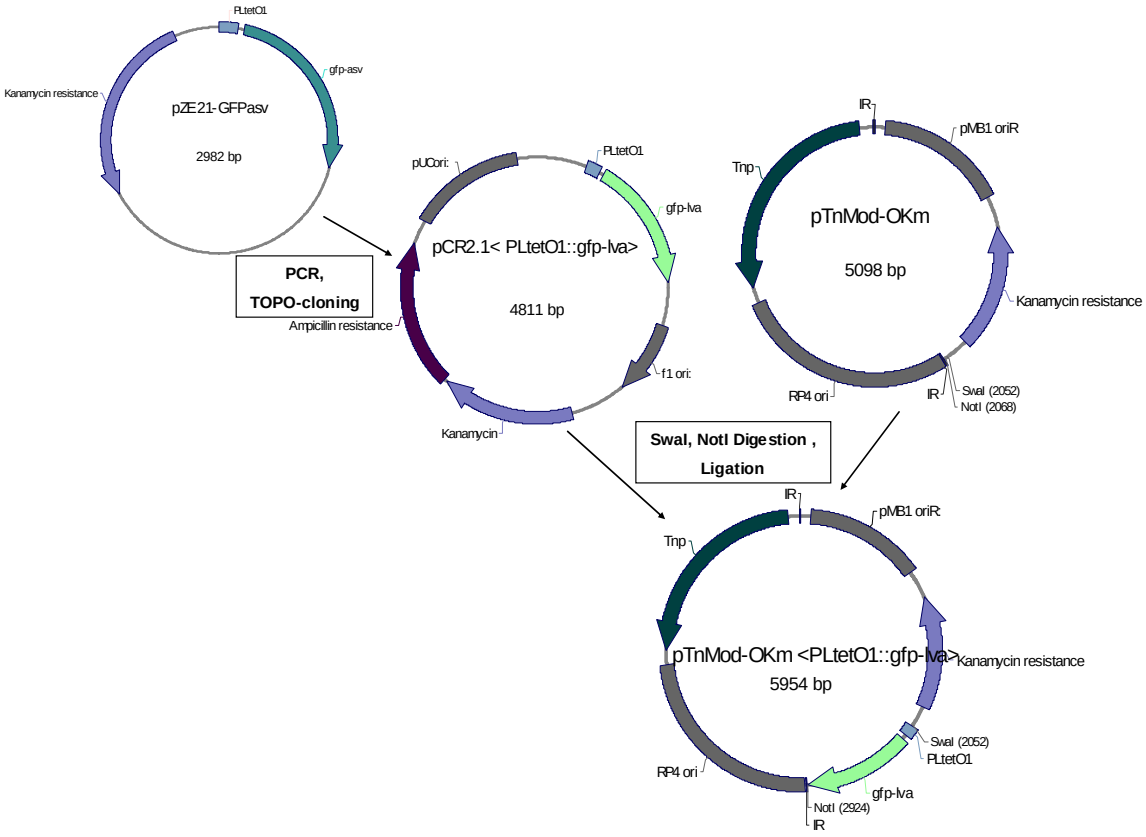

Fig. VII.1. Construction of pTn*Mod*-Okm<PLtetO1::gfp-lva>. Arrows and boxes indicates the construction manipulations. IR: Inverted repeat. Plasmids are not drawn to scale.

5’-ATTTAAATTCGAGTCCCTATCAGTGATAGAGA-3’ and 5’-GCGGCCGCTTA TTAAGCTACTAAAGCGTAGTTTTCGTC-3’. The PCR product was Topo-cloned in the cloning vector pCR2.1 (Invitrogen). Based on sequencing analyses, a clone with the appropriate P_L*tetO1*::*gfp*-lva insertion was selected. The P_L*tetO1*::*gfp*-lva DNA fragment was then isolated from the pCR2.1 as *Swa*I/*Not*1 fragment and inserted into *Swa*I/*Not*1 digested pTnMoD-OKm, resulting in the minitransposon delivery vector pTn*Mod*-OKm < P_L*tetO1*::*gfp*-lva>. The *gfp* gene is indeed expressed under control of the P_L*tetO1* promoter which is known to be a strong promoter in bacteria lacking the Tet repressor protein (Lutz and Bujard, 1997).

3.2. Chromosomal insertion of the $P_L$tetO1::gfp-lva fragment into the chromosome of P. putida FeLux

The pTn*Mod*-OKm < P_L*tetO1*::*gfp*-lva> vector was introduced into *E. coli* SV 17 by chemical transformation. All transformants were screened for GFP expression (as fluorescent colonies). One clone was selected based on sequencing analysis and was subsequently used as donor strain. The pTn*Mod*-OKm < P_L*tetO1*::*gfp*-lva> plasmid was transformed into *P. putida* Felux through tri-parental mating (Dennis and Zylstra, 1998). Transformants displaying both luciferase and GFP phenotype were further screened by PCR amplifying the target P_L*tetO1*::*gfp*-lva DNA fragment from genomic DNA and plasmid DNA. The transformant sample displayed a single band of the expected size when the *gfp* construct was amplified from genomic DNA, while amplification from plasmidial DNA yielded negative results. Unfortunately, we have not had the opportunity to localize the miniTn5 < P_L*tetO1*::*gfp*-lva> cassette within the genome of *P. putida* FeLux.

3.3. Preliminary investigation

The *P. putida* FeLux/gfp transformants were analyzed by microscopy upon exposure to UV (Fig. VII.2). Although the GFP-lva variant is only weakly excited by

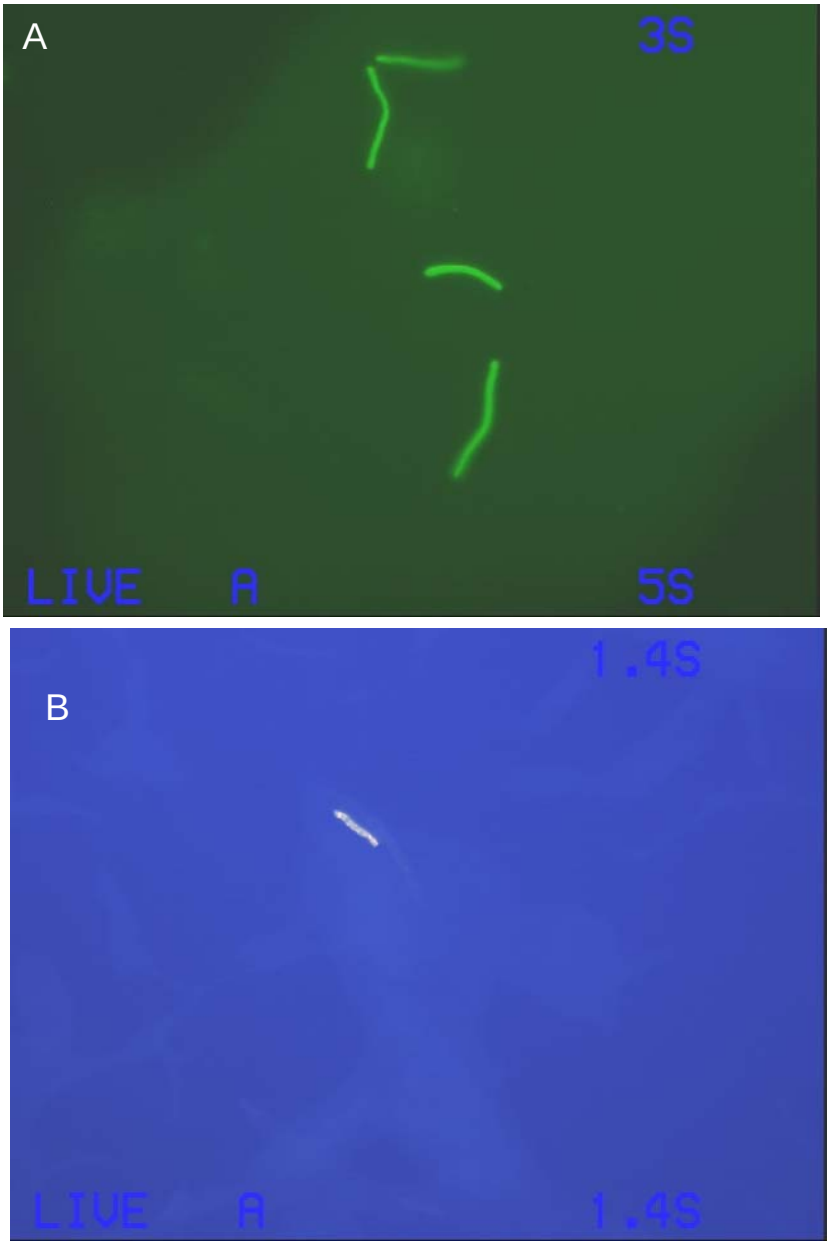

Fig. VII.2. Photomicrograph of *P. putida* FeLux cells as observed by microscopy (× 1,000). A: photograph of *P. putida* FeLux cells after treatment with Acridine orange dye (for comparison); B: Photomicrograph of a GFP-fluorescing *P. putida* FeLux/gfp cell, with a 1.4 s exposure. (some other cells can be seen in the background but the fluorescence was very weak).

short UV light several cells were glowing. The number of cells glowing upon excitation by UV were however far less numerous than the total cells number.

4. Future directions

Before applying this system to field monitoring, several points need to be taken into consideration. Although the characterization of this new bioreporter is beyond the scope of this Ph.D. project, several future directions are suggested and discussed below as friendly advice.

4.1. Detection of the fluorescent signal

Theoretically, single cells tagged with a single chromosomal copy of the *gfp* reporter gene can be visualized by epifluorescence microscopy or flow cytometry (Suarez et al., 1997; Tombolini et al., 1997). Although more investigations are required to draw definitive conclusions, our bioreporter does not appear to fulfill this promise. Previous reports have already pointed out this problem and it has been estimated that cells will appear dark as compared to the background if a threshold number of cellular GFP molecules is not met because the detector cannot differentiate below this threshold (van der Meer et al., 2004). Such a disadvantage appears to be accentuated in the case of unstable GFP-based bioreporter as it has previously been reported that much higher analyte concentrations were required to obtain the same signal intensity than that of stable GFP-based bioreporters (Leveau and Lindow, 2001). However, several solutions have been suggested to overcome this inconvenient (please note that this list is certainly not exhaustive):

(i) it has been suggested that the use of a charge-coupled device camera could enhance a weak signal or could compensate for autofluorescence (Tombolini et al., 1997),

(ii) it has also been suggested to use fluorescence correlation spectroscopy, as this detection technique could detect a single molecule in a focal volume comparable to that of a bacterial cell, *ca.* 1 fL (van der Meer et al., 2004),

(iii) it is also possible to increase the intensity of the cellular fluorescence by chromosomal integration of two tandem copies of *gfp* (Unge et al., 1999). This solution would unfortunately imply the construction of a new bioreporter.

(iv) the synchronization of the cell growth at the beginning of the experiment may enhance the intensity of the fluorescence signal at the appropriate time point,

(v) Another solution would be to redesign the construct by using the stable wild type of the GFP protein marker or an unstable GFP variant with a slower turn over (Andersen et al., 1998) or a faster maturation rate. An optimized *gfp* cassette fused to a strong constitutive with optimized ribosome binding site has previously been used successfully in *Pseudomonas fluorescens* (Tombolini et al., 1997). As well, Dr Gerben Zylstra has in his possession a plasposon containing already a stable *gfp* reporter gene that he would be pleased to share. Using such a construct would save time if the bioreporter construct has to be unfortunately redesigned, as such it would only be required to fuse an appropriate promoter upstream to the preexisting *gfp* reporter gene.

It should also be noted that fluorescent cells were only visualized here upon UV exposure while the GFP variant protein used as a marker in this study is only weakly excited by UV light. Indeed, Andersen et al. (1997) reported that the fluorescent signal intensity is enhanced by a factor 20 when the GFP variants are excited at *ca.* 488 nm relative to UV. Although these authors did not report that the fluorescent signal could be detected at the single-cell level, they were able to obtain a fluorescent signal from broth cultures of *P. putida* cells expressing the GFP-lva variant using a fluorometer (excitation: 475 nm; emission: 515 nm) as well as by fluorescence microscopy (excitation: 470-490 nm; emission: 515 – 565 nm). Therefore, if more investigations are required, the present bioreporter may be suitable for environmental applications.

4.2. Timeframe for the detection of the fluorescent signal

Another issue to be considered for the applicability of this new dual marker bioreporter is the incubation time required before to record the fluorescent signal. Indeed, this bioreporter as been designed specifically to allow simultaneous monitoring of high-affinity transport system gene expression and cell density. Therefore, it is required that

the detection limit for GFP detection be reached before the optimal time point for monitoring of the *fepA-fes* promoter activity. This optimal time-point is generally of *ca.* 6 to 12 h (depending on the environment in which the bioreporter cells are grown) and coincides with the exponential phase of the bioreporter growth curve, i.e. when the bioreporter cells are metabolically active. However, under environmental stress, not all viable cells may be metabolically active. Since our dual *gfp-lux* marker bioreporter is based on an unstable GFP protein which is produced only in metabolically active cells, cell density may be underestimated in such conditions. Previous works have however shown that the *lux* gene expression was affected more severely than the *gfp* gene expression under environmental stress (Unge et al., 1999). A decoupling between the information provided by both markers may indeed affect the results if the cellular metabolic activity of the bioreporter cells is severely impacted (*e.g.*, by nutrient starvation, change in growth phase, etc.). Ultimately, since the bacterial bioreporter will be subjected to Fe stress, it would be prudent to check the impact of Fe depletion on the GFP signal and determine the range of Fe concentrations in which this system can be used with accuracy.

Acknowledgements

CEM is very grateful to Dr Michael Allen and Amy Allen for their guidance, their inexorable patience and outstanding friendship, not only with regard to this part of her research work, but also all along her dissertation work. CEM is also very grateful to everybody at CEB (especially Aysu, Amanda, Leslie, and Victoria) for sharing tips and advice and for being so supportive all along this project. We would like to thank Dr Gerben J. Zylstra and Dr Michael B. Elowitz who kindly provided the pTnMod plasposon and the repressilator plasmid respectively. Last CEM would like to thank Matt Smith for his help at the beginning of this project.

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Part VIII

SUMMARY AND CONCLUSIONS

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In this study, we fully characterized and successfully applied to freshwater and marine samples a bioluminescent heterotrophic bacterial bioreporter that was developed to assess Fe bioavailability at trace levels. Although other Fe-dependent prokaryotic bioreporters had been previously reported in the literature, they were either limited to laboratory use (Khang et al., 1997) or to soil (Loper and Henkels, 1997; Joyner and Lindow, 2000) or freshwater (Durham et al., 2002; Porta et al., 2003) samples. Moreover, these bioreporter strains were based on reporter proteins necessitating either specific technical requirements (Loper and Henkels, 1997; Joyner and Lindow, 2000) or addition of exogenous substrate (Durham et al., 2002; Porta et al., 2003) for the recovery of the reporter signal. Indeed, believe that the Fe-dependant bacterial bioreporter described in the present study is not only the first that can be applied in both freshwater and marine environments but also the easiest to handle when compared to preexisting bioreporters.

Based on the findings of this study, the following conclusions can be drawn:

(i) The expression of the bacterial luciferase operon (*luxCDABE*) itself is not affected by exogenous Fe within the experimental range of concentrations in this trace metal. This hypothesis was validated since, at Fe concentrations which are known to be limiting, the constitutive expression of the *luxCDABE* reporter gene cassette was not altered.

(ii) The energetic cost of the luciferase reaction does not affect the sensitivity of the reporter system in an environmentally relevant range of Fe concentrations. *P. putida* FeLux is sensitive enough to be used in aquatic environments where Fe generally occurs at trace level. Using an incubation time of 12 h and a fully defined synthetic medium, the heterotrophic bacterial bioreporter *P. putida* FeLux responded in a dose-dependant way to the level of bioavailable Fe within a range spanning between pFe 18.6 and 15.9. These values correspond to total Fe concentrations of 200 nM to 50 μM in presence of 100 μM

of the complexing agent EDTA. Although these concentrations appear high, environmental samples generally fell within this linear range. Moreover, we demonstrated that *P. putida* FeLux was sensitive enough to respond to changes in Fe bioavailability at subnanomolar level (*ca.* 0.69 nM).

(iii) The bioreporter system is selective enough to determine the fraction of bioavailable Fe rather than the total concentration in the cell environment within an environmentally relevant range of concentrations. Several convincing evidence support this conclusion. First, at similar total Fe concentration, *P. putida* FeLux and *E. coli* FeLux bioreporter cells responded differently to the addition of various commercialized siderophores or synthetic chelators. A decrease in Fe bioavailability was generally perceived when Fe-complexing agents which are known to bioavailable to either bioreporters were added. Second, concomitant equimolar additions of DFB (a fungal siderophore which is known to be unavailable to *P. putida* FeLux and to bind Fe in a 1:1 molar ratio) and Fe do not result in variation of the bioluminescent signal. Indeed, although total Fe concentrations vary, no change in Fe bioavailability was perceived by the bioreporter *P. putida* FeLux. Last, the bioluminescence signal of *P. putida* FeLux is directly correlated to the Fe assimilation rates. Indeed, the bioreporter *P. putida* FeLux can be used as a supplement to traditional analytical analyses, which do not allow resolving the fraction of bioavailable Fe from the total Fe pool.

(iv) Fe biovailability in aquatic systems is strongly influenced by the resident biological component. Investigations in both marine and freshwater natural systems suggested that the colloidal and particulate Fe size-classes, which are thought to derive at least in part from resident biota, contribute to a substantial fraction of bioavailable Fe. However, we have also shown that the small particulate size-class (< 0.8 μm) in environmental samples buffer artificial manipulation of Fe bioavailability in a different fashion depending on the geographic location. Laboratory experiments have demonstrated that the degree of bioavailability of exogenous ferri-siderophores to *P. putida* FeLux differ among ligands. This observation also implies that bacterioplankton may both act as competitor and cooperator in natural environment. It is indeed possible that, as suggested previously, the resident microbial community structure and the

geographic location influence Fe bioavailability in natural ecosystems (Weaver et al., 2003). Laboratory evidences also suggest that Fe released from virus-mediated lysis of bacterial cells is significantly more available than inorganic Fe at comparable concentrations, and is also more available at similar concentration than cognate siderophores (putative tri-catechol(s) siderophores). As pointed out previously, viruses may play an important role in the recycling of Fe in aquatic environments (Poorvin et al., 2004)

(v) Last, heterotrophic bacteria such as *P. putida* FeLux appear to mediate Fe acquisition through several transport systems. Our experiments indicate that other systems that FUR-regulated transport systems are involved in Fe uptake. Based on our observation, we generated a model which differentiates two Fe acquisition pathways: a pathway based on specific recognition of Fe-siderophores complexes and a nonspecific pathway in which Fe may enter the cells through porins or ferrireductase-Fe shuttling systems.

The different points enumerated above validate the suitability of using a bioanalytical tool such as the bioreporter *P. putida* FeLux to complement chemical analyses. Fe-dependent bioreporters, such as *P. putida* FeLux, are presently the only tools available to assess Fe bioavailability in aquatic systems. Future studies using the present bioreporter device under various conditions (*e.g.* assessment of Fe bioavailability in natural seawater samples *versus* samples treated with ultra violet or sunlight) or using other host strains (*e.g.* oceanic and coastal heterotrophic bacterial isolates) should improve not only our knowledge with regard to the bioavailable Fe pool in natural aquatic systems but also our understanding of Fe-acquisition strategies used by microbial organisms.

This study also highlights the complexity of the concept of Fe bioavailability in aquatic systems. Fe bioavailability is both dependent on Fe physical and chemical speciation but also on the Fe-acquisition strategies of the ambient microbial community (Bachmann, 2003). The idea of fertilizing the oceans with Fe to reduced atmospheric pCO_2 has emerged from the brain of apprentice-sorcerers concomitantly to the “iron age of the oceanography”. Our results here confirm the reserves of some oceanographers with

regard to the consequences of such geoengineering (Chisholm et al., 2001). Fe addition will not always result in an increase of Fe bioavailability to the planktonic communities. Understanding the consequences of 200 years of industrial activity on the global carbon cycle is becoming critical (Falkowski et al., 2000). However, the evolution of pCO_2 in the environment is not only dependent on human activities but also on how the oceans, especially the components of the biological pump, will react and on how key factors such as Fe bioavailability will impact such a reaction. It appears therefore evident that the actual solution is not to dump Fe in the oceans but to dump "nickels" in it instead. The anticipation of the inherent changes induced by human activities requires a better understanding of the biological and physical processes governing the biological carbon pump.

"We are the stewards, passing just a moment on the land, the air and the water which must feed the hosts to come. We should therefore bequeath their lands in good order."

Anita Conti (1935) – The first French female oceanographer

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VITA

Cécile Élise Mioni was born in Le Mans (France) on March 3, 1977. She attended Bernard Palissy High School (Agen, France) and received a General Scientific Baccalauréat, with a major in Life and Earth Sciences in July 1995 (*Cum Laude*).

She entered the University of Bordeaux I (France) as a freshman in Fall of 1995. She received a Bachelor of Science degree (*Magna cum Laude*, salutatorian) in Biology and Earth Sciences from the University of Bordeaux I (Talence, France) in 1998. Upon graduation, she was performed a training in marine ecology at the affiliated marine station (Marine Station of Arcachon, France) under the direction of Dr. Pierre-Jean Labourg and Dr. Xavier de Montaudouin. She then went on to earn a Maîtrise degree in Marine Sciences (Valedictorian, *Magna cum Laude*) at the European Institute of Marine Studies (Plouzané, France) in 1999. She performed her research work at the Institute de Recherche pour le Dévelopement (ex-ORSTOM) under the direction of Dr. Éric Morize. Her thesis "title" addressed the evolution of coastal fish stocks in the waters of Guinea (Africa). Upon graduation, she was awarded a scholarship from the French minister of Research and Education which allowed her to complete her academic education with a "Diplome d'Etude Approfondie" (DEA, *i.e.* Master) in marine biogeochemistry at the University of Pierre and Marie Curie, Paris (France). She performed her research work at the European Institute of Marine Studies (Plouzané, France) under the direction of Dr. Stéphane Blain (Professor Paul Tréguer's lab), her thesis work focused on the effect of iron limitation on marine diatoms. She graduated with honors (*Cum Laude*) in 2000 and continued her research on carbon-iron colimitation in Professor François M. Morel's lab at Princeton University, NJ during summer 2000.

After working for several months in the LEGOS/CNES laboratory (Toulouse, France) under the direction of Dr. Véronique Garçon, she began a Ph.D. program at The University of Tennessee, Knoxville in Fall 2001 with Dr. Steven W. Wilhelm. This project was a joint undertaking between the Center of Environmental Technology and the Microbiology Department of the University of Tennessee, Knoxville.