FEMS MICROBIOLOGY LETTERS

RESEARCH LETTER

A method for the use of the radiotracer ^{55}Fe for microautoradiography and CARD-FISH of natural bacterial communities

Marion Fourquez, Ingrid Obernosterer & Stéphane Blain

Univ Paris 06, CNRS, Laboratoire d'Océanographie Microbienne, Banyuls-sur-mer, France

Correspondence: Marion Fourquez, Univ. Paris 06, CNRS, Laboratoire d'Océanographie Microbienne, Observatoire océanologique, avenue du Fontaule, 66650 Banyuls-sur-mer, France. Tel.: +33 (0) 646 275 122; fax: +33 (0)468 887 395; e-mail: marion.fourquez@obs-banyuls.fr

Received 11 July 2012; revised 31 August 2012; accepted 27 September 2012. Final version published online 2 November 2012.

DOI: 10.1111/1574-6968.12022

Editor: Stefan Sievert

Keywords
microautoradiography; ^{55}Fe; CARD-FISH; marine bacteria.

Abstract

Heterotrophic bacteria are key players in the biogeochemical cycle of iron (Fe) in the ocean, but the capability of different bacterial groups to access this micronutrient is ignored thus far. The aim of our study was to develop a protocol for the combined application of microautoradiography (MICRO) and catalyzed reporter deposition–fluorescence *in situ* hybridization (CARD-FISH) using the radioisotope ^{55}Fe. Among the different washing solutions tested, Ti-citrate-EDTA was the most efficient for the removal of extracellular ^{55}Fe providing sufficiently low background values. We further demonstrate that the washing of cells with Ti-citrate-EDTA and the fixation with paraformaldehyde or formaldehyde do not induce leakage of intracellular ^{55}Fe. Incubating natural bacterial communities collected from contrasting environments, the NW Mediterranean Sea and the Southern Ocean, with ^{55}Fe revealed that 3–29% of bacterial cells were associated with silver grains. Combining microautoradiography with CARD-FISH, we demonstrate that the contribution of different bacterial groups to total ^{55}Fe-incorporating cells was overall reflected by their relative contribution to abundance. An exception to this pattern was the proportionally higher contribution of *Gammaproteobacteria*, SAR86 and *Alteromonas*. Our study demonstrates the feasibility of MICRO-CARD-FISH using the radiotracer ^{55}Fe and provides the first description of marine bacterial assemblages actively incorporating Fe.

Introduction

Iron is a rare resource for microorganisms in the ocean. In surface waters, the iron demand of heterotrophic bacteria can be as high as that of phytoplankton, leading to a strong competition among microorganisms (Tortell *et al.*, 1996). Concurrently, heterotrophic bacteria are key players in the remineralization of particulate biogenic and lithogenic iron, thereby contributing to the production of regenerated bioavailable iron (Tortell *et al.*, 1999; Poorvin *et al.*, 2004). Our understanding of the role of heterotrophic bacteria in iron cycling relies mainly on bulk measurements, such as the contribution of bacterial biomass to the biogenic iron stock and bacterial iron uptake rates (Strzepek *et al.*, 2005). By contrast, links between bacterial diversity and biogeochemical functions involving iron are still lacking. Single-cell approaches were proven a powerful tool to study the role of bacterial groups in biogeochemical cycles of the major elements carbon, phosphorus, and sulfur. Among the different single-cell approaches, fluorescence *in situ* hybridization (FISH) coupled to microautoradiography was applied with a suite of radiolabeled substrates to study their utilization by phylogenetically broad or more specific bacterial groups (Cottrell & Kirchman, 2003; Vila-Costa *et al.*, 2004; Herndl *et al.*, 2005; Alonso-Sáez & Gasol, 2007). The commonly used radiotracers are ^{3}H, ^{14}C, and ^{35}S coupled to organic or inorganic compounds. In a recent study, ^{33}P-labeled phosphate was successfully used to assess the bacterial groups contributing to the phosphorus cycle (Longnecker *et al.*, 2010).

In the case of iron, the radioisotope ^{55}Fe has been widely applied for autoradiographic analyses in cellular biology or biochemistry (Orlic, 1968; Parry & Blackett,

1973). By contrast, only two studies have thus far applied ^{55}Fe microautoradiography to investigate the uptake of iron by different aquatic microorganisms on a single-cell level. Paerl (1982) demonstrated the feasibility of ^{55}Fe microautoradiography with cultures of the nitrogen-fixing Cyanobacterium *Anabaena* spp. isolated from a eutrophic lake. The cultures used by Paerl (1982) were not axenic, they therefore provided also microautoradiographic evidence for the utilization of ^{55}Fe by free-living bacteria or bacteria attached to filaments. The two major challenges pointed out by Paerl (1982) were the exposure time of several weeks to develop the silver grains and the abiotic adsorption of ^{55}Fe to filters or particulate matter, which resulted in a high number of nonspecific silver grains. In the marine environment, the only study applying ^{55}Fe microautoradiography to determine cell-specific activity is based on phytoplankton cells (Hutchins *et al.*, 1993). These authors demonstrated the incorporation of ^{55}Fe by different members of the phytoplankton community, in particular by the diatom *Thalassiosira weissflogii* and by the Cyanobacterium *Synechococcus* spp. (Hutchins *et al.*, 1993). The contribution of different bacterial groups to the utilization of iron in the marine environment has, however, not been addressed thus far.

The objective of this study was to elaborate a protocol for the use of ^{55}Fe as a radiotracer for bacterial single-cell analysis, applying microautoradiography coupled to FISH.

Materials and methods

Chemical reagents and preparation of the wash solutions

The $^{55}FeCl_3$ stock solution (1.86×10^3 Ci mol^{-1}; Perkin-Elmer) was diluted 10 000 times in 0.012 M suprapur HCl to obtain the working solution. Preparation of the wash solutions oxalate-Ethylenediaminetetraacetic acid (EDTA) and Ti-citrate-EDTA was performed following the protocols described in Tovar-Sanchez *et al.* (2003) and in Hudson & Morel (1989), respectively. Solutions were 0.2-μm-filtered (syringe filter; Acrodisc) before use.

Cleaning protocol

For sampling and incubations, we used polycarbonate (PC) bottles and plastic ware soaked in 10% HCl for at least 24 h and subsequently rinsed with Milli-Q (MQ) water before being used. Labware was sterilized three times by microwaving (5 min, power 750W), dried, and stored under a laminar flow hood. This cleaning procedure was performed in a clean room.

Incubation of bacterial cells with ^{55}Fe

In a first set of experiments, we used the bacterial strain *Alteromonas macleodii* (MOLA60, GenBank accession number: AM990835). Before incubation with ^{55}Fe, *A. macleodii* was acclimated for 7 days to iron-limited conditions, by daily transfer in AQUIL medium (Price *et al.*, 1989) containing 5.4 nM of non-radioactive Fe-EDTA. The experiments were conducted with cells transferred into freshly prepared AQUIL medium containing 5.4 nM of ^{55}Fe-EDTA. Triplicate live incubations (20 mL) were performed in the dark, at 20 °C and under agitation. For triplicate controls, formaldehyde (FA, 2% final concentration) was added to the *A. macleodii* culture and kept for 1 h before the addition of ^{55}Fe.

A second set of experiments was performed with natural bacterial communities. In the NW Mediterranean Sea, seawater samples were collected five nautical miles offshore at Station POLA (42°28′300N – 03°15′500E, 90 m overall depth). Seawater (2 L) was pumped at 5 m using a trace metal clean Teflon pump (ASTI) connected to an acid-washed PVC tube. The samples were stored in 1 L acid-washed PC bottles in the dark until arrival to the laboratory about 1 h later. In the Southern Ocean, samples were collected during the Kerguelen Ocean and Plateau Compared Study 2 cruise (KEOPS2, October–November 2011) at Station E-4W (48°45′900S – 71°25′500E, 1384 m overall depth). Samples were collected at 20 m using trace metal clean 12L modified Niskin bottles and further processed in a clean laboratory. At both sites, seawater was filtered at low pressure (< 200 mm Hg) through 0.8 μm acid-washed PC filters (47 mm; Millipore). Subsamples (100 mL) of filtered seawater were spiked with ^{55}Fe at a final concentration of 15 nM. This concentration was chosen to limit isotope dilution as determined by saturation curves (data not shown) and to allow a maximum number of cells to obtain the critical amount of ^{55}Fe for silver grain production (see Results and discussion Section). Samples from the NW Mediterranean Sea were incubated on a rotary shaking platform at *in situ* temperature (20 °C), in the dark, for periods ranging between 24 h and 7 days. Experiments were carried out in triplicate. In the Southern Ocean, the PC bottles were incubated at 1% PAR irradiance in an on-deck incubator supplied with circulating surface seawater. For both sites, control treatments of the seawater samples were killed with formaldehyde and kept for 1 h before the addition of ^{55}Fe.

Collection and washing of bacterial cells

Following incubation with ^{55}Fe, subsamples for the determination of the radioactivity incorporated into bacterial cells were collected on nitrocellulose filters (NC, 25 mm

diameter, 0.2 μm pore size; Nuclepore), and subsamples for microscopic observations were collected on 0.2-μm PC filters (25 mm diameter; Millipore) (Fig. 1). To investigate the efficiency of eliminating extracellular ^{55}Fe, two rinsing solutions and 0.2-μm-filtered seawater were tested. Subsamples of the *A. macleodii* culture (1 mL) and the natural seawater (10 mL) were filtered onto 0.2-μm NC filters. In the case of *A. macleodii*, cells were filtered after five generations to ensure a homogenous incorporation of ^{55}Fe and triplicate filters for radioactivity counting was performed. The filters were left on the filter holder and immediately rinsed with the freshly prepared oxalate-EDTA or the Ti-citrate-EDTA solution, followed by a rinse with 0.2-μm-filtered seawater (Fig. 1, steps a and d). For the oxalate-EDTA rinse, filters were kept in contact with 1.5 mL of the solution during 5 min before filtration. This washing step was repeated three times. For Ti-citrate-EDTA, the washing step was applied once with 1.5 mL of solution during 2 min. For both treatments, the filters were subsequently rinsed 10 times with 1 mL of 0.2-μm-filtered seawater sitting on the filters for 1 min before filtration. In addition, triplicate filters were rinsed with 0.2-μm-filtered seawater only. Controls were treated in the same way, except that the filtered volume was adjusted to account for the dilution of bacterial cells due to fixation. For live samples and controls, a set of filters remained unwashed (Fig. 1 step c). Filters were placed in scintillation vials, and 10 mL of Filter-Count scintillation cocktail from PerkinElmer was added. The vials were agitated overnight, and the radioactivity was counted by liquid scintillation (Beckman Coulter LS 6500).

For catalyzed reporter deposition–fluorescence *in situ* hybridization (CARD-FISH) and microautoradiography (Fig. 1, steps a, e and f), the volume of sample filtered was adjusted to obtain roughly 5×10^7 cells per filter.

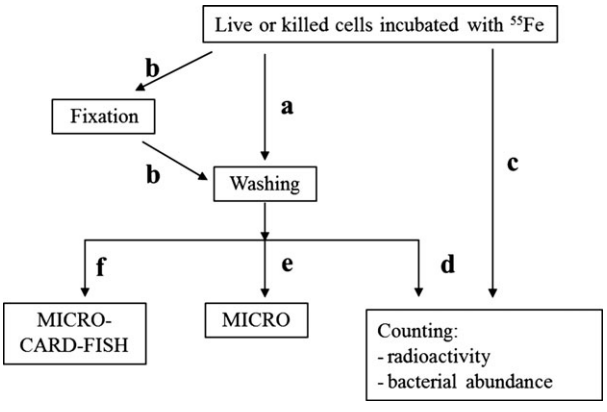

Fig. 1. Schematic presentation of the washing and cell fixation tests performed in this study. MICRO-microautoradiography; MICRO-CARD-FISH – microautoradiography coupled to Catalyzed reporter deposition – Fluorescence *In situ* Hybridization.

After filtration, cells were immediately fixed by deposition of filters on absorbent pads saturated with paraformaldehyde (PFA, 2% final concentration). Following 4 h of fixation at 4 °C, the filters were rinsed three times with 1 mL of 0.2-μm-filtered MQ water and washed with the Ti-citrate-EDTA reagent as described above. Finally, the filters were dried and kept at −20 °C until processed.

CARD-FISH was performed prior to microautoradiography on filter sections from the seawater samples following the incubation with ^{55}Fe. CARD-FISH was performed as described in Sekar *et al.* (2003), using the probes detailed in the Supporting Information, Table S1.

Microautoradiography was performed following the protocol described in Cottrell & Kirchman (2003). We used a photographic emulsion (type NTB2; Kodak, Rochester, NY) diluted at a ratio 50 : 50 (vol : vol) with 0.2-μm-filtered MQ water. Slides were observed under the semiautomatic Olympus BX61 epifluorescence microscope using an image analysis system (Microbe Counter software; Cottrell & Kirchman, 2003). Total cells (DAPI stained) and cells hybridized with the probes (FITC labeled) were counted from 10 fields of view. For the enumeration of silver grains, 12 images, spaced vertically by 0.5 μm, were acquired under visible light–transmitted illumination. To determine the contribution of different bacterial groups to total iron-incorporating cells, the number of probe-positive cells associated with silver grains was divided by the number of DAPI cells associated with silver grains.

Bacterial abundance

Bacterial abundance in natural samples was determined by counting cells stained with DAPI (2 μg mL^{-1} in a 4 : 1 mixture of Citifluor-Vectashield for 10 min) by microscopy using image analysis. The abundance of *A. macleodii* was determined by flow cytometry (FACS Calibur; Marie *et al.*, 1997).

Results and discussion

Removal of extracellular iron

The application of microautoradiography to investigate the activity of heterotrophic bacteria at a single-cell level requires that the cells associated with silver grains are representative of cells that actively incorporate the radioisotope used. In the case of iron, the complete elimination of extracellular iron is challenging, especially in aquatic environments, where iron is present as FeO_x, which are easily adsorbed at the surface of biogenic or lithogenic particles. An efficient washing step of bacterial cells is therefore necessary to remove extracellular iron before exposure of the cells to the photographic emulsion.

Several washing methods were proposed previously, and two of them were used for seawater samples. The Ti-citrate-EDTA method (Hudson & Morel, 1989) is based on the reduction of Fe (III) with $TiCl_3$. In the case of oxalate-EDTA (Tovar-Sanchez *et al.*, 2003), the dissolution is very likely due to a ligand-promoted process (Tang & Morel, 2006). In the present study, we tested the efficiency of Ti-citrate-EDTA, of oxalate-EDTA and of 0.2-μm-filtered seawater (Fig. 1, steps a + d and c). An almost complete removal of extracellular iron was only obtained with the Ti-citrate-EDTA reagent (96 ± 0.3%, $n = 3$). Oxalate-EDTA and 0.2-μm-filtered seawater were less efficient with 88 ± 1% ($n = 3$) and 76 ± 0.2% ($n = 3$) of ^{55}Fe removed, respectively (data not shown). These results are consistent with Hutchins *et al.* (1999) who report the removal of up to 97% of surface-adsorbed ^{55}Fe using the Ti-citrate-EDTA solution for phytoplankton cells. Tang & Morel (2006) also concluded that the oxalate-EDTA wash is not as efficient as the Ti-citrate-EDTA wash in dissolving extracellular FeO_x in phytoplankton cultures.

Effect of washing and fixatives on intracellular iron

When a washing step is applied, it is also important to assure that no intracellular iron release occurs due to the damage of the cell membrane. Contrasting results are reported for phytoplankton cultures. Tang & Morel (2006) did not observe any membrane damage when using Ti-citrate-EDTA and oxalate-EDTA. By contrast, other studies report that Ti-citrate-EDTA could produce leakage of the intracellular content (Sunda & Huntsman, 1995; Tovar-Sanchez *et al.*, 2003). To our knowledge, only one study tested whether the wash protocol with Ti-citrate-EDTA alters the integrity of bacterial cell membranes, which could result in the release of intracellular Fe (Chase & Price, 1997). These authors tested the release of radioactivity after washing with Ti-citrate-EDTA using *A. macleodii* (jul88 strain) incubated concurrently with ^{14}C-glucose and ^{55}Fe. Washing with Ti-citrate-EDTA induced < 10% loss of ^{14}C incorporated by bacteria, but the loss of ^{55}Fe was not considered here (Chase & Price, 1997). To address this issue, we incubated live or killed *A. macleodii* cells with ^{55}Fe, and we subsequently tested whether the Ti-citrate-EDTA wash induces ^{55}Fe leakage (Fig. 1, steps a + d and c). We therefore determined the cellular ^{55}Fe quota (i.e. the activity per cell) for washed and unwashed live and killed cells, based on the radioactivity measured on the filter and the bacterial abundance determined by flow cytometry (Table 1a). For each biological replicate, we calculated the difference in the ^{55}Fe quota between unwashed and washed cells and we compared by *t*-test these differences obtained for live and killed cells. No significant difference between live and killed cells (*t*-test, $P = 0.06$) was detectable. These results demonstrate that the washing step with the Ti-citrate-EDTA solution does not induce leakage of intracellular ^{55}Fe.

The application of CARD-FISH requires fixation of bacterial cells with PFA. In the present study, this fixation step was performed prior to the washing with Ti-citrate-EDTA (Fig. 1, step b). The loss of intracellular radiotracers due to the treatment of cells with fixatives was reported in several studies (Silver & Davoll, 1978; Larsen *et al.*, 2008). Tang & Morel (2006) observed that the fixation of diatoms (*T. weissflogii*) with glutaraldehyde resulted in a loss of 90% of ^{14}C-labeled methylamine, a substrate that is taken up, but not assimilated by diatoms. By contrast, negligible loss of intracellular ^{55}Fe was observed in the same study (Tang & Morel, 2006). To investigate whether fixation results in the loss of intracellular ^{55}Fe of bacterial cells, we tested the two different fixatives PFA and FA on *A. macleodii* cells labeled with ^{55}Fe (Fig. 1, steps b + d and a + d). Our results demonstrate that the fixation of bacterial cells for 4 h does not induce any significant loss of intracellular ^{55}Fe as compared to cells that were not exposed to these fixatives (Table 1b, paired *t*-test, $P = 0.05$ and 0.11 for PFA and FA, respectively).

Ti-citrate-EDTA was thus selected as the suitable reagent for ^{55}Fe, because in addition to an excellent removal of extracellular iron without loss of radioactivity, it did not interfere with the procedure of *in situ* hybridization, as described below.

Microautoradiography

To determine the maximum amount of cells associated with silver grains, time series were performed for each experiment. As illustrated for two time series (Fig. 2), a minimum of 4 weeks of exposure to the NTB2 emulsion was required to reach a saturation level in the fraction of DAPI cells associated with silver grains. The maximum percent cells with silver grains varied among experiments between 3% and 29% of total DAPI cells. In the control treatments, the percent DAPI cells associated with silver grains remained low (< 0.5% of total DAPI cells) over the exposure period. These microscopic observations further demonstrate the efficient removal of nonspecifically bound ^{55}Fe. The exposure time we determined in the present study is higher than the 2–4 weeks required for autoradiography with *Anabaena* spp. (Paerl, 1982) and higher than the 3 weeks reported by Hutchins *et al.* (1993) for microautoradiograms with natural phytoplankton communities. In these previous studies, enough silver

Table 1. Effect of the Ti-citrate-EDTA wash and cell fixation on the cellular iron quota of *Alteromonas macleodii*. The Ti-citrate-EDTA wash (a) was tested on cells incubated live (Replicate 1–3) and on cells killed prior to the incubation with ^{55}Fe (Replicate 4–6). For each paired replicate, the difference between the washed and unwashed treatment was calculated and results from live cells were compared to those from killed cells. The effect of fixatives (b) was evaluated on cells incubated live with ^{55}Fe and then fixed with either paraformaldehyde (PFA fixed) or formaldehyde (FA fixed) prior to the washing procedure. The intracellular iron quota of PFA- or FA-fixed cells was compared pairwise to the cells without fixatives. Mean values ± SD of triplicate filters of each biological replicate are given. All 55Fe cell quota are in 10-4 dpm per cell.

(a) Washing and ^{55}Fe leakage

Live cells			Killed cells		
Replicate	Unwashed	+Ti-citrate-EDTA	Replicate	Unwashed	+Ti-citrate-EDTA
1	2.2 ± 0.4	1.5 ± 0.2	4	2.0 ± 1.0	0.17 ± 0.01
2	4.2 ± 0.2	2.5 ± 0.1	5	3.0 ± 0.4	0.15 ± 0.01
3	3.9 ± 0.2	2.4 ± 0.1	6	3.1 ± 0.2	0.16 ± 0.01

(b) Fixatives and ^{55}Fe leakage

Cells washed with Ti-citrate-EDTA			
Replicate	No fixative	PFA fixed	FA fixed
1	1.5 ± 0.2	1.22 ± 0.02	1.25 ± 0.04
2	2.5 ± 0.1	1.83 ± 0.06	1.62 ± 0.06
3	2.4 ± 0.1	1.87 ± 0.03	1.98 ± 0.04

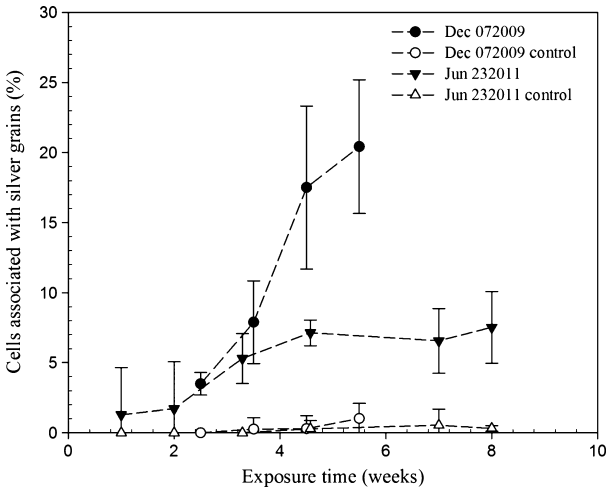

Fig. 2. Changes in the percent DAPI cells associated with silver grains as a function of exposure time. For both experiments, natural bacterial communities from POLA site (NW Mediterranean Sea) were incubated with ^{55}Fe for 7 days. Black symbols denote live treatments, and white symbols denote treatments fixed with formaldehyde prior to incubation. Mean values of 10 counted fields ± SD are given.

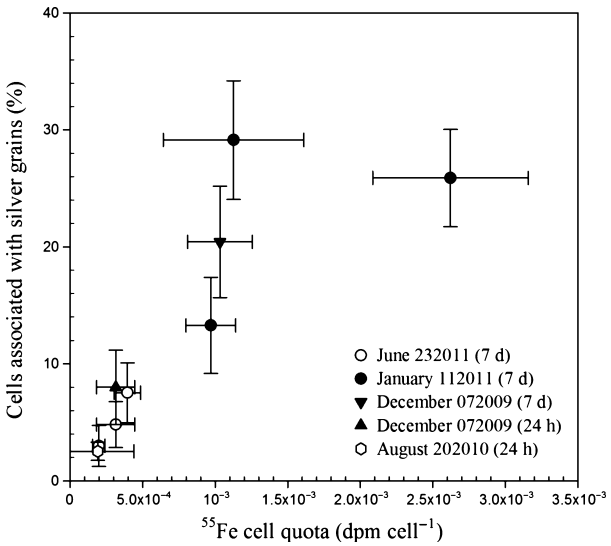

Fig. 3. Maximum fraction of DAPI cells associated with silver grains obtained during time series experiments vs. ^{55}Fe cell quota. Each symbol represents an independent experiment performed on samples from the POLA site at different dates and with variable ^{55}Fe incubation times in parenthesis. Filled and open symbols denote sampling performed in winter and summer, respectively. Filled triangle: December 7, 2009 (24 h); filled inversed triangle: December 7, 2009 (7 day); filled circle: January 14, 2011 (7 day); open circle: June 23, 2011 (7 day); diamond: August 20, 2010 (24 h). Mean ± SD of triplicate incubations of each experiment are given. For all experiments, exposure time in emulsion was 7 weeks.

grains for useful microautoradiograms developed after a shorter exposure time than in our study. However, the maximum of cells associated with silver grains might not have been reached, as no detailed time series are reported in these previous studies.

Combining our results from the different dates and incubation times reveals a linear increase in the maximum fraction of DAPI cells with silver grains and the cellular ^{55}Fe quota up to about 1×10^{-3} dpm per cell (Fig. 3). The highest fraction of cells associated with silver grains was observed in winter at Station POLA, and it was also linked to the duration of the ^{55}Fe incubation.

The environmental conditions, overall bacterial activity, in particular the bacterial iron demand, and the bacterial community composition were most likely different between sampling dates and could have influenced the amount of ^{55}Fe incorporated by the bacterial cells. In several experiments, the maximum percent DAPI cells with silver grains were < 5%, suggesting that only a subset of iron-incorporating bacteria contained the critical cellular ^{55}Fe quota for silver grain production. Our results strongly suggest that the ^{55}Fe quota is a critical parameter for the production of useful microautoradiograms of heterotrophic bacteria, as was already pointed out by Fuhrman & Azam (1982). For ^{3}H, a frequently used radioisotope that emits electrons with similar energy as ^{55}Fe, this issue was never problematic because the activity per cell is estimated in the range 7–14 $\times$ 10^{-3} dpm per cell, based on data from the same study area (Laghdass *et al.*, 2010), and therefore much higher than the per cell activity observed in the present study.

Table 2. Environmental characteristics in surface waters at the sample sites.

	NW Mediterranean Sea (POLA Station)	Southern Ocean (E-4W Station)
Date of sampling	Jun 23 2011	Nov 12 2011
T (°C)	20	2.7
$NO_3^- + NO_2^-$ (μM)	0.15	25.3
PO_4^{3-} (μM)	0.07	1.74
Chla (μg L^{-1})	0.28	0.56
DFe* (nM)	> 1	0.2

*At Station POLA, no direct measurements are available, however, in the NW Mediterranean Sea, dissolved iron concentrations (DFe) are > 1 nM during summer due to the accumulation of dust deposition in the shallow well stratified mixed layer (Bonnet *et al*. 2006). DFe concentrations in surface waters at Station E-4W were 0.2 nM (Quéroué, pers. com).

Combining microautoradiography with CARD-FISH

We applied our protocol in combination with CARD-FISH to natural bacterial communities collected at two contrasting sites. Samples from the NW Mediterranean Sea, at Station POLA, were collected during the summer period, when concentrations of major inorganic nutrients and chlorophyll *a* are low (Table 2). Station E-4W sampled in the Southern Ocean has characteristic features of high-nutrient, low-chlorophyll waters. To compare the within-assemblage distribution of ^{55}Fe between the bacterial communities at these contrasting sites, experiments were carried out with the same incubation time and the same concentration of ^{55}Fe. In addition, samples for microautoradiography coupled to catalyzed reporter deposition–fluorescence *in situ* hybridization (MICRO-CARD-FISH) have been chosen to harbor roughly the same amount of ^{55}Fe per cell above the minimum ^{55}Fe quota discussed previously. The percent total DAPI cells with silver grains in these experiments were on average 5.1 ± 2.7 (n = 12) and 3.4 ± 1.2 (n = 8) for the NW Mediterranean Sea and the Southern Ocean site, respectively. We applied a suite of oligonucleotide probes to verify the compatibility of the different steps of our protocol with CARD-FISH and concomitantly to provide a first overview of the application of the method. At both sites, 83–90% of DAPI cells were EUB positive. Of the DAPI cells associated with silver grains, 83–100% of them were also EUB positive. By contrast, the fraction of cells that hybridized with the control probe remained low (< 1% of DAPI cells).

We determined that the relative contribution of the bacterial groups to total ^{55}Fe-incorporating cells was reflected in their respective contributions to abundance (Fig. 4). The percent DAPI cells with visible silver grains were overall low for both experiments, this pattern could therefore reflect the most active iron-incorporating cells. It was, however, interesting to note that the contributions

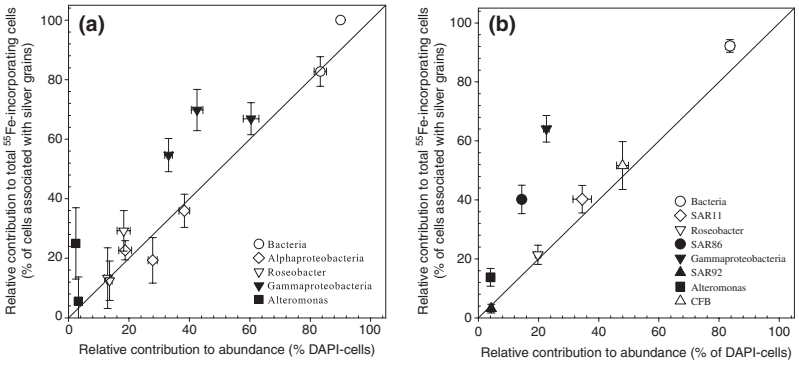

Fig. 4. Relative contribution of bacterial groups to total ^{55}Fe-incorporating cells vs. the relative contribution to abundance in NW Mediterranean Sea at Station POLA (a) and in Southern Ocean at Station E-4W (b). At Station POLA, samples were collected on June 23, 2011. Black symbols denote groups of *Gammaproteobacteria*. Mean values ± SE of 10 counted fields are given. Line denotes 1:1.

of *Gammaproteobacteria*, SAR86 and *Alteromonas* to ^{55}Fe uptake were higher than their respective contributions to abundance. Members of the *Gammaproteobacteria* are frequently reported to develop in incubation experiments due to their opportunistic lifestyle. Even though we did not observe any major changes in the relative abundance of the bacterial groups over the 7 days of incubation time (Fig. S1), this group could have strategies to efficiently respond to the iron addition. Alternatively, it is also possible that members of the *Gammaproteobacteria* have higher iron cell quota. Additional work should aim to address these issues further. Thus, despite the contrasting environmental conditions at the two study sites, we observed a similar pattern in the response of the bacterial community to iron uptake. To the best of our knowledge, our study provides the first description of the bacterial community, on different phylogenetic levels, that contributes to iron uptake in different ocean regimes.

Taken together, the method described here demonstrates that MICRO-CARD-FISH using the radiotracer ^{55}Fe can be successfully applied to the study of marine bacterial groups involved in iron uptake. Our study highlights the potential of the method in future studies. A promising application would be to investigate iron bound to various organic ligands, which could provide insights into the capability of heterotrophic bacteria to acquire iron from different sources.

Acknowledgements

We thank Matthew Cottrell for invaluable advice in image analysis processing. We thank the constructive comments of two anonymous reviewers and the editor that helped improve a previous version of the manuscript. This work was funded by Agence Nationale de la Recherche (Project BACCIO, Biomolecular Approach of the Cycling of Carbon and Iron in the Ocean, ANR-08-BLAN-0 309).

References

Alonso-Sáez L & Gasol JM (2007) Seasonal variations in the contribution of different bacterial groups to the uptake of low molecular weight-compounds in NW Mediterranean coastal waters. *Appl Environ Microbiol* **73**: 3528–3535.

Bonnet S & Guieu C (2006) Atmospheric forcing on the annual iron cycle in the western: a 1-year survey. *J Geophys Res* **111**: 1–13.

Chase Z & Price NM (1997) Metabolic consequences of iron deficiency in heterotrophic marine protozoa. *Limnol Oceanogr* **42**: 1673–1684.

Cottrell MT & Kirchman DL (2003) Contribution of major bacterial groups to bacterial biomass production (thymidine and leucine incorporation) in the Delaware estuary. *Limnol Oceanogr* **48**: 168–178.

Fuhrman JA & Azam F (1982) Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. *Mar Biol* **66**: 109–120.

Herndl GJ, Reinthaler T, Teira E, Aken HV, Veth C, Pernthaler A & Pernthaler J (2005) Contribution of *Archaea* to total prokaryotic production in the deep Atlantic Ocean. *Appl Environ Microbiol* **71**: 2303–2309.

Hudson RJM & Morel FMM (1989) Distinguishing between extra- and intracellular iron in marine phytoplankton. *Limnol Oceanogr* **34**: 1113–1120.

Hutchins DA, DiTullio GR & Bruland KW (1993) Iron and regenerated production: evidence for biological iron recycling in two marine environments. *Limnol Oceanogr* **38**: 1242–1255.

Hutchins DA, Wang W-X, Schmidt MA & Fisher NS (1999) Dual-labeling techniques for trace metal biogeochemical investigations in aquatic plankton communities. *Aquat Microbiol Ecol* **19**: 129–138.

Laghdass M, West N, Batailler N, Caparros J, Catala P, Lantoine F, Oriol L, Lebaron P & Obernosterer I (2010) Impact of lower salinity water masses on bacterial heterotrophic production and community structure in the offshore NW Mediterranean Sea. *Environ Microbiol Rep* **2**: 761–769.

Larsen A, Tanaka T, Zubkov MV & Thingstad TF (2008) P-affinity measurements of specific osmotroph populations using cell-sorting flow cytometry. *Limnol Oceanogr Methods* **6**: 355–363.

Longnecker K, Lomas MW & Van Mooy BAS (2010) Abundance and diversity of heterotrophic bacterial cells assimilating phosphate in the subtropical North Atlantic Ocean. *Environ Microbiol* **12**: 2773–2782.

Marie D, Partensky F, Jacquet S & Vaulot D (1997) Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry using the nucleic acid stain SYBR Green I. *Appl Environ Microbiol* **63**: 186–193.

Orlic D (1968) The use of ^{55}Fe in high-resolution radioautoradiography of developing red cells. *J Cell Biol* **39**: 201–208.

Paerl HW (1982) Feasibility of ^{55}Fe autoradiography as performed on N_2-fixing *Anabaena* spp. populations and associated bacteria. *Appl Environ Microbiol* **43**: 210–217.

Parry DM & Blackett NM (1973) Electron microscope autoradiography of erythroid cells using radioactive iron. *J Cell Biol* **57**: 16–26.

Poorvin L, Rinta-Kanto JM, Hutchins DA & Wilhelm SW (2004) Viral release of iron and its bioavailability to marine plankton. *Limnol Oceanogr* **49**: 1734–1741.

Price NM, Harrison GI, Hering JG, Hudson Robert J, Nirel PMV, Palenik B & Morel FMM (1989) Preparation and chemistry of the artificial algal culture medium aquil. *Biol Oceanogr* **6**: 443–461.

Sekar R, Pernthaler A, Pernthaler J, Warnecke F, Posch T & Amann R (2003) An improved protocol for quantification of freshwater *Actinobacteria* by fluorescence

in situ hybridization. *Appl Environ Microbiol* **69**: 2928–2935.

Silver MW & Davoll PJ (1978) Loss of ^{14}C activity after chemical fixation of phytoplankton: error source for autoradiography and other productivity measurements. *Limnol Oceanogr* **23**: 362–368.

Strzepek RF, Maldonado MT, Higgins JL, Hall J, Safi K, Wilhelm SW & Boyd PW (2005) Spinning the "Ferrous Wheel": the importance of the microbial community in an iron budget during FeCycle experiment. *Global Biogeochem Cycles* **19**: 1–14.

Sunda WG & Huntsman SA (1995) Iron uptake and growth limitation in oceanic and coastal phytoplankton. *Mar Chem* **50**: 189–206.

Tang D & Morel FMM (2006) Distinguishing between cellular and Fe-oxide-associated trace elements in phytoplankton. *Mar Chem* **98**: 18–30.

Tortell PD, Maldonado MT & Price NM (1996) The role of heterotrophic bacteria in iron-limited ocean ecosystems. *Nature* **383**: 330–332.

Tortell PD, Maldonado MT, Granger J & Price NM (1999) Marine bacteria and biogeochemical cycling of iron in the oceans. *FEMS Microbiol Ecol* **29**: 1–11.

Tovar-Sanchez A, Sañudo-Wilhelmy SA, Garcia-Vargas M, Weaver RS, Popels LC & Hutchins DA (2003) A trace metal clean reagent to remove surface-bound iron from marine phytoplankton. *Mar Chem* **82**: 91–99.

Vila-Costa M, Simó R, Kiene RP, Pinhassi J, González JM, Moran MA & Pedrós-Alió C (2004) Use of microautoradiography combined with fluorescence *in situ* hybridization to determine dimethylsulfoniopropionate incorporation by marine bacterioplankton taxa. *Appl Environ Microbiol* **70**: 4648–4657.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Relative abundance of bacterial groups at the beginning (white bars) and at the end (grey bars) of the 7-day incubation period.

Table S1. List of the probes used in this study for CARD-FISH.