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Identification and Quantification of Siderophore Type Chelators in the Incubation Samples

Abstract

Determination of siderophore type chelates in nutrient's enrichment incubation samples was conducted during this study by applying the current developed method on seawater from high-latitude North Atlantic. Seawater samples were enriched with a few nutrient combinations to stimulate production of siderophores during the incubation period. Five different siderophore type chelates which comprised two groups; the ferrioxamines (ferrioxamine B (FOB) and ferrioxamine G (FOG)) and the amphibactins (amphibactin D, E and unknown amphibactin) were identified in our samples. Their concentration was ranged between 0.024-3.814 pM and this indicated that the bacteria capable of producing these siderophores are present in this region. Our data also suggested that the combination nutrients (GNP and GNO3P) enrichment produced more abundance of heterotrophic bacterial and more diversity of siderophore type chelates, compared to single nutrient (G) addition. Moreover, the diversity of siderophore type chelates also affected by nitrogen source, with $NH4^+$ (GNP) is being more optimal for the production of siderophores.

Keywords: Siderophore; Heterotrophic bacterial; Nutrient's enrichment; Ferrioxamine; Amphibactins

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Introduction

Prokaryotes are known to have high cellular Fe:C ratios and therefore, higher iron (Fe) requirements than phytoplankton [1]. The Fe:C ratios of eukaryotic phytoplankton and heterotrophic bacteria are 3.7 ± 2.3 and 6.1 ± 2.5 µmol Fe mol C^{-1}, respectively [2,3]. In response to Fe deficiency marine prokaryotes secrete siderophores to solubilise and facilitate the acquisition of Fe(III) in the environment. Both cyanobacteria and heterotrophic bacteria have been found to produce siderophores under Fe limited conditions [4-7]. However, production of siderophores by phytoplankton has been the subject to much research, and up until now, there has been no evidence that phytoplankton is actively producing siderophores [5,6].

In a recent study, Mawji et al. [10] reported an existing correlation between total ferrioxamine siderophores concentration and heterotrophic bacterial abundance (r=0.47, n=19, p<0.05) in the low-latitude North Atlantic Ocean. On the other hand, these workers did not observe a significant correlation between the total ferrioxamine concentration and autotrophic bacteria or picoeukaryote phytoplankton (<2 µm) abundances. Recently, our understanding of siderophore production by heterotrophic bacteria in the marine environment has been largely based on bacteria. These bacteria can either be cultured in the laboratory [11-14] or grown successfully in nutrient enriched seawater samples [10,15,16], as this allows for the production of sufficient quantities of siderophores for further characterisation. The influence of different sources of carbon (glucose, glycine and chitin) along with nitrogen (ammonium) and phosphorus (phosphate) on the siderophore production has been examined by Mawji et al. [16]. These workers found that the easily available carbon source (glucose; $C_6H_{12}O_6$) produced highest concentration and diversity of hydroxamate siderophores, compared to other carbon sources (glycine; $C2H_5NO_2$ and chitin; $(C_8H_{13}NO_5)_n$) [16]. The total concentration of siderophores produced in glucose incubations ranged between 0.2-69.0 nM with 12-14 different siderophores identified in waters from the low latitude of Atlantic Ocean (43.7°N – 31.8°S) [16].

Furthermore, these workers observed a positive correlation between siderophore concentrations and bacterial cell abundance in the glucose incubations. In contrast, there was no relationship between these variables in the chitin and glycine incubations. A high number of siderophore type chelates (10-12) was determined in the chitin incubation, but at low concentrations (0.1-0.6 nM) [16]. In the glycine incubations, a constant number of siderophore type chelates (3-8) was observed at low concentrations, suggesting that the lack of readily available nitrogen in glycine incubations might have affected siderophore production [16], since glycine was used as a source of both nitrogen and carbon by the bacteria.

In this study, the influence of different sources of nitrogen and iron concentrations on siderophore's production and types of siderophore secreted by heterotrophic bacteria was examined in order to identify the role of different nitrogen sources on marine siderophore's productions and diversity.

Methodology

Chemical preparation

Glucose solution: A 0.1 M glucose solution was prepared by diluting 4.502 g of glucose stock ($C_6H_{12}O_6$, 180.080 g/mol, Fisher Scientific) into 250 ml MQ water. A final concentration of 100 µM of glucose was added to seawater samples. All nutrients were prepared in acid cleaned 250 ml LDPE bottle (Nalgene) in a laminar flow hood.

Ammonium chloride solution: A 2.675 g of ammonium chloride stock (NH_4Cl, 53.49 g/mol, Fisher Scientific) was diluted into 250 ml MQ water (final concentration 0.2 M). Ammonia was added to obtain a final concentration of 200 µM for the incubated seawater.

Sodium nitrate solution: A 4.250 g of Sodium nitrate stock ($NaNO_3$, 84.99 g/mol, Fisher Scientific) was diluted into 250 ml MQ water to get 0.2 M concentration solution. Nitrate was added to obtain a final concentration of 200 µM in the incubated seawater.

Di-sodium hydrogen orthophosphate solution: A 0.02 M di-sodium hydrogen orthophosphate solution was prepared by diluting 0.760 g of stock (Na_2HPO_4, 156.01 g/mol, Fisher Scientific) into 250 ml MQ water. Phosphate was added to obtain a final concentration of 200 µM in the incubated seawater.

Paraformaldehyde: The 10% paraformaldehyde solution was prepared from paraformaldehyde stock ($(C_1H_2O)_n$, 30.03 g/mol, Sigma Aldrich) in the fume hood. A 5.0 g of paraformaldehyde added to 40 ml MQ water. Then 0.5 ml of 1.0 M NaOH (Fisher Scientific) was added and heated to 60°C in water bath. The solution was allowed to cool, and the final volume was adjusted to 50 ml with MQ water. The solution was stored at -80°C. A final solution of 1% paraformaldehyde was used to fix the flow cytometry samples.

Nutrient cleaning

Iron and other trace metal contaminants were removed from nutrient solutions using chelex-100 (Sigma). The chelex-100 column was cleaned with 50 ml MQ water and 50 ml 1.0 M HCI (Fisher Scientific), followed by another 50 ml MQ water. The column was conditioned with 250 ml of 0.05 M NaOH (Fisher Scientific). The pH of each nutrient was adjusted to 8 using 0.05 M NaOH or 1.0 M HCl before loading on to the column. The first 50 ml of the nutrient eluant was discharged, and the remainder was collected into 250 ml acid cleaned LDPE bottle (Nalgene) and stored at 4 °C. The column was rinsed with 150 mL MQ water between nutrients.

Incubation conditions

Seawater samples for the enrichment experiments were collected in the high-latitude North Atlantic Ocean (Figure 1) into 2 L polystyrene tissue culture flasks (Becton Dickinson, USA) during the RRS Discovery cruise D350 and D354 in April-May 2010. Unfiltered seawater was added to 2 L polystyrene tissue culture flask (Becton Dickinson) and was enriched with nutrients in a laminar flow hood. Incubation conditions are given in Table 2. The nutrient solutions were filter sterilized (10 mL BD Discardit™ II syringe, 0.2 µm Minisart RC-membrane, Sartorius stedim filter) on addition to the seawater. The nutrient enrichment experiments represent an assay for siderophores that may be detected in seawater.

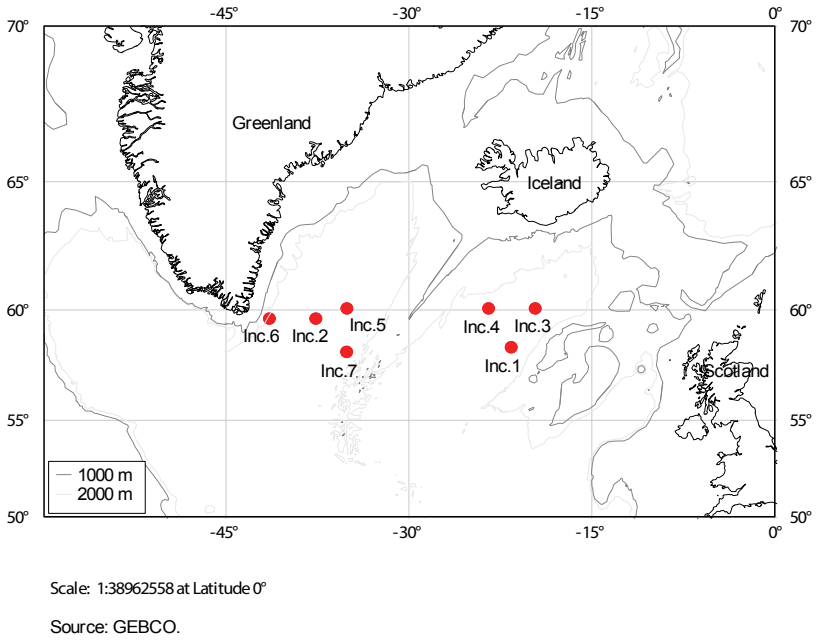


Figure 1: Location for enriched seawater experiments during RRS Discovery cruise D350 and D354 in the high- latitude North Atlantic Ocean.

Table 1: The enriched seawater sample treatments used during this study. All treatments were done in duplicate and untreated seawater was used as control.

Date	Station	Treatments	
29/04/2010	Inc. 1	Control	
	58.34ºN, 21.51ºW	GNP	100 µM glucose + 200 µM NH_4^+ + 20µM PO_4^{3-}
	(3 m depth)	GNP+Fe	100 µM glucose + 200 µM NH_4^+ + 20µM PO_4^{3-} + 9 nM Fe(III)
		GNP++Fe	100 µM glucose + 200 µM NH_4^+ + 20 µM PO_4^3 + 90 nM Fe(III)
03/05/2010	Inc. 2	Control	
	59.59ºN, 37.55ºW	GNP	100 µM glucose + 200 µM NH_4^+ + 20µM PO_4^{3-}
	(27 m depth)	GNP+Fe	100 µM glucose + 200 µM NH_4^+ + 20µM PO_4^{3-} + 9 nM Fe(III)
		GNP++Fe	100µM glucose + 200 µM NH4+ + 20µM PO43- + 90 nM Fe(III)
12/07/2010	Inc.3	Control	
	60.02ºN, 19.58ºW	GNP	100 µM glucose + 200 µM NH_4^+ + 20µM PO_4^{3-}
	(3 m depth)	GNO3P	100 µM glucose + 200 µM NH_4^+ + 20µM PO_4^{3-}
		G	100 µM glucose
15/07/2010	Inc.4	Control	
	60.02ºN, 23.37ºW	GNP	100 µM glucose + 200 µM NH_4^+ + 20µM PO_4^{3-}
	(20 m depth)	GNO3P	100 µM glucose + 200 µM NH_4^+ + 20µM PO_4^{3-}
		G	100 µM glucose
18/07/2010	Inc. 5	Control	
	60.02ºN, 35.00 ºW	GNP	100 µM glucose + 200 µM NH_4^+ + 20µM PO_4^{3-}
	(3 m depth)	GNO3P	100 µM glucose + 200 µM NH_4^+ + 20µM PO_4^{3-}
		G	100 µM glucose
19/07/2010	Inc. 6	Control	
	59.59 ºN, 41.35ºW	GNP	100 µM glucose + 200 µM NH_4^+ + 20µM PO_4^{3-}
	(3 m depth)	GNO3P	100 µM glucose + 200 µM NH_4^+ + 20µM PO_4^{3-}
		G	100 µM glucose
26/07/2010	Inc. 7	Control	
	58.13ºN, 35.02 ºW	GNP	100 µM glucose + 200 µM NH_4^+ + 20µM PO_4^{3-}
	(40 m depth)	GNO3P	100 µM glucose + 200 µM NH_4^+ + 20µM PO_4^{3-}
		G	100 µM glucose

The enriched seawater was incubated in the dark on deck in incubators at ambient surface ocean temperature, with un-enriched seawater used as a control. The samples were incubated until the bacteria had reached the late exponential or stationary growth phase (4-5 days). Bacterial growth was monitored every day by using absorption measurements (Red Tide USB 650 visible spectrophotometer, Ocean Optics) at a wavelength of 600 nm. Samples were collected daily for enumeration of bacteria (flow cytometric analysis) and freeze at -80°C after adding 1% (v:v) paraformaldehyde.

At the end of incubation period, samples were sequentially filtered through 3.0 and 0.2 µm cellulose acetate filters to remove bacterial cells (Sartorius polycarbonate filter unit, 45

mm nitrocellulose membrane filter, Millipore). In a laminar flow hood, the filtered supernatant was passed over pre-washed polystyrene- divinylbenzene solid phase extraction (SPE) cartridges (Isolute ENV+, 200 mg × 3 ml) under gentle vacuum (Supelco Visiprep™) for extraction of siderophores. Cartridges loaded with sample were frozen at -20°C until further processing and analysis on shore. Prior to analysis, SPE cartridges were defrosted and eluted with 5 mL of 81:14:5:1 (v/v/v/v) acetonitrile: propan-2-ol: water: formic acid [17].

Determination of siderophores

The quantification of siderophores in the incubated seawater samples was carried out as for seawater samples [17], except for the concentration of added gallium. In order to ensure complete exchange of iron with gallium, a higher final concentration of 14 mM gallium (ICP–MS standard, VWR) was added to extracts from nutrient enrichment experiments and left overnight before analysis by HPLC-ICP-MS.

The identifications of siderophore type compounds in the incubation samples were carried by HPLC-ESI- MS method in the full scan's mode (m/z 200-1500) on both unamended samples, and samples pre-equilibrated with 14 mM gallium [16,17]. The analysis of samples after addition of excess Ga allows unknown siderophores to be identified in the complex mass chromatograms [18]. In the samples with added Ga, the Ga complexes of siderophores were determined through the distinctive isotopic ratio of gallium ($^{69}Ga/^{71}Ga$ ratio 3:2) in the full mass spectrum chromatograms. The identity of the siderophores was compared to the retention time for the potential Ga complex peak to peaks at similar retention times in the unamended sample, that were m/z 13 units less than the most abundant isotope in the added Ga sample (equivalent to the difference in mass between ^{56}Fe and ^{69}Ga). Siderophores identified by gallium exchanges were then characterised by collision induced dissociation (CID) analysis of the selected parent ions as described by Mohamed et al. [17].

Flow cytometry analysis

Flow cytometric analysis used to enumerate heterotrophic bacterial was based on their fluorescence and light scattering properties. It does not possess detectable auto-fluorescence, and therefore, fluorescent probes are added, such as DNA or protein stains. During this study, the nucleic acid stains SYBR Green I was used (Sigma-Aldrich) to determine its abundance of in the samples. 10 µl of SYBR Green I was added to samples (1 ml) and the solution was incubated for 1 hour in the dark at room temperature [17].

Results and Discussion

Bacterial growth in the nutrients enrichment samples

On day 1, the bacterial abundances in each seawater enrichment varied between 0.9-1.1 × 10^6 cells ML^{-1} and 0.9-

2.0 × 10^6 cells mL^{-1} for Inc. 1 and Inc. 2 (Figure 2), respectively. An initial abundance in the control was 0.9 × 10^6 cells mL^{-1} in both incubation experiments.

The bacterial abundance during the day 5 varied by the type of nutrient enrichment, in Inc. 1. The highest abundance was 5.5 × 10^6 cells mL^{-1} in the GNP+Fe treatment (Table 2, Figure 2), which is nearly three times higher than its abundance in the control of Inc. 1 (1.9 × 10^6 cells mL^{-1}). While, there were 2.4 × 10^6 cells mL^{-1} and 2.8 × 10^6 cells mL^{-1} in the GNP and GNP++Fe treatment (Table 2), respectively, at the end of the incubation period in the Inc. 1. On the other hand, there was no significant difference in its abundances in the treatments of Inc. 2 (Figure 2). At the end of the incubation period (day 5), its abundance varied between 3.0 × 10^6 cells mL^{-1} (in the control) and 4.3 × 10^6 cells mL^{-1} (in the GNP treatment) (Table 2). This indicated that the addition of extra Fe likely did not increase the abundance of bacterial in the high-latitude North Atlantic seawater sample. The bacterial growth in these incubations is thus to be more strongly influenced by other factors, e.g. temperature [19-21].

An addition of 100 µM glucose (G) to the samples was sufficient to result in a significant increase in the bacterial abundance (3.5-7.5 × 10^6 cells mL^{-1}, Table 2) at the end of the incubation for the cruise in July-August 2010 relative to the control (1.7-4.7 × 10^6 cells mL^{-1}, Table 2, Figure 3). The addition of other nutrients NH_4^+ and PO_4^{3-} or NO_3^- and PO_4^{3-} along with glucose further increased the abundance in the GNP and GNO_3P treatment (Figure 3). The final abundance in the GNP and GNO3P treatments was ranged between 13.8-17.6 × 106 cells mL^{-1} and 7.7-10.2 × 10^6 cell's mL^{-1} (Table 2), respectively. This suggested that the combination of nitrogen, phosphate and

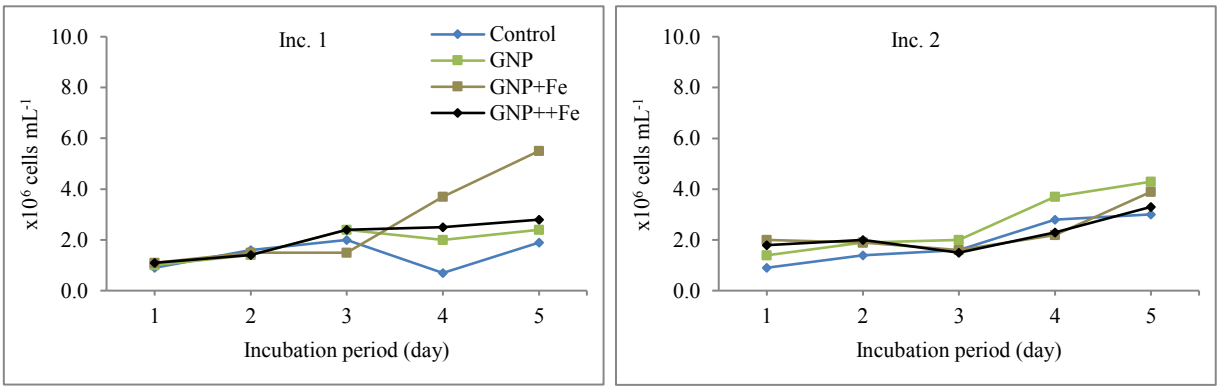


Figure 2: Bacterial abundance in the nutrient enriched samples during RRS Discovery cruise D350 in the Inc. 1 and Inc.2. GNP represents the addition to the samples of glucose (100µM), NH4+ (200 µM) and PO43- (20µM) to the samples. GNP+Fe and GNP++Fe represent addition of Fe at concentration 9 nM and 90 nM, respectively, along with GNP.

Table 2: Concentrations and diversity of siderophore type chelates determined in nutrient enriched seawater in the high-latitude North Atlantic Ocean.

Station	Incubation period	Nutrient	Final bacteria	Siderophores determined	
	(days)	enrichment	(x106 cells mL-1)	Type chelates	Conc. (pM)
Inc. 1	5	Control	1.9	-	-
		GNP	2.4	-	-
		GNP+Fe	5.5	-	-
		GNP++Fe	2.8	-	-
Inc. 2	3	Control	1.6	-	-
		GNP	2.0	FOB	-
		GNP+Fe	1.6	FOB	-
		GNP++Fe	1.5	FOB	-
	5	Control	3.0	-	-
		GNP	4.3	FOB	2.039
		GNP+Fe	3.9	FOB	-
		GNP++Fe	3.3	FOB	-
Inc.3	4	Control	2.9	-	-
		GNP	14.8	FOB	0.024
				FOG	-
				Amph. (883)	-
				Amph. D (885)	-
				Amph. E (911)	-
		GNO3P	8.4	FOB	-
				Amph. (883)	-
				Amph. E (911)	-
		G	3.5	FOB	-
				FOG	-
Inc. 4	4	Control	4.7	-	-
		GNP	13.7	FOG	0.849
				Amph. E (911)	-
		GNO3P	10.2	FOG	-
				Amph. E (911)	-
		G	6.0	Amph. E (911)	-
Inc. 5	4	Control	1.7	-	-
		GNP	13.8	FOB	-
				FOG	0.072
				Amph. E (911)	-
		GNO3P	7.7	FOB	-
				Amph. (883)	-
		G	3.5	FOB	-
				FOG	-
				Amph. E (911)	-
Inc. 6	5	Control	3.6	-	-
		GNP	14.0	FOB	-
				FOG	-
				Amph. (883)	-
				Amph. D (885)	-
				Amph. E (911)	-
		GNO3P	8.5	FOB	-
				FOG	-
				Amph. E (911)	-
		G	4.2	FOB	-
				Amph. E (911)	-
Inc. 7	5	Control	4.0	-	-
		GNP	17.6	FOB	3.814
				FOG	0.133
		GNO3P	8.0	FOB	-
		G	5.5	FOB	-

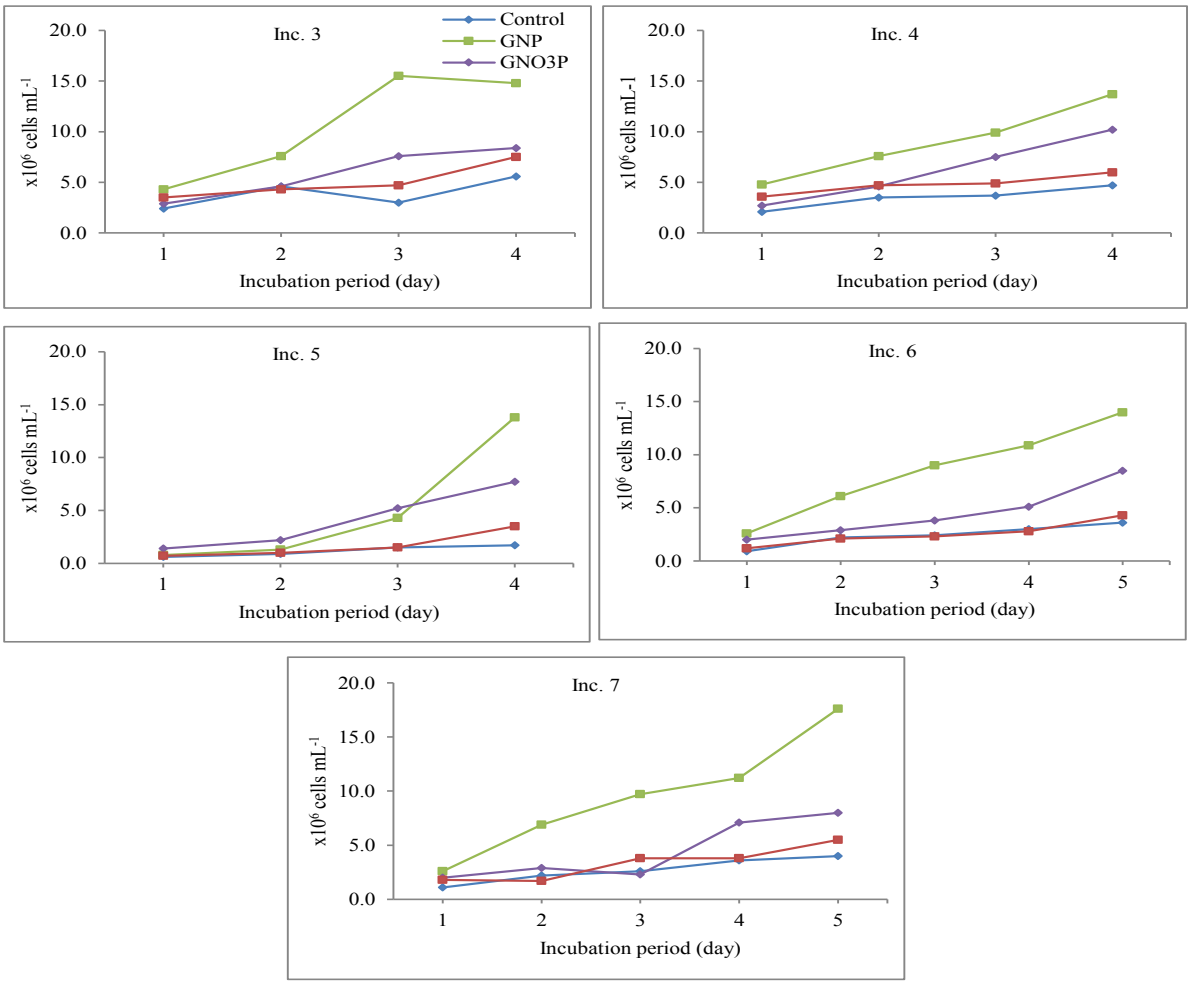


Figure 3: Bacterial abundance in the nutrient enriched incubations during RRS Discovery cruise D354 in the high- latitude North Atlantic Ocean. Two different sources of nitrogen (GNP and GNO3P) were added to the sample along with glucose and phosphate. G represents the addition of glucose (100 µM) alone to the samples.

glucose (GNP or GNO_3P) resulted in the greatest enhancement of bacterial abundance. These results hint on that the growth on glucose alone may have been less efficient than the growth in the addition nitrogen source. Carbon-rich substrates such as glucose provide energy for cellular maintenance but do not provide all the essential nutrients needed to facilitate growth for the bacteria [22,23].

However, the GNP treatment produced higher bacterial abundance compared with the GNO_3P treatment (Figure 3). It indicated a high uptake of NH_4^+ compared to the NO_3 form for its growth. In fact, NH_4 is invariably the preferred nitrogen source for bacteria growth, although its concentration is less than NO_3 concentrations in the oceans [24]. According to Kirchman and Wheeler [25], the nitrogen uptake by heterotrophic marine bacterial was 78% and 32% of the total NH4 and NO3 uptake, respectively. The uptake of NO_3 is unusual because assimilatory NO3 reduction is thought to be too energetically expensive to be carried out by heterotrophic bacterial that is carbon and energy limited [26].

During this study, a higher bacterial abundance was observed in the GNP treatment during July-August 2010 compared to April-May 2010. In July-August 2010, the highest final bacterial abundance in the GNP treatment was ranged between 13.7 × 10^6 cells Ml^{-1} and 17.6 × 10^6 cells Ml^{-1} with an average 14.8 × 10^6 cells Ml^{-1} (n=5).

During this study, the highest bacterial abundance was 4.3 × 10^6 cells Ml^{-1} in the Inc. 2 (Table 2). The different of seawater temperature during both cruises D350 (7-10°C) and D354 (8-13°C) in the high-latitude North Atlantic Ocean may have contributed to the different of bacterial abundances in the nutrients enrichment samples. In fact, the bacterial abundances in the nutrients enrichment samples in this region (ranged between 2.4-17.6 × 10^6 cells Ml^{-1}, with an average 11.5 × 10^6 cells Ml^{-1}, *n*=7) were lower than reported in the low-latitude North Atlantic Ocean (ranged between 8.4-18.0 × 10^6 cells Ml^{-1}, with an average 13.4 × 10^6 cells Ml^{-1}, *n*=6) [16].

Diversity and concentration of siderophore type chelates

Siderophore type chelates were isolated from the nutrient enriched seawaters collected in the high-latitude North Atlantic Ocean. Five different siderophore type chelates have been detected during this study (Table 2). The compounds comprised two groups; the ferrioxamines (ferrioxamine B (FOB) and ferrioxamine G (FOG)) and the amphibactins (amphibactin D, E and unknown amphibactin). These two ferrioxamine siderophores have been detected in the dissolved phase in this

region [17]. But, the amphibactins D and E (Figure 4) and an unknown amphibactin were not detected. However, these amphibactin siderophores have been observed in nutrient enriched incubations, which were conducted in the open ocean [16] and in near-shore waters [15]. These amphibactins and FOG have previously been reported to be produced by gram-negative bacteria such as Vibrio species [27,28]. On the other hand, desferrioxamines B and G are produced by gram-positive Actinomycetes species [29,30]. Unfortunately, the distribution of the specific bacterial species was not been determined during this study.

The siderophore type chelates were identified by reanalysis of the samples using LC-ESI-MS analysis after overnight equilibration with excess (14 mM) Ga. A peak for Ga complexed with FOB (GaFOB) was observed at Rt=7.35 minute in the chromatogram (Figure 5). The mass to charge ratios (*m/z*) of 627 and 629, which indicated protonated complexes of ^{69}GaFOB and ^{71}GaFOB (Figure 5), respectively, were observed at the retention time of

7.35 min. Mass chromatograms for other Ga-siderophore complexes present in the samples were shown in Figure 6 for Ga-ferrioxamine G (GaFOG) and Figure 7-8 for Ga-amphibactin complexes.

The collision induced dissociation (CID) analysis of the selected ions has confirmed the presence of ferrioxamine (*m/z* 614, 672) and amphibactin (*m/z* 885, 911, 883) siderophores in our nutrient enrichment incubation samples (Figure 9). The amphibactin siderophores were characterised by a peptide head group containing the amino acids (L-serine, D ornithine and L-ornithine) [28]. Each amphibactin has initially fragmented through the loss of water (*m/z* 18), followed by fragmentation of *m/z* 190 (terminal hydroxamate chelating group) and *m/z* 277 (Figure 9). An identical *m/z* 503 in all spectra indicated a second fragmentation pathway, involving the loss of the third hydroxamic acid group together with the fatty acid tail. For the FOB and FOG fragmentation was discussed previously by Mohamed and Gledhill [17] in the dissolved phase.

Since, most of the amphibactin siderophore complexes eluted between R_t ~20-21 min in 100% organic solvent, this has suggested that this group of siderophores are hydrophobic in nature. The masses of each amphibactin differed by an extension of saturated or unsaturated carbon chains, which ranges from C-14 to C-18 [28]. Thus, two peaks of amphibactin were obtained in the mass chromatogram shown in Figure 7. The first peak (Rt = 19.58 min) was identified as an unknown amphibactin (*m/z* 883) and second peak (Rt = 20.38 minute) was identified as amphibactin D (*m/z* 885).

During this study, the number of siderophore type chelates detected by LC–ICP–MS was lower than that determined by LC–ESI–MS analysis due to the very low siderophore concentrations. Only ferrioxamine siderophores (FOB and FOG) (Figure 10) were determined by LC–ICP–MS (Table 2, Figure 10). Furthermore, FOB and FOG were only determined in the nutrient enriched incubations with GNP treatment. The

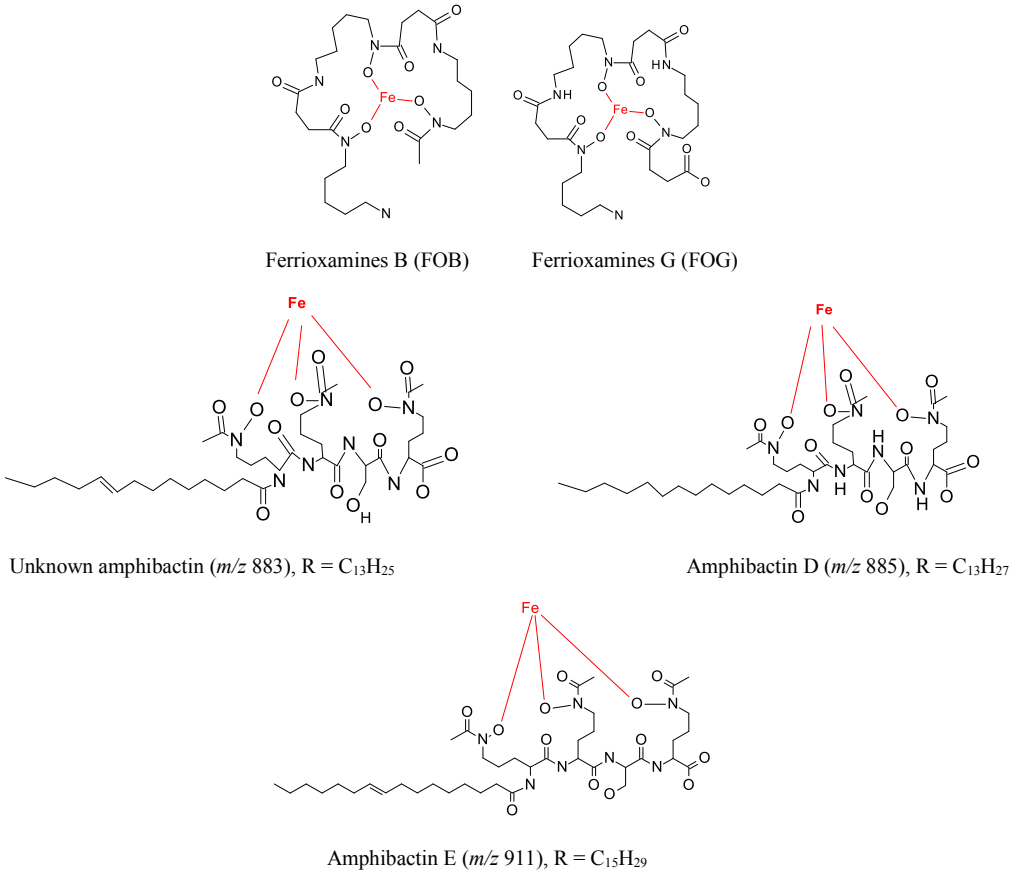


Figure 4: Structure of ferrioxamines and amphibactins in the nutrients enrichment incubation samples from high- latitude North Atlantic Ocean during this study.

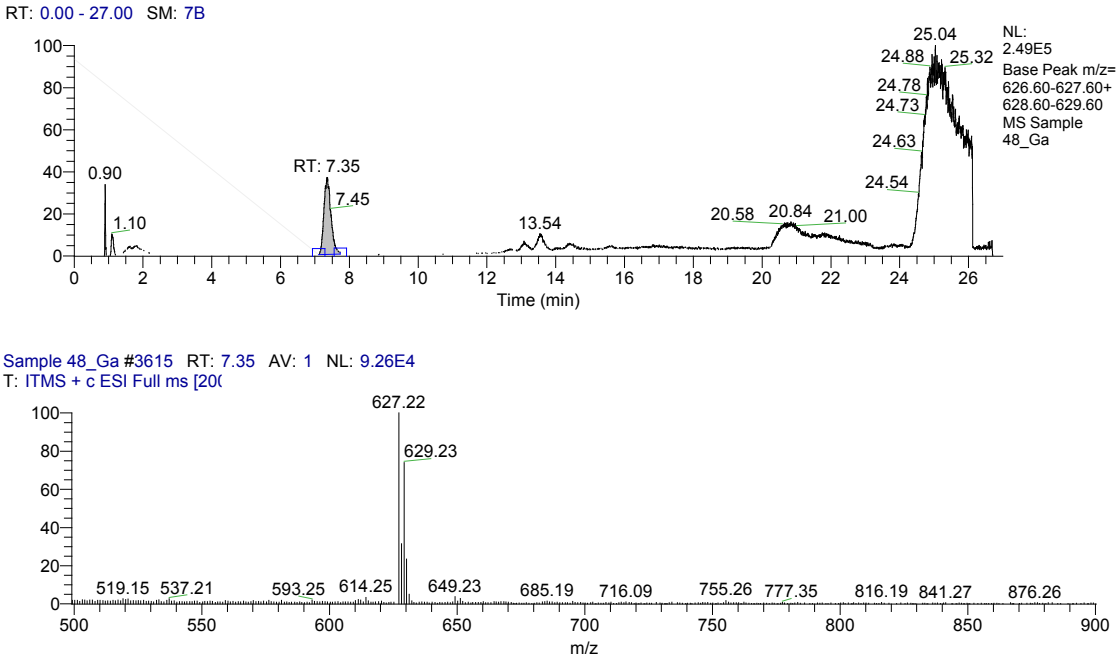


Figure 5: Extracted mass spectra for Ga complexed siderophore type compound (Ga-ferrioxamine B (GaFOBH+), *m/z* 672/629). This siderophore was identified in the high-latitude North Atlantic Ocean in Inc. 3 which was amended with glucose (100 µM), NH_4^+ (200 µM) and PO_4^{3-} (20 µM) (GNP).

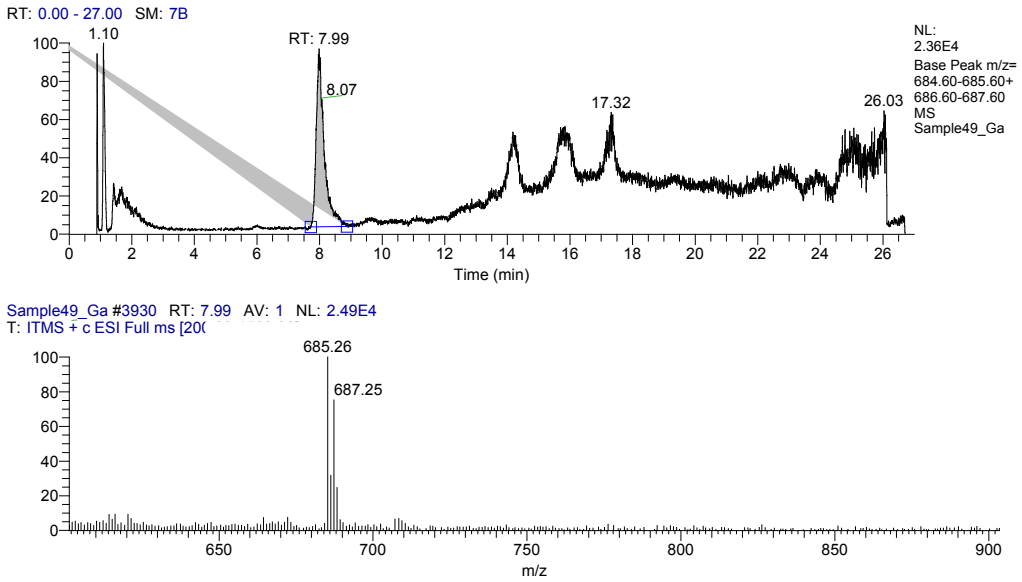


Figure 6: Extracted mass chromatograms for Ga-ferrioxamine G complexed (GaFOGH+) identified in at Rt = 7.99 (*m/z* 685/687) obtained from Inc. 3 which was amended with glucose (100 µM), NH_4^+ (200 µM) and PO_4^{3-} (20 µM) (GNP).

concentrations for both FOB and FOG varied between 0.024-3.814 pM and 0.072–0.849 pM (Table 2), respectively.

The diversity and concentrations of siderophore type chelates determined during this study was less than reported for the low-latitude of Atlantic Ocean (43.74ºN – 31.83ºS) [16] and in coastal waters [15]. Both of these studies have determined more than 7 different siderophore type chelates [15,16]. Furthermore, Mawji et al. [16] found a higher diversity of siderophore type chelates in the Western Tropical Atlantic (12-14 siderophore type chelates). Although the concentrations (0.024-3.814 pM) and diversity (5 siderophore type chelates) of siderophore produced by bacteria during this study are much lower than previously reported (0.1- 69.0 nM) [16], siderophores were nevertheless detected in the incubations carried out in the high-latitude North Atlantic Ocean. This indicated that the bacteria capable of producing these siderophores are present in this region, but that either they are naturally less abundant, or the production of siderophore's type chelates was limited by a so far unidentified factor such as low seawater temperatures [31-33].

Effect of iron and nitrogen on siderophore production

The effect of enhanced Fe concentrations on siderophores production was investigated in Inc. 1 and Inc. 2 (Figure 1). There were no siderophore type chelates detected in any treatment conditions in Inc. 1 after 5 days (Table 2) and only FOB was identified in the Inc. 2 (Table 2) in all treatments after 3 days

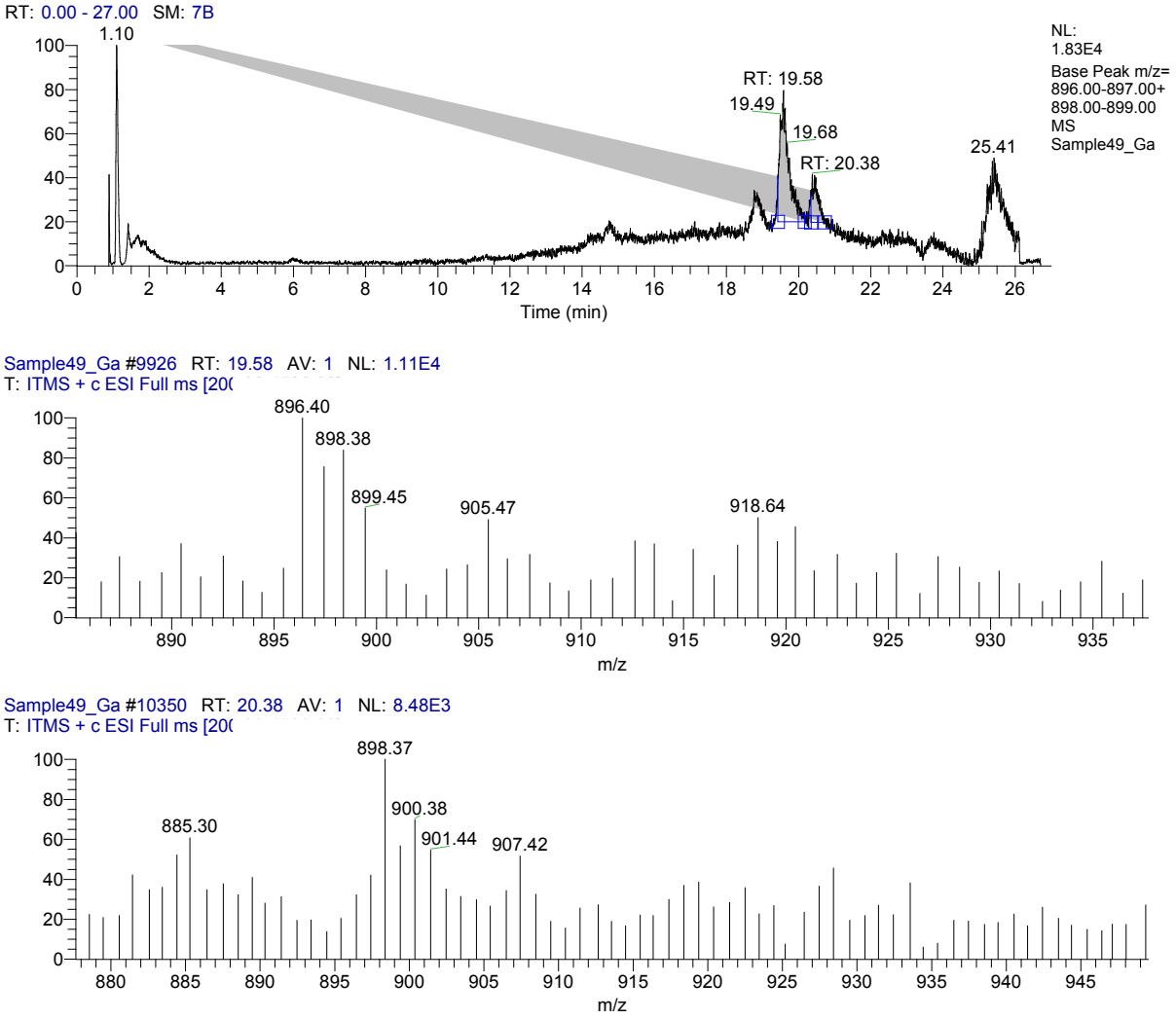


Figure 7: Extracted mass chromatograms for a Ga complexed siderophores identified in an extract from Incubation 3 which was amended with glucose (100 µM), NH4+ (200 µM) and PO43- (20µM) (GNP). Peak at Rt = 19.58 min (*m/z* 896/898) was identified as the protonated Ga complex of the unknown amphibactin, and peak at Rt=20.38 min (*m/z* 898/900) was identified as the protonated Ga complex of amphibactin D.

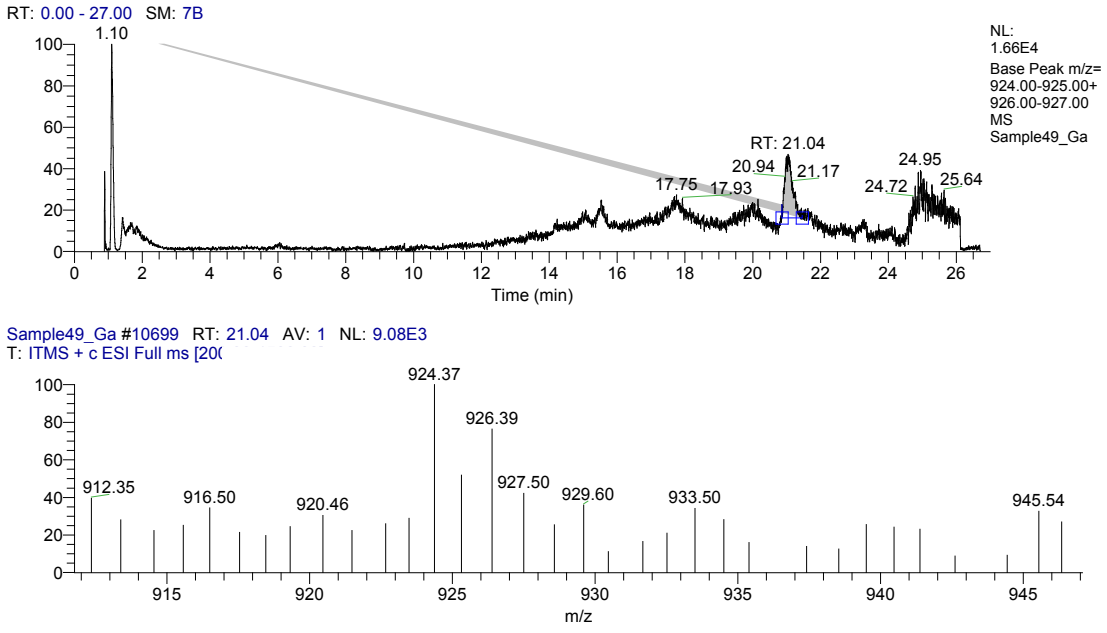
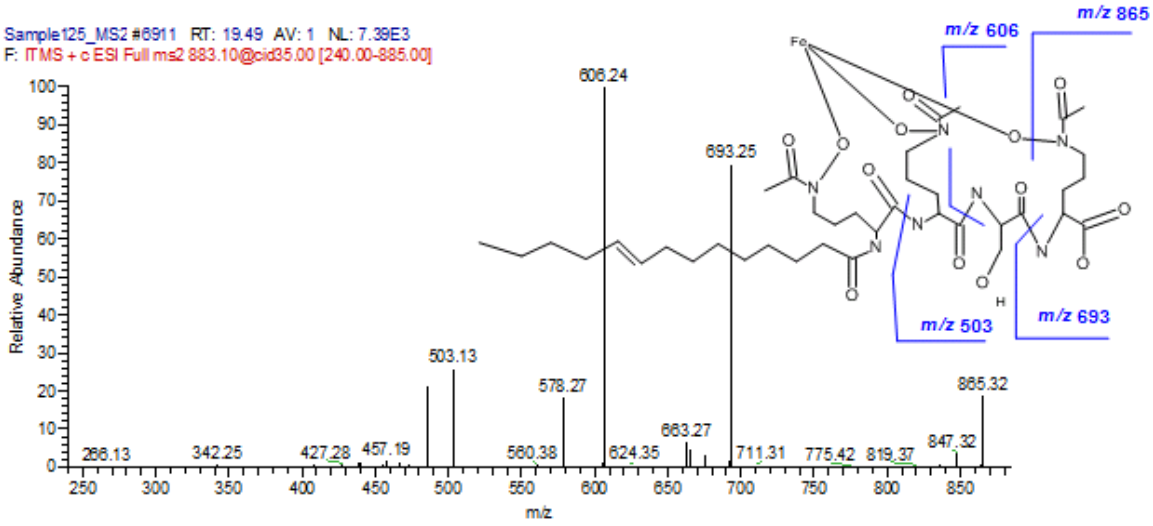
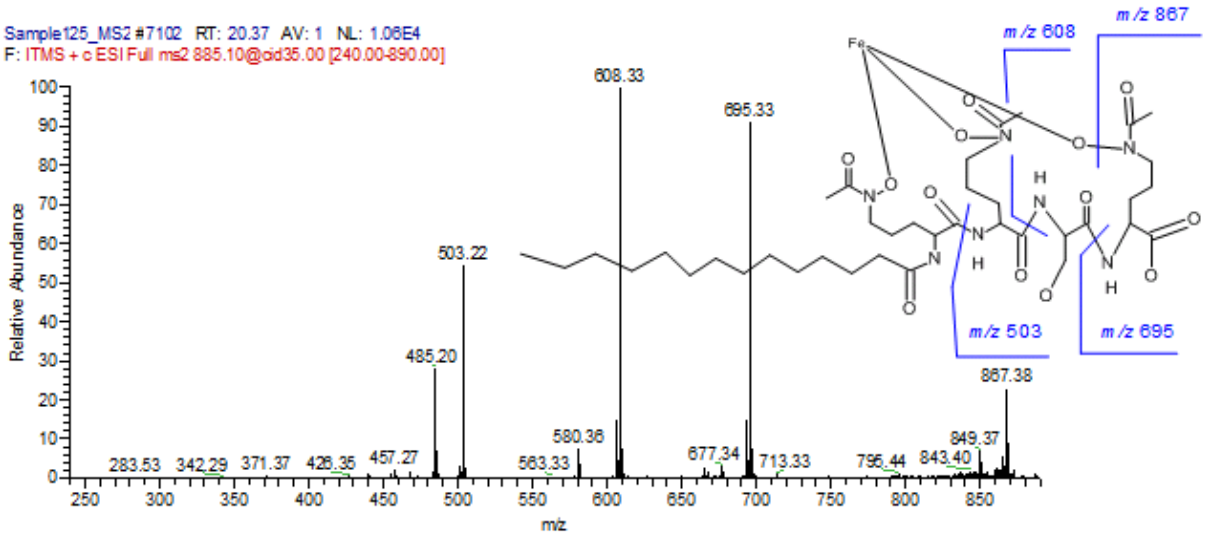


Figure 8: Extracted mass chromatograms for the protonated Ga-amphibactin E complex in the Inc. 3 which was amended with glucose (100 µM) plus PO43- (20µM) plus NH4+ (200 µM), at Rt=21.04 min (*m/z* 924/926).

a) Unknown amphibactin (*m/z* 883)



b) Amphibactin D (*m/z* 885)



c) Amphibactin E (*m/z 911*)

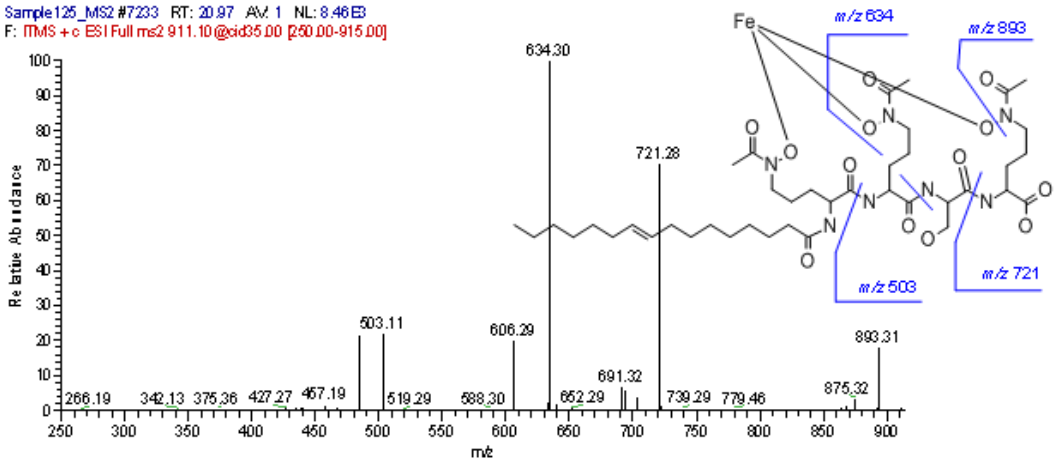


Figure 9: Mass spectra obtained on CID analysis of amphibactin D (m/z 885), E (m/z 911) and unknown amphibactin (m/z 883) in Inc. 6which was amended with glucose (100 µM), NH4+ (200 µM) and PO43- (20 µM) (GNP).

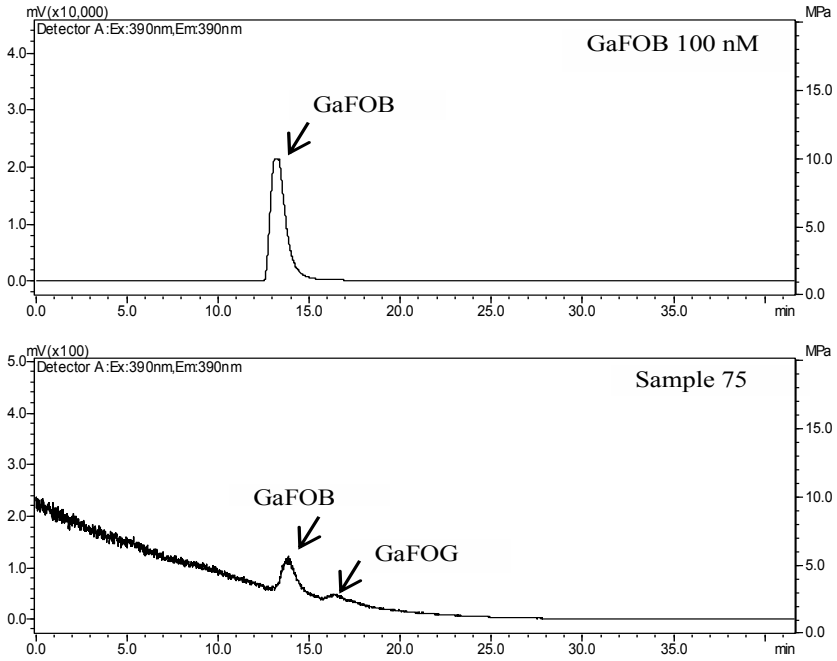


Figure 10: An example of a ^{69}Ga chromatogram from the HPLC-ICP-MS analysis. This chromatogram shows the peaks for the Ga-siderophore complexes in the working standard solution (GaFOB 100 nM) and nutrient enriched seawater sample for the GNP treatment (Sample 75, Inc. 7).

and 5 days, except in the control (Table 2). Thus, it appeared that addition of extra Fe does not necessarily alter siderophore production in seawaters of high-latitude North Atlantic.

Seawater enriched with only glucose (G) produced a low diversity of siderophore type chelates compared to samples which were amended with combination of GNP or GNO_3P (Table 2). This was consistent with the observed lower heterotrophic bacterial abundance in the glucose treatment when compared with treatments that include added nitrogen and phosphate (Figure 3).

Samples amended with GNO3P produced a lower diversity of siderophore type chelates when compared to GNP treatments (Figure 3, Table 2). This indicated that siderophore diversity was also affected by nitrogen source, with NH_4^+ is being more optimal for the production of siderophores. It is interesting to note that uptake of NH_4^+ was reported to be less temperature dependent than NO_3 uptake [34,35]. Thus, it was possible that NH_4^+ is more important as a nitrogen source for bacterial growth and siderophores production in the high-latitude region.

The addition of GNP to the sample produced the highest diversity and concentrations of siderophores type chelates, and hence is the best condition for siderophore type chelates production by bacteria in the marine environment. However, in this region, the siderophore production may be strongly affected by the low temperature which reduces its production by the heterotrophic bacteria.

Conclusions

Two types of ferrioxamine siderophores and amphibactin siderophores, produced by heterotrophic bacteria, were determined by HPLC-ESI-MS analysis in nutrient enriched seawater experiments in the high-latitude North Atlantic Ocean. The siderophore type chelates detected in these experiments all belong to the tris-hydroxamate family and may reflect the selectivity of the chromatographic method used. It is thus possible that other siderophore type chelators may be present in the samples which were not detected due to methodological constraints. Since the Ga exchange analysis depends strongly on complexation of siderophore with Ga at low pH (~2), siderophore complexes that are unstable or insoluble at low pH (e.g. catecholate siderophores) [36] will not be detected using the conditions applied in this study. In addition to the pH effect, further method selectivity will be introduced by the pre-concentration process, as a result of a loss of some hydrophilic siderophores. This study also highlighted the importance of nutrient type to the production of siderophores in nutrient enrichment experiments. Further studies are necessary in order to examine the relationship between siderophore production and geographical location.

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