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Determination of specific iron chelator by using LC-ICP-MS and LC-ESI-MS

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Abstract

Identification and quantification of siderophore type chelate was undertaken using recently developed high-performance liquid chromatography-mass spectrometry methods. Five different siderophore type chelates were detected and the compounds comprised of two groups; ferrioxamines. In the dissolved phase, three types of hydroxamate siderophore have been identified; Ferioxamine B (FOB), Ferrioxamine G (FOG) and ferrioxamine E (FOE). Concentration of dissolved FOB, FOG and FOE are extremely low between 0–135 x 10^{-18} M, and their distribution is spatially and temporally variable in this region. The concentration and diversity of siderophore type chelates determined during this study, was lower than those reported previously for dissolved ferrioxamine siderophore concentrations at lower latitudes in the Atlantic Ocean. The initial data from this study suggested that dissolved siderophores in this region is not an important fraction (< 0.1%) of the natural organic Fe(III) binding ligand pool.

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Keywords: Speciation; iron chelator; LC-ICP-MS; LC-ESI-MS; siderophores

1. Introduction

Iron plays a special role in marine food chains, as it is an essential ingredient for growth and functioning of organisms.

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In phytoplankton cells, iron plays a major role in the electron transfer processes in photosynthesis and respiration. Iron is essential for the synthesis of the photosynthetic pigment chlorophyll a along with a range of enzymes [1]. Atmospheric dust accounts for a major portion of the global iron input to the world's ocean [2], that away from river inputs. Dust is considered the principal source of soluble and bio-available Fe to remote open ocean surface water [3]. Deposition of dust occurs via dry and wet deposition, and is strongly seasonal and episodic in nature [4]. In certain regions, this deposition represents a significant source of iron to the ocean and can alleviate iron limitation [3]. In high-latitude areas, the atmospheric Fe deposition is very low and insufficient to compensate for the ambient Fe concentrations. It was resulting in depleted dissolved Fe concentrations in the open ocean [5].

Iron was an important factor in controlling phytoplankton growth in the high nutrient low chlorophyll (HNLC) regions. The manipulations of phytoplankton communities in bottle experiments and in situ physiological measurements in the North Atlantic Ocean (~40°N) have indicated the formation of iron limited conditions (2). Thus, this region has been suggested as an iron limited conditions [6]. Furthermore, the low concentration of iron (<0.1 nM) in the surface waters has shown to coincide with excess organic Fe(III) binding ligands (0.4-0.5 nM, log K´FeL= 22-23) [7]. The presence of organic Fe(III) binding ligands in this Fe limited region will work towards prevention of pronounced Fe stress in a microbial surface water community by maintaining Fe in the soluble phase.

The source of these ligands is thought to be biological [8]. There is a prominent hypothesis that an important fraction of natural organic Fe(III) binding ligands are bacterial siderophores [9] which alter Fe bioavailability to marine organisms [10]. Siderophores are produced by prokaryotes as part of a specific Fe uptake mechanism [11]. These compounds make iron more available for biological uptake by bacteria by enhancing its solubility [12]. Marine heterotrophic bacteria and cyanobacteria have been shown to produce siderophores to acquire iron and take up both their own siderophores as well as those produced by other organisms when iron limited [13].

The direct determination of siderophores in natural seawater has been reported in only a limited number of locations [10; 14]. The total concentrations of siderophores ranged between 3-20 pM, which was much lower than the dissolved Fe concentrations (0.58±0.25 nM, n =118) [14]. In this study, the presence of dissolve hydroxamate siderophore in the seawater has been investigated in order to extend the knowledge of the spatial distribution of siderophores in the high-latitude region.

2. Materials and methods

2.1. Seawater sampling

The seawater samples were collected during two separate sampling campaigns on board RRS Discovery cruises D350 (May 2010) and D354 (July/August 2010) in the high-latitude North Atlantic Ocean (Table 1, Fig. 1). Seawater samples were collected in a trace metal clean titanium CTD frame with 20 L trace metal clean Teflon coated OTE (Ocean Technology Equipment) bottles, fitted with silicone O rings and plastic-coated springs. In this analysis two different studies were conducted, dissolved siderophore pre-concentrate analysis in field, and identification and quantification of dissolved siderophores in laboratory at National Oceanography Centre Southampton, United Kingdom.

Table 1 The coordinate for each station during RRS Discovery D350 and D354 cruise in the high latitude North Atlantic Ocean. Seawater sample at each station was filtrated and dissolved siderophores was extracted by SPE technique

Date	St.	Lat. (W°)	Long. (N°)	Depth (m)	Extraction volume (L)
01/05/2010	A1	60.56	34.52	25	19
02/05/2010	A2	60.02	34.57	27	19
03/05/2010	A3	59.59	37.55	27	7.5
04/05/2010	A4	59.58	29.10	24	15
05/05/2010	A5	59.54	26.02	30	16
06/05/2010	A6	60.50	21.44	30	17
07/05/2010	A7	61.57	20.01	20	17
08/05/2010	A8	63.05	19.52	23	12

11/07/2010	B1	60.00	19.58	20	18
14/07/2010	B2	60.00	23.00	20	18
16/07/2010	B3	60.02	29.00	20	12
19/07/2010	B4	60.02	41.00	20	10
22/07/2010	B5	63.00	35.00	20	17
24/07/2010	B6	63.00	30.00	20	18
26/07/2010	B7	58.00	35.00	40	19
30/07/2010	B8	63.49	35.04	20	19
31/07/2010	B9	63.30	33.23	3	17
02/08/2010	B10	63.25	23.35	20	18
03/08/2010	B11	61.47	24.27	30	19
04/08/2010	B12	61.14	24.45	3	18
06/08/2011	B13	61.45	24.00	3	20

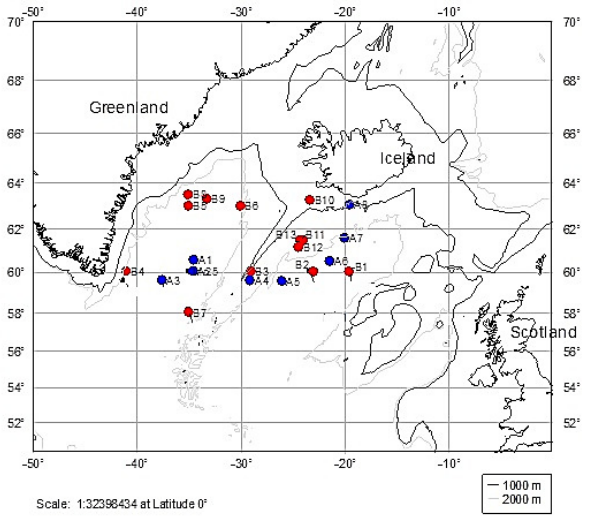

Fig. 1. The stations during D350 and D354 sampling, respectively, for dissolved siderophores determined during this study. The black filled circle represents the station for the nutrients enrichment experiment

2.1.1 Dissolved siderophores pre-concentrate analysis

Seawater samples (20 L) were collected from the chlorophyll a maximum layer. The samples were filtered through 3.0 and 0.2 µm filters using cellulose nitrate membrane filters. The dissolved siderophores in the samples were concentrated onto polystyrene-divinylbenzene solid-phase extraction (SPE) cartridges (Isolute ENV+) [14] with a reservoir capacity of 3 mL and 200 mg packing. The cartridges were pre-washed with 1 mL methanol (LC-MS grade, Riedel-de Haen) before pre-concentrating the samples. The volume of sample extracted through the cartridge was recorded from the carboy scale measurement. The cartridge was frozen at -20 °C until further processing and analysis on shore.

2.2. Quantification of dissolved siderophores

Siderophores in the dissolved samples were quantified using high-performance liquid chromatography-inductively coupled plasma mass spectrometry (LC-ICP-MS) (X series, Thermo Scientific). It was done by monitoring the gallium-69 (^{69}Ga) isotope [14]. The ^{69}Ga (ICP–MS standard, VWR) with final concentration 10 µM – 14 mM was added to a 200 µL sub-sample and allowed to equilibrate for overnight at room temperature before

measurement by LC-ICP-MS. The chromatographic separation was performed by using a polystyrene divinyl benzene stationary phase (PRP-H1, 100×2.1 mm 5 µm, Hamilton) column.

A standard gradient of 95% solvent A to 100% solvent B over 20 minutes at the beginning of the chromatogram was followed by isocratic elution with 100% solvent B for 5 minutes [14].

2.3. Identification of dissolved siderophores

The types of siderophores present in our samples were carried out using high-performance liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) [14].

Samples and standard solutions were automatically injected (25 µL volume) onto the separation column using an auto sampler (Accela, Thermo Scientific). The chromatography was started with a standard gradient of 100% solvent A to 100% solvent B over a period of 20 minutes. Then an isocratic elution with 100% solvent B was conducted for 2 minutes. The mass to charge ratio of each type of siderophores was determined using an ion trap mass spectrometer (LTQ Velos, Thermo Scientific) in positive ion mode. Data was collected and interpreted using Xcalibur 2.0 software (Thermoquest). The full mass spectra (MS) chromatogram was obtained between m/z 200-1500, while the ferric complexes of the siderophores were detected by selective ion monitoring (SIM) of the most abundant ion (parent ion) in the total ion mass spectra.

A collision induced dissociation (CID) analysis was carried out the parent ion to confirm the present of siderophores' complexes. The parent ion in each total ion mass spectra undergoes fragmentation on bombardment with helium atoms at activation amplitude of 35% [5]. The fragmentation pattern that obtained from CID analysis was compared to the previous published pattern for the confirmation of the siderophore's identity.

3. Results and discussion

During this study, three types of hydroxamate siderophore have been identified; Ferioxamine B (FOB), Ferrioxamine G (FOG) and ferrioxamine E (FOE) (Fig. 2). Apply of selective ion monitoring (SIM) allowed for the detection of very low concentrations of known individual siderophores. FOB, FOG and FOE produce a single charged protonated ion ($[M+H]^+$) m/z 614, m/z 672 and m/z 654 at 7.53 minutes, 8.11 minutes and 9.86 minutes, respectively.

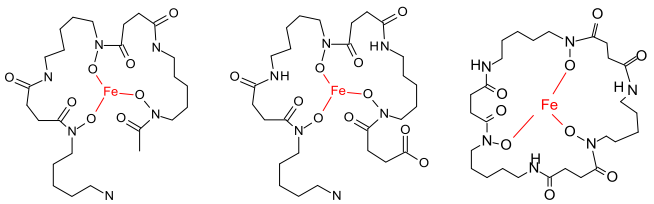

Fig. 2: Structure of ferrioxamine B (FOB), ferrioxamine G (FOG) and ferrioxamine E (FOE) determined during this study in the high latitude North Atlantic Ocean

Most of the seawater samples showed the present of FOB at a concentration between 0.63×10^{-18} M and 135.56×10^{-18} M (Table 2), with an average concentration of 16.65×10^{-18} M (n=17). The concentration for FOG and FOE was in the range 0.54-6.27x10^{-18} M and 1.24-2.79 x10^{-18} M (Table 2), respectively.

Table 2 Types of dissolved siderophores present in the seawater of high latitude North Atlantic Ocean during RRS *Discovery cruise* D350 (May 2010) and D354 (July/August) cruise. Quantification and identification were carried out by HPLC-ICP-MS and HPLC-ESI-MS methods, respectively. Concentration for each siderophore is represented by the values in brackets. n/a is below the detection limit of ICP-MS

Date	St.	Depth (m)	[dFe] nM	Conc. of dissolved siderophore (x10^{-18} M) [FOB]	[FOG]	[FOE]
01/05/2010	A1	25		√ (135.56)		
		85		√ (1.54)		
02/05/2010	A2	27		√ (1.77)		
		93		√ (0.63)		
04/05/2010	A4	24		√ (7.79)		
05/05/2010	A5	30		√ (44.54)		
06/05/2010	A6	30		√ (13.49)	√ (n/a)	
07/05/2010	A7	20		√ (3.26)	√ (n/a)	
08/05/2010	A8	23		√ (5.96)	√ (n/a)	
11/07/2010	B1	20	0.27	√ (14.10)		
		70	0.24	√ (2.44)		
24/07/2010	B6	20	0.05	√ (1.99)	√ (n/a)	
		60	0.09	√ (7.79)	√ (6.27)	
26/07/2010	B7	40	0.12	√ (4.17)	√ (0.90)	
31/07/2010	B9	3	0.05	√ (2.64)	√ (n/a)	
03/08/2010	B11	30	0.10	√ (33.82)	√ (n/a)	√ (1.63)
04/08/2010	B12	3	0.08	√ (n/a)	√ (n/a)	√ (1.24)
06/08/2011	B13	3	0.07	√ (1.58)	√ (0.54)	√ (2.79)

The FOE was also detected in this region with concentration between 1.24–2.79 x10^{-18} M in the chlorophyll a maximum layer (Table 2). According to 15 and 16, several microbes produce more than one siderophore, and in many cases the siderophores may be produced by different bacteria. A more powerful chelator with high stability constant with iron (FOE; log K=$10^{32.5}$), may only be produced when the first less powerful chelator (FOB; log K=$10^{30.6}$), fails to provide enough iron to the cell due to very low iron condition at these stations (<0.1 nM) (Table 2). This type of siderophore production has been observed in Azotobacter vinelandii [15; 16].

Hydroxamate siderophores are known to be produced by gram-negative bacteria such as Vibrio species [17] and by gram-positive Actinomycetes species [18]. In the previous studies [21], most authors have suggested that the production of siderophores was stimulated by low concentrations of dissolved iron. In fact, the measurement of surface (<150 m depth) dissolved iron concentration (0.2 µm fraction) (Steigeneurger et al., unpublished data) in this region has shown the concentration was between 0.11-0.32 nM (Table 2). However, due to the formation of the spring bloom with a rapid increase in the chlorophyll biomass [20] in these regions, the dissolved iron concentration decreased to 0.07 - 0.10 nM by the end of July to August 2010 (Table 2). As a response to the iron limited condition, it is possible that heterotrophic bacteria will actively produce siderophores [21] to sequester iron from the various iron pools [22] due to their high iron requirements for growth compared to the phytoplankton in open-ocean [9].

4. Conclusion

This initial study on the determination of iron (III) specific chelator (hydroxamate siderophores) has confirmed the presence of ferrioxamine in the high latitude region. Very low concentrations of dissolved siderophores (0–135 x 10^{-18} M) and a relatively low diversity of siderophore type chelates were observed by using this powerful tools (combination of LC-ICP-MS and LC-ESI-MS). The extremely low concentration of total dissolved siderophores detected during this study, may thus be affected by a low seawater temperature that potentially plays an important role in influencing siderophores production and growth of a potential heterotrophic bacterial producing siderophores.

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