Production of siderophore type chelates in Atlantic Ocean waters enriched with different carbon and nitrogen sources.

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Abstract

Siderophore type chelates were detected in nutrient enriched, incubated seawater collected from different biogeographical regions of the Atlantic Ocean. Seawater was enriched with glucose and ammonium, glycine (as a source of carbon and nitrogen) or chitin and ammonium at different concentrations and incubated for up to 3 – 4 days in the dark. Siderophore type chelates were detected using high performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC-ICP-MS) after complexation with Ga. Samples were subsequently analysed by HPLC - electrospray ionisation mass spectrometry (HPLC-ESI-MS) in order to confirm the identity of the known siderophores, and to obtain the pseudo-molecular ions of unknown siderophore type chelates. A total of 22 different siderophore type chelates were resolved in the HPLC-ICP-MS chromatograms. Ten different siderophore type chelates were identified by HPLC-ESI-MS, 3 of which had not previously been identified in nutrient enriched seawater incubations. The concentration and diversity of siderophore type

chelates was highest in seawater amended with glucose. The concentrations and diversity of siderophore type chelates also varied with biogeographical area in the Atlantic Ocean, with the North Atlantic Sub-tropical Gyre yielding highest concentrations in incubations, and the South Atlantic Sub-tropical Gyre and Western Tropical Atlantic yielding the highest diversity.

Keywords Iron biogeochemistry, siderophores, seawater, bacteria, Atlantic Ocean

Introduction

Siderophores are metal-binding chelates produced by prokaryotes as part of a highly specific Fe uptake mechanism (Vraspir and Butler, 2009). Siderophores are considered important in the bacterial acquisition of Fe in seawater and consequently influence Fe biogeochemistry in the ocean (Hopkinson and Morel, 2009; Mawji et al., 2008a; Vraspir and Butler, 2009). In addition to Fe uptake, siderophores are reported to have additional roles such as the acquisition of other metals (Duckworth et al., 2009a; Duckworth et al., 2009b), antimicrobial activity (Girijavallabhan and Miller, 2004) and quorum sensing (Amin et al., 2007). Siderophores are chemically diverse, utilizing hydroxamate, catecholate and carboxylate groups to chelate Fe. Bacteria and Archea produce a range of different types of siderophores (e.g. Homann et al., 2009; Neilands, 1995; Winkelmann, 2007). Recently characterised groups of siderophores produced by marine Bacteria and Fungi (e.g. Holinsworth and Martin, 2009; Homann et al., 2009; Vraspir and Butler, 2009) occur as siderophore suites containing a peptide head group and a fatty acid tail (Homann et al., 2009). The peptide head group chelates the Fe while the fatty acid tail increases the hydrophobicity of the siderophore and is thought to prevent loss from the cell via diffusion (Volker and Wolf-Gladrow, 1999; Vraspir and Butler, 2009). Our current knowledge of marine siderophores is largely based on microorganisms that can be grown successfully in the laboratory as this allows for the production of sufficient quantities of siderophores for complete characterisation (Homann et al., 2009; Vraspir and Butler, 2009). In this study enrichment of seawater samples with different sources of carbon was examined with the aim of increasing our knowledge of novel and uncharacterised siderophore type chelates produced by marine bacterioplankton. The study was carried out in different biogeographical regions of the Atlantic Ocean to allow the assessment of spatial variations in the production of siderophore type chelates. Glucose,

glycine and chitin were selected as carbon (and nitrogen) sources with different availabilities. Glucose is the most abundant dissolved free natural sugar and is preferentially utilised by oceanic prokaryotes relative to other free sugars (Carlson et al., 2002). Opportunistic gamma proteobacteria such as *Alteromonas* sp. and *Vibrio* sp. are known to respond well to enrichments with glucose (Carlson et al., 2002), and siderophores (ferrioxamines and amphibactins) produced by marine *Vibrio* sp. (Martinez et al., 2003; Martinez et al., 2001) have been readily identified in glucose enriched seawater incubations (Gledhill et al., 2004; Mawji et al., 2008a). Glycine was used as a supply of both nitrogen and carbon in incubation experiments. Amino acids such as glycine are taken up by many bacteria, and (Zubkov et al., 2008) showed that amino acid uptake rates were higher than glucose uptake rates in Atlantic gyre waters. In addition, amino acids have been suggested as the preferred form of nitrogen for oligotrophic prokaryotes (Mary et al., 2008). Furthermore, it has recently been shown that glycine is required for growth by SAR11 alpha proteobacteria (Tripp et al., 2009). Chitin is an insoluble biopolymer composed of linear chains of linked N-acetyl-D-glucosamine residues and consists of approximately 47% carbon by weight. Chitin is abundant as a structural component of cell walls and a constituent of shells and exoskeletons. Chitin was used as a representative of high molecular weight organic matter, which requires enzymatic cleavage before uptake.

High performance liquid chromatography – inductively coupled plasma – mass spectrometry (HPLC-ICP-MS) and high performance liquid chromatography – electrospray ionisation – mass spectrometry (HPLC-ESI-MS) were used to detect siderophore type chelates in seawater. HPLC-ICP-MS offers superior detection limits compared to HPLC-ESI-MS due to the absence of interferences from other co-eluting organic compounds. In addition, detection with ICP-MS allows quantification of siderophore type chelates, according to their metal content. However, identification and characterisation of siderophore type chelates is not possible with ICP-MS because ICP is a hard ionisation technique which destroys the organic part of the molecule. In addition, although HPLC allows the siderophore type chelates to be separated prior to ICP-MS detection it is possible for more than one siderophore type chelate to co-elute from the chromatographic column. This particularly occurs in natural samples, where siderophore type chelates can have similar molecular weights and chemical characteristics and where there may be many different siderophore type chelates. In contrast, HPLC-ESI-MS allows molecular masses of prospective siderophore type chelates to be identified and enables further characterisation of compound structure on collision induced

dissociation (CID) (Gledhill et al., 2004; Mawji et al., 2008a; Mawji et al., 2008b). Whilst co-eluting compounds cannot be differentiated by ICP-MS, in principle they can be deconvoluted in the chromatogram by ESI-MS. Thus the two techniques offer complimentary information and potentially provide a powerful tool for the investigation of the production of siderophore type chelates in environmental samples.

Methods

Sample collection.

Samples for the incubation experiments were collected in the period between October 15 and November 28 (2005) in four different biogeographical provinces of the Atlantic Ocean on-board the *RRS Discovery* as part of the Atlantic Meridional Transect (AMT) cruise No. 17 (Fig. 1). Samples were obtained at station 6 in the North Atlantic Subtropical Gyre (NAST), station 17 in the Tropical North Atlantic (NATL), station 31 in the Western Tropical Atlantic (WTRA) and stations 52 and 57 in the South Atlantic Subtropical Gyre (SATL) (Longhurst, 1998). Seawater was collected in the mixed layer from a depth with a 55% light level relative to surface water irradiation. Sample collection was conducted using a CTD rosette frame (Seabird) fitted with 20 L Niskin (General Oceanics) samplers. Seawater was transferred to an acid washed 20 L polyethylene carboy (Nalgene) and subsequently to acid washed, autoclaved 1 L polycarbonate culture vessels (Nalgene).

Incubation conditions

The aliquots of unfiltered seawater (1000 ± 20ml) were enriched with carbon, nitrogen and phosphorus (Table 1) and incubated for 3-4 days in a growth chamber (Sanyo MLR 315, Loughborough, UK) in the dark at ambient seawater temperature. Iron and other trace metal contaminants were removed from nutrient stock solutions using chelex-100 (Sigma), and the nutrient solutions (except for chitin, which is insoluble) were sterilised by filtration (0.2 µm pore size) prior to addition. Chitin was added directly as particles. Unenriched seawater was used as a control at sea. Potential siderophore production from bacterial contamination when using chitin was subsequently examined in the laboratory by the same techniques employed for the incubations at sea except for the use of autoclaved seawater (collected on AMT17). As chitin could not be chelexed, it is possible that addition of chitin also resulted in Fe

contamination. Incubations were prepared in a Class 100 laminar flow hood. Bacterial growth was monitored daily using absorption measurements at a wavelength of 600 nm. Samples for flow cytometry were also collected every 24 hours.

Sample Analysis

Enumeration of bacteria

Samples for flow cytometry analysis of the microbial community were preserved with paraformaldehyde (1% w/v final concentration, Sigma), frozen in liquid nitrogen and subsequently stored at -80 °C. Prior to analysis in the laboratory, the samples were incubated with SYBR green I according to the method of Heywood et al. (2006). The flow cytometric analysis was optimised for enumeration of heterotrophic prokaryotes (hereafter referred to as bacteria) with high nucleic acid and low nucleic acid content. A single analysis was performed on each sample as analytical errors for flow cytometry are normally in the region of 1–2 % (R. Holland, pers. comm.). Average growth rates were calculated as ln(cell concentration)- ln(intitial cell concentration)/(duration of experiment).

Identification and quantification of siderophore type chelates

All sample handling was carried out in a Class 100 laminar flow hood. Chemicals used were purchased from Fisher (UK) unless otherwise stated. High purity water (Milli-Q, Millipore) and LCMS grade solvents (Rheidel de Haan) were used throughout.

After the 3-4 days incubation period, bacterial cells were removed by filtration (0.2 μm cellulose acetate, Sartobran, Sartorius) and the supernatant reserved for the analysis of siderophore type chelates (Gledhill et al., 2004; Mawji et al., 2008a). Siderophore type chelates were preconcentrated onto pre-washed (5 mL methanol, 5 mL Milli Q water) polystyrene-divinylbenzene solid phase extraction (SPE) cartridges (Isolute ENV+, 200 mg x 3 mL). Cartridges loaded with sample were rinsed with 11.2 mM ammonium carbonate (5 mL) and frozen (-20 °C) until further processing and analysis in the laboratory. Cartridges were defrosted and eluted with 5 mL of 81:14:5:1 (v/v/v/v) acetonitrile: propan-2-ol: water: formic acid. The eluent was blown down under nitrogen to aproximately 100 μL and then made up to 500 μL with 0.1 % (v/v) formic acid (Sigma).

Siderophore type chelates were detected and identified by a combination of HPLC-ICP-MS and HPLC-ESI-MS according to the scheme shown in figure 2. Elemental mass spectrometry was performed first as it provides information with respect to likely retention times of

siderophore type chelates, which is useful when examining HPLC-ESI-MS chromatograms. Chromatography was performed using a polystyrene divinyl benzene stationary phase (100 × 2.1 mm 3 μm, Hamilton, Reno, NA, USA). For detection by ESI-MS, samples were analysed both before and after addition of Ga (Gledhill et al., 2004), while for detection by ICP-MS, only samples with added Ga were analysed. Gallium ($Ga(NO_3)_3$ 10,000 ppm ICP-MS standard, VWR) was added to a 100 μL sub-sample at a concentration of 14 mM and the sub-sample allowed to equilibrate overnight at room temperature.

Siderophore type chelates in the incubations were detected using HPLC-ICP-MS (Thermo Elemental PQ2) interfaced via a desolvating nebuliser (MCN 6000, Cetac Technologies) to binary HPLC pumps (Shimadzu LC10ADvp) which were controlled by a system controller (Shimadzu SCL10Avp). Gallium (Ga^{69}) rather than Fe was used to quantify siderophore type chelates because of its lower background contamination and reduced interferences in ICP-MS analysis (Moberg et al., 2004). Chromatographic conditions were as described previously (Mawji et al., 2008a). Briefly, the mobile phase consisted of (A) 95 % water: 5 % methanol: 0.1 % formic acid (v:v:v) and (B) 100 % methanol: 0.1 % formic acid (v:v). The flow rate was 150 μl min^{-1}. An isocratic step of 100 % A for 15 min was followed by a standard gradient of 100% A to 100 % B over 20 min and another isocratic step at 100 % B for 5 min. The system returned to the starting conditions over 5 min and the HPLC column was re-equilibrated with 100 % A for 10 min. Samples and standards (5 μL) were manually injected onto the column using a metal-free injector (Rheodyne 9725i). The injector was suction loaded in order to avoid contact between the sample and the glass syringe. Mixed standards (1.5 nM to 1.5 μM) of ferrioxamine B, ferrichrome (Sigma, Poole, UK) and triacetylfusarinine C (EMC Microcollection, Tubingen, Germany) were used to calibrate the instrument on a daily basis. Reproducibility for the standards was <10%. Instrument drift was checked with a Ga standard after every third sample. A post-sample injection of 5 μL 0.5 M nitric acid and a 15 min isocratic step at 5 % methanol, 95 % water and 0.1 % formic acid (v:v:v) were employed at the beginning of the chromatographic run in order to allow the high concentrations of free Ga to be washed out of the system. Omission of this step resulted in high background Ga counts during the ICP-MS analysis. The eluant line of the HPLC pump was connected to the ICP-MS after the 15 min isocratic step.

Identification of siderophore type chelates (where possible) and further characterization of siderophore type chelates was carried out using HPLC-ESI-MS with a Triple Quadrupole

Mass Spectrometer (ThermoQuest Finnigan TSQ 7000) equipped with electrospray ionization interface (Mawji et al., 2008a). The source and capillary voltage were set at +4.5 kV and 0-50 V (autotune), respectively with a capillary temperature of 250 °C. Nitrogen sheath gas flow rate was 80 (arbitrary units) and auxiliary gas flow rate 40 (arbitrary units). The positive ion scan range was fixed at *m/z* 400-1800 throughout and data was processed with Xcalibur 1.0 software. Chromatographic separation was performed using a binary HPLC pump (ThermoQuest Finnigan TSP P4000). The conditions were the same as for analysis by HPLC-ICP-MS except that the flow rate was 200 µl min^{-1} and the post sample injection of 0.5 M nitric acid and 15 min isocratic step at 100% A were omitted. Collision induced dissociation (CID) (Gledhill et al., 2004; Mawji et al., 2008b) was used to confirm the identity of known siderophores. The instrument was set up for data dependant acquisition of CID spectra where the most abundant ion in each total ion mass spectra undergoes fragmentation. An activation amplitude of 35 % and an activation time of 30.0 ms were used for CID.

HPLC-ESI-MS is a soft ionisation mass spectrometry technique that can be used for the identification and partial characterisation of siderophores e.g. (Berner et al., 1991; Mawji et al., 2008a; Mawji et al., 2008b; McCormack et al., 2003; Vraspir and Butler, 2009). HPLC-ESI-MS has been successfully applied to the detection and identification of siderophore type chelates in seawater incubations and ambient seawater samples (Mawji et al., 2008a; McCormack et al., 2003). Collision induced dissociation of pseudo-molecular ions formed in HPLC-ESI-MS has the advantage of potentially providing structural information. However, in the chromatograms of crude (particulate or dissolved) extracts there are many interfering compounds, and therefore Ga is added as pseudo molecular ions containing Ga have a distinctive isotopic ratio. Gallium is complexed by hydroxamate siderophores, allowing their identification even in complex matrices (Mawji et al., 2008a; McCormack et al., 2003). However, it is nonetheless theoretically possible for other organic compounds to produce an isotopic ratio similar to the Ga complexes. Thus unamended samples were also analysed and examined for major ions that have a mass/charge ratio corresponding to the Fe or apo (metal free) complex with a similar retention time to that of the putative Ga complex. M/z ratios are thus reported only for peaks which satisfy the following criteria:

1) Major ions in the mass spectra have two isotopes which differ by m/z = 2 and have a relative abundance close to that of Ga 69 and 71 (3:2)

2) A peak for the molecular ion also occurs at approximately the same retention time in the sample containing no Ga, that is m/z 13 units less than the most abundant isotope in the + Ga sample (equivalent to the difference in mass between Fe^{56} and Ga^{69}), or m/z 66 units less (representing the uncomplexed siderophore type chelate)

3) No molecular ions for the putative Ga complex at the relevant retention time are observed in the unamended sample.

In order to compensate for the different chromatographic conditions and HPLC pumps used in HPLC-ICP-MS and HPLC-ESI-MS analysis, retention times are expressed relative to the retention time of ferrioxamine B (FOB), according to the equation

$$t_r = (t_i - t_0)/(t_{FOB} - t_0),$$

where t_r is the relative retention time of peak *i*, t_i is the retention time of peak *i*, t_0 is the void volume of the chromatographic system and t_{FOB} is the retention time of FOB.

Results and Discussion

In this study results are reported and discussed for the production of siderophore type compounds in nutrient enriched incubations conducted across the Atlantic Ocean. The study builds on previous work on the production of siderophore type chelates in nutrient enriched seawater (Gledhill et al., 2004; Haygood et al., 1993; Mawji et al., 2008a) through the use of different carbon sources and preconcentration of larger sample sizes (1 L as opposed to 500 mL used in previous studies; Gledhill et al., 2004; Mawji et al., 2008a). The aim of the study was to widen the number of siderophore type chelates detectable by the combined application of HPLC-ICP-MS and HPLC-ESI-MS. Siderophore type chelate production in glucose incubations is compared with production in glycine and chitin incubations. Glycine and chitin are more refractory organic substrates than glucose, and potentially more representative of the type of organic matter present in seawater. However, it should be highlighted that other aspects of the methodology will also influence the type of siderophore detected. The method depends strongly on the complexation of the siderophore type chelate with Ga. Gallium exchange is carried out at low pH (~2) to ensure that gallium remains in solution for the 16 hr equilibration period. Although this has the advantage of ensuring that only strong Ga complexes are detected and thus increases the likelihood that these complexes are siderophores, the addition of Ga could result in the loss of siderophore type chelates that are

unstable or insoluble at low pH (e.g. catecholate siderophores: Harris et al., 1979a; Loomis and Raymond, 1991). Preconcentration is undertaken at the pH of the sample (~8), so siderophores that are hydrophilic at pH 8 (e.g. negatively charged siderophores such as ferric aerobactin: Harris et al., 1979b) might not be efficiently retained by the SEP column. Indeed, preliminary analysis of both ferric enterobactin and ferric aerobactin has indicated that neither of these types of siderophores will be detectable using the current methodolody (Gledhill, unpublished data). This study thus focuses on the detection of hydroxamate siderophore type compounds and how this varies with carbon source.

Growth of bacteria in nutrient enriched Atlantic seawater incubations.

Initial (day 0) numbers of bacteria in the incubations varied between 0.16×10^6 mL^{-1} and 0.4×10^6 mL^{-1} (Table 2). Bacteria counts (Table 2) showed similar geographical trends to previous studies (see e.g. Heywood et al., 2006; Mawji et al., 2008a). Densities were highest in the equatorial region, lowest in the South Atlantic Gyre and intermediate in the remaining regions. Growth rates and final bacterial densities varied with carbon source and concentration (Table 2). For glucose and chitin amended incubations, the average growth rates were 0.85 ± 0.15 and 0.8 ± 0.25 day^{-1} respectively, while growth rates for glycine were lower at 0.5 ± 0.2 day^{-1}. Final densities of bacteria showed a similar trend with averages of $1.1 \pm 0.5 \times 10^7$ mL^{-1} and $1.1 \pm 0.9 \times 10^7$ mL^{-1} for glucose and chitin and $3.4 \pm 3 \times 10^6$ mL^{-1} for glycine. The addition of chitin, a complex polysaccharide, resulted in very similar growth rates and final bacteria densities to glucose, a highly available carbon source. On the other hand glycine, an amino acid, produced the lowest growth rates and final bacteria densities of the three carbon sources. For glucose amended incubations, growth rates were similar in the different geographical provinces of the Atlantic Ocean, but final heterotrophic bacteria concentrations were lower in the SATL (Table 2). Greater variability in growth rate and final heterotrophic bacteria concentrations were observed for both chitin and glycine, with lowest values of both observed at station 52 in the SATL. The concentrations of carbon added in these experiments were very high in order to ensure successful detection of as many siderophores as possible. Despite this, consistent differences were observed between incubations, particularly between glycine and the other carbon sources. The community structure was not examined, however, with such high concentrations of carbon the bacterial communities at the end of the experiment would not have been representative of the original communities (Carlson et al., 2002). Manipulations of oceanic bacterial communities often

result in community composition changes even when trace concentrations of available carbon sources are added (Carlson et al., 2002; Fuchs et al., 2000). The flow cytometry data showed that the communities shifted towards dominance by high nucleic acid bacteria during the incubations (results not shown) however without further data on community structure it is not possible to interpret this data more fully.

Concentration and diversity of siderophore type chelates detected in nutrient enriched Atlantic seawater incubations.

Example Ga^{69} chromatograms obtained from HPLC-ICP-MS analysis of the incubation extracts and controls are presented in figure 3. The number of siderophore type chelate peaks (labelled a-v) detected by ICP-MS from different incubations varied both with carbon source and geographical location (Table 2, Fig 4), but not with the concentration of added carbon, possibly indicating that added carbon concentrations were too high to limit siderophore production. Siderophore type chelates were not detected in control incubations. At least 2 siderophore type chelates were identified in every incubation sample. The highest numbers of siderophore type chelates were for glucose (12-14) at stations 31 in the WTRA, and stations 41 and 52 in the SATL (Fig. 3). High numbers of siderophore type chelates (10-12) were also observed in chitin incubations at stations 41 and 52. For glycine amended incubations, the number of siderophore type chelates was lower (3–8) and showed less variability between stations. The lower number of siderophore type chelates in glycine incubations was likely to be linked to the lower final bacteria concentrations and lower growth rates observed in glycine incubations.

Concentrations of siderophore type chelates were not corrected for preconcentration effects as recoveries of the non-ferrioxamine siderophore type chelates from ENV+ SPE cartridges have not been determined. Total siderophore type chelate concentrations for glucose incubations varied between 0.2 and 69 nM. The latter concentration was an order of magnitude higher than that of any other station or any other carbon source, and was observed on addition of the highest concentration of glucose (9 mM) at station 6 in the NAST. The high concentrations of siderophore type chelates in the glucose amended incubations at stations 6 and 17 were mainly due to increases in the concentrations of siderophore type chelates i and j. In particular, siderophore type chelate j dominated glucose incubations at station 6, 17, 41 and 52. There was a general trend of lower siderophore type chelate concentrations in the SATL, and in incubations with lower glucose concentrations. For the glucose incubations, a

significant relationship ($r^2 = 0.42$, $n = 15$, $p < 0.01$) was observed between –log[siderophore] and final heterotrophic bacteria density (Fig. 5). The positive correlation between siderophore type chelate concentrations in glucose incubations and final bacterial densities implies that the siderophore type chelate producing bacteria in these incubations formed a significant fraction of the total bacterial population, although as mentioned above, this is unlikely to be a reflection of the original community composition. For chitin, total siderophore type chelate concentrations were lower, varying between 0.1 and 0.6 nM. The highest siderophore type chelate concentration in incubations with added chitin was observed in the NAST and again a general trend of lower siderophore type chelate concentrations was observed in the SATL. The concentration of added chitin did not influence the siderophore type chelate concentration and in these incubations there was no relationship between siderophore type chelate concentration and final heterotrophic bacteria concentrations. Siderophore type chelate concentrations produced in glycine incubations were similar to those produced in chitin incubations, ranging between 0.1 and 0.6 nM. In contrast to glucose and chitin, total siderophore type chelate concentrations in glycine incubations did not vary consistently across the different regions of the Atlantic Ocean. There was no major difference in siderophore type chelate concentrations when different glycine concentrations were used and, as for chitin, there was no relationship between siderophore type chelate concentration and final heterotrophic bacterial densities (Fig. 5). Carbon source clearly influenced the concentration and diversity of siderophore type chelates produced in these incubations, with glucose representing the best source of carbon for siderophore type chelate production of those tested in this study. The somewhat erratic relationship between carbon concentrations and siderophore production indicates that other factors may also influence siderophore production. For example, it is possible that a lack of readily available nitrogen source also influenced siderophore type chelate production in the glycine incubations, and that Fe contamination may have influenced siderophore production in the chitin incubations. Environmental factors that control siderophore production in marine bacteria have received little attention to date and much is inferred from laboratory studies of bacterial monocultures. These studies have largely focused on characterizing siderophores and their transport mechanisms e.g.(Crosa et al., 2004; Vraspir and Butler, 2009), while potential environmental controls on siderophore production, including the presence of competitor organisms, concentrations of nutrients and even Fe to carbon ratios, have received much less attention (Winkelmann, 2004; Winkelmann, 2007).

HPLC-ICP-MS chromatograms showed that geographical sampling location had an important influence on siderophore type chelate production, with higher diversity of siderophore type chelates produced in the South Atlantic. This trend was consistently observed in incubations with all three sources of carbon examined. Furthermore, the character of siderophore type chelates produced in the incubations showed a contrast between the North and South Atlantic (Fig. 2), with siderophore type chelates of a more hydrophobic nature (Fig 2. peaks o – v) being more abundant in the South Atlantic, while hydrophilic siderophore type chelates were dominant in the North Atlantic. However, in contrast to the result reported here, where increased siderophore diversity was observed in incubations carried out in SATL waters, a study on AMT-16, carried out in May - June of the same year (2005), showed a slight decrease in siderophore type chelate diversity in incubations carried out in SATL waters compared to other regions of the Atlantic Ocean (Mawji et al., 2008a). Thus the diversity of siderophore type chelates produced in nutrient enriched seawater incubations appears to vary both temporally and spatially. This study showed that the concentration of siderophore type chelates produced in the incubations (although not quantified absolutely) also varied across the Atlantic Ocean, with higher concentrations produced in the North Atlantic. In these incubation studies, where C, N and P are added in excess, another possible influence on the concentration of siderophore type chelates produced is the concentration of Fe in the incubations. Siderophore transport mechanisms are known to be induced under Fe limitation (Winkelmann, 2004). However, once induced, siderophore production is thought to continue even under Fe replete conditions, with the production likely to be tightly coupled to uptake, thereby reducing excess Fe free siderophore levels in the environment (Neilands, 1995; Winkelmann, 2007) and linking the siderophore concentration to the Fe concentration. Surface concentrations of Fe observed across the AMT17 transect (Moore et al., 2009) averaged 0.5 nM in the WTRA, NATL and NAST, and decreased to 0.03 nM in the SATL (Moore et al., 2009), consistent with the hypothesis that concentrations of Fe in the sampled waters could have influenced the siderophore type chelate concentrations produced in the incubations.

Identification of siderophore type chelates in nutrient enriched Atlantic seawater incubations.

Results from HPLC-ESI-MS analysis of samples are given in Table 3. Relative retention times of peaks detected by HPLC-ICP-MS and HPLC-ESI-MS were compared and used to

assign identities to HPLC-ICP-MS peaks (Table 3), however the different chromatographic conditions used for each technique, combined with the total number of peaks observed made it difficult to assign individual identities to peaks in HPLC-ICP-MS chromatograms with absolute certainty. The abundance of the siderophore type chelates detected by HPLC-ESI-MS was consistent with the concentrations determined by HPLC-ICP-MS despite suppression of the ESI-MS ionisation signal observed between a t_r of between 2.11 and 2.75, caused by uncharacterised co-eluting compounds. For example, higher numbers of siderophore type chelates were detected at stations in the SATL by both HPLC-ESI-MS and HPLC-ICP-MS. It was notable that the majority of the siderophore type chelates detected in this study by HPLC-ESI-MS (Table 3) were present at concentrations greater than approximately 200 nM in the concentrated extracts, which is equivalent to concentrations of > 100 pM as shown on Figure 3. Thus very few siderophore type chelates were detectable in chitin and glycine incubations by HPLC-ESI-MS.

Siderophores previously identified in nutrient enriched seawater incubations were identified in this study, including ferrioxamine B, G and E (m/z = 614, 672, 654; t_r 1, 1.1 and 1.5 respectively). Collision induced dissociation of these compounds (results not shown) produced MS^2 spectra as observed previously (Mawji et al., 2008b). The relative retention times, co-occurrence in incubations and relatively high concentration in both ESI-MS and ICP-MS chromatograms allowed identification of peaks c,d and j in the ICP-MS chromatograms as ferrioxamine B, G and E, respectively. All three compounds were identified in glucose amended incubations, while only ferrioxamine G was identified in chitin and glycine incubations. It seems likely therefore that soluble ferrioxamine type siderophore production is linked to or stimulated by the presence of enhanced concentrations of a dissolved labile carbon source. The ferrioxamine type siderophores are known to be produced by marine *Vibrio* sp. (Martinez et al., 2001). Gamma proteobacteria such as *vibrio* sp. are also known to grow very well in glucose enrichment experiments (Fuchs et al., 2000). The high concentration of the ferrioxamines (peaks c,d and j) observed in glucose incubations is therefore consistent with enhanced growth of opportunistic γ proteobacteria in glucose incubations. It was thus interesting to note that these siderophores were not present at such high concentrations in chitin or glycine incubations. An unidentified siderophore type chelate (ferric complex m/z = 640) was observed at t_r of 1.38, eluting immediately prior to ferrioxamine E (Fig. 6). The t_r for this compound and its pseudo-molecular ion were identical to that observed for ferrioxamine D_2 (Mawji et al., 2008b). However, the low signal in the

unamended samples for m/z 640 and a co-eluting compound (m/z 625 for the protonated adduct and m/z = 647 for sodium adduct, Fig. 6) meant that the identity of this compound in this study could not be confirmed by the CID experiments conducted during this study. Amphibactins with m/z = 883 (t_r 2.27), 885 (amphibactin D; t_r 2.33) and 911 (amphibactin E, t_r 2.38) were detected in glucose incubations. These amphibactins corresponded with peaks r-t in HPLC-ICP-MS chromatograms, respectively. Collision induced dissociation of these compounds (results not shown) produced MS^2 spectra as observed previously (Gledhill et al., 2004).

In addition to ferrioxamines and amphibactins, this study detected unreported and uncharacterised siderophore type chelates. Thus an unknown siderophore type chelate with m/z = 1044 (Fe complex), and m/z 1057 and 1059 (Ga complex) and a relative retention time of 1.64 was observed in the 9 mM glucose incubation at station 52 (Fig. 7). The relative retention time for this siderophore type chelate corresponded with peak l in the HPLC-ICP-MS chromatograms. This peak was also present at a relatively high concentration in the HPLC-ICP-MS chromatogram of the same sample (station 52, Fig. 2). An unknown siderophore type chelate with a pseudo-molecular ion m/z = 675 (Fe complex) and m/z 688 and 690 (Ga complex) (Fig. 8), eluted with a t_r of 0.16 (Fig. 8), implying a chelate with hydrophilic characteristics. This siderophore type chelate was observed in 9 mM glucose incubations at station 52 and 57 in the SATL. The retention time t_r of the compound did not correspond with any peak observed in the ICP-MS chromatograms. This may be due to the post-sample injection of 0.5 M nitric acid and inclusion of the 15 min isocratic step at 100 % A when detection by ICP-MS is used. This step was necessary to ensure complete removal of uncomplexed excess free Ga from the HPLC system prior to connection of the HPLC eluant to the ICP-MS detector. However, a post sample injection of 0.5 M nitric acid also has the potential to decrease the retention time, or cause the complete loss of hydrophilic siderophore type chelates from the column.

Conclusion

A total of 23 different siderophore type chelates were identified in nutrient enriched seawater incubations from the Atlantic Ocean by a combination of HPLC-ICP-MS and HPLC-ESI-MS. The concentration and diversity of siderophore type chelates produced varied across the Atlantic Ocean and was influenced by the source of carbon used in the incubation. Glucose, a

highly available carbon source, produced the highest concentrations and most diverse range of siderophore type chelates, while glycine and chitin, carbon sources more representative of naturally occurring organic material, produced lower siderophore type chelate concentrations and diversity. This finding highlights the importance of considering the composition of organic material and the influence of nutrients other than Fe on the production of siderophore type chelates, although it is difficult to fully expand on the implications of these findings without knowledge of the changes in bacterial community structure induced as a result of the different carbon enrichments. The concentration of carbon source was found to have little or no effect on siderophore concentrations or diversity. This was potentially due to a lack of carbon limitation as a result of the high concentrations of carbon added. Spatial variability in siderophore type chelate production was also observed, with highest siderophore type chelate concentrations and lowest diversity observed in nutrient enriched incubations conducted in water sampled from the North Atlantic, and lowest concentrations and highest diversity in incubations carried out in the South Atlantic. Seven of the siderophore type chelates were positively identified by HPLC-ESI-MS, two were identified as the linear hydroxamates ferrioxamine B and G, two were identified as cyclic hydroxamates ferrioxamine E and D_2, and three were identified as amphibactins. Mass/charge ratios of two further, so far uncharacterised, siderophore type chelates were also obtained. The results indicate that there is potential for a wide variety of siderophore type chelates to be present in surface waters of the Atlantic Ocean. The study represents a first step in developing protocols to examine the influence of nutrient source and concentration on the production of siderophore type chelates in mixed bacterial populations.

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Figure captions

Fig. 1. Map of the Atlantic Ocean showing the locations from which the water was collected for the incubation experiments.

Fig. 2. Schematic describing the analysis of siderophore type chelates in the incubation extracts. Aliquots of extracts are derivatised with Ga and first quantified by High Performance Liquid Chromatography – Inductively coupled – Mass spectrometry (HPLC-ICP-MS) then analysed by HPLC-ESI-MS to obtain the ($M-3H^{+}+Ga^{3+}+H^{+}$) pseudo molecular ions. Finally the extract is analysed in an underivatised form by HPLC-ESI-MS, with data dependant collision induced dissociation (CID). The final step allows putative Ga compounds detected by HPLC-ESI-MS to be also identified as ferric chelates and for identities to be assigned from comparison of fragmentation patterns with those in the literature.

Fig. 3. Example Ga^{69} chromatograms obtained by High Performance Liquid Chromatography – Inductively coupled – Mass spectrometry from analysis of extracts of incubations carried out at station 41 in the South Atlantic. (a) control incubation (b) 9 mM glucose incubation (c) 100 mg L^{-1}chitin incubation and (d) 2 mM glycine incubation. Peaks are labelled according to table 3. Incubation and chromatography conditions are as described in the text. Note scale changes for the y axis.

Fig. 4. Concentrations of individual siderophore type chelates (labelled a-v) extracted from nutrient enrichment incubations carried out on water sampled from the Atlantic Ocean. Concentrations were determined by HPLC-ICP-MS after Ga exchange. Data represent an average (plus or minus the standard deviation) for each glucose concentration used. For glycine and chitin incubations all treatments at each station were pooled as no relationship between the concentration of these carbon sources and siderophore type chelate production was observed (see text). The siderophore type chelate concentrations were not corrected for preconcentration effects as recoveries of the non ferrioxamine type siderophore type chelates from ENV+ SPE cartridges have not been determined. Note scale changes for the y axis.

Fig. 5. Plots of final heterotrophic bacteria counts versus –log(total siderophore type chelate concentration) for the three types of carbon added to incubations. a) Glucose incubations (n=15) b) Glycine incubations (n = 10) and c) chitin incubations (n = 9). A positive correlation ($p < 0.01$) was observed between the heterotrophic bacterial concentrations and –log(siderophore concentration) in the glucose incubations ($y = 9.82 - 7.9 \times 10^{8}x$, $r^2 = 0.42$). See text for enrichment and incubation conditions.

Fig. 6. a) Mass spectra for a Ga complexed siderophore type compound eluting at $t_R = 17.2$ min showing the two isotopes for the Ga complex (protonated adduct m/z 653.1, 655.1, sodium adduct 675.1, 677.1) and b) mass spectra for the Ga free sample, showing the Fe complex (m/z 640.1, $t_R = 17.34$) and interfering ions at m/z 625 and m/z 647 which prevented collision induced fragmentation of this ion. c) Chromatogram for masses m/z = 653, 655 (Ga derivatised sample) and (d) chromatogram for m/z 640 (underivatised sample) obtained from the total ion

chromatogram. The chromatograms were obtained from an incubation with 9 mM glucose added to water collected from station 6 in the NAST.

Fig. 7. a) Mass spectra for the a Ga complexed siderophore type compound (protonated adduct m/z 1057, 1059, sodium adduct 1079, 1081) eluting at t_R = 20.5 min and b) mass spectra for the Fe complex (protonated adduct m/z 1044).c) Chromatograms obtained from the total ion chromatogram for masses m/z 1057, 1059 for Ga complexed siderophore type chelate observed at a retention time relative to ferrioxamine B of 1.64. The chromatogram was obtained from an incubation with 9 mM glucose added to water collected from station 52 in the SATL.

Fig. 8 a) Mass spectra for a Ga complexed siderophore type compound eluting at t_R = 3.34 min (protonated adduct m/z 688.1, 690.1, sodium adduct 710.1, 712.1) and b) mass spectra for the Fe complex (m/z 675.1). c) Chromatograms obtained from the total ion chromatogram for masses m/z 687, 689 for Ga complexed siderophore type chelate observed at a retention time relative to ferrioxamine B of 0.18. The chromatogram was obtained from an incubation with 9 mM glucose added to water collected from station 52 in the SATL. The peak at RT 18.64 min represents elution of another unrelated compound.

Table 1. Concentrations of carbon, nitrogen and phosphorus used in nutrient enrichment incubations in the Atlantic Ocean.

Glucose ($C_6H_{12}O_6$)			Glycine ($C_2H_5NO_2$)		Chitin $(C_8H_{13}NO_5)_n$		
Glucose (μM)	NH_4^+ (μM)	PO_4^{3-} (μM)	Glycine (μM)	PO_4^{3-} (μM)	Chitin (mg L^{-1})	NH_4^+ (μM)	PO_4^{3-} (μM)
9	20	10	20	10	1	20	10
90	100	10	200	10			
9000	200	20	2000	20	100	200	20

Table 2. Initial bacteria concentrations, growth rate, final bacteria concentrations, total siderophore type chelate concentrations and number of siderophore type chelates detected by HPLC-ICP-MS analysis of preconcentrated incubated, nutrient enriched seawater collected from six stations in the Atlantic Ocean. The siderophore type chelate concentrations are not corrected for preconcentration effects as recoveries of the non ferrioxamine type siderophore type chelates from ENV+ SPE cartridges have not been determined.

Station	Position and depth of sampling	Carbon Source	Conc. of carbon added	Initial bacteria ($\times 10^5$ mL^{-1})	Growth rate (day^{-1})	Final bacteria ($\times 10^5$ mL^{-1})	Total siderophore type chelate conc. (nM)	Total N^o of sidero-phores identified
6	26.5° W 37.34 °N (NAST), 12 m	Glucose	54 mM	1.7	0.91	160	69 ± 1.8	5
			540 µM		0.81	96	2.5 ± 0.9	6
			54 µM		0.74	69	1.3	6
		Glycine	4 mM		0.51	21	0.2	7
			400 µM		0.79	85	0.1	3
			40 µM		0.06	2.2	0.2	2
		Chitin	4 mM		1	330	0.1 ± 0.09	6
			40 µM		0.68	51	0.2	3
17	36.35 °W 23.14 °N (NATR), 24 m	Glucose	54 mM	3.2	0.95	140	2.9 ± 2.8	8
			540 µM		1	180	1.9 ± 1.1	7
			54 µM		0.75	65	7 ± 2	4
		Glycine	4 mM		0.8	78	0.1	3
			400 µM		0.7	5	0.6	3
		Chitin	4 mM		0.82	87	0.6 ± 0.4	4
			40 µM		0.71	56	0.4 ± 0.2	3

1 Table 2. continued

Station	Position and depth of sampling	Carbon Source	Conc. of carbon added	Initial bacteria ($\times 10^5$ mL^{-1})	Growth rate (day^{-1})	Final bacteria ($\times 10^5$ mL^{-1})	Total siderophore type chelate conc. (nM)	Total N^o of sidero-phores identified
31	27.36 °W 4.95 °N (WTRA), 8 m	Glucose	54 mM	4	0.79	96	1.2 ± 0.7	14
			540 µM		0.83	110	0.4 ± 0.2	6
41	25.01 °W 15.5 °S (SATL), 19 m	Glucose	54 mM	2.6	086	84	0.3 ± 0.3	7
			540 µM		0.6	29	0.2 ± 0.07	6
			54 µM		0.8	6	0.6 ± 0.1	13
		Glycine	4 mM		0.41	14	0.1	6
			400 µM		0.3	8.6	0.1	6
		Chitin	4 mM		1	160	0.4 ± 0.3	12
			40 µM		0.94	120	0.3 ± 0.03	11
52	7.22 °W 27.95 °S (SATL), 14 m	Glucose	54 mM	1.6	0.9	54	2.5 ± 2.1	12
		Glycine	4 mM		0.4	7.5	0.3	3
		Chitin	4 mM		0.73	30	0.3	10
57	4.44 °E 32.53 °S (SATL), 9 m	Glucose	54 mM	2.7	1.3	130	0.9	7
			540 µM		0.81	68	0.2 ± 0.1	11
			54 µM		0.71	46	0.2 ± 0.02	8
		Glycine	4 mM		0.72	48	0.3	8
			400 µM		0.48	19	0.2	7
		Chitin	4 mM		1	170	0.1	7
			40 µM		0.5	19	0.3 ± 0.1	8

2

Table 3. Retention time - relative to ferrioxamine B - and mass to charge ratio for ferric and Ga siderophore type chelates identified by HPLC-ESI-MS. Identities are indicated where possible. FOB: ferrioxamine B, FOG: ferrioxamine G, FOE: ferrioxamine E, amph: uncharaterised amphibactin, amph D, E: amphibactins D and E respectively. Stations where the siderophore type chelates were detected are also indicated. Relative retention times and peak labels for peaks obtained by HPLC-ICP-MS are given for comparison.

Peaks detected by ESI-MS				Peaks detected by ICP-MS	
Relative retention time	m/z molecular ion unamended sample	m/z molecular ion sample + Ga	Stations where siderophore type chelate detected	Relative retention time	peak label
0.18	675	688/690	52,57		
				0.70	a
				0.92	b
1.0	614 (FOB)	627/629	6,31,52	1.03	c
1.10	672 (FOG)	685/687	6,17,31,52	1.13	d
				1.19	e
1.22	624	637/639	6	1.24	f
				1.28	g
				1.31	h
1.38	640	653/655	6	1.38	i
1.47	654 (FOE)	667/669	6,17,31,52,57	1.47	j
				1.55	k
1.64	1044	1057/1059	52	1.64	l

1 Table 3. continued.

Peaks detected by ESI-MS				Peaks detected by ICP-MS	
Relative retention time	m/z molecular ion unamended sample	m/z molecular ion sample + Ga	Stations where siderophore type chelate detected	Relative retention time	peak label
				1.74	m
				1.84	n
				1.97	o
				2.05	p
				2.19	q
2.27	883 (amph)	896/898	52,57	2.28	r
2.33	885 (amph D)	898/900	52,57	2.37	s
2.38	911 (amph E)	924/926	52	2.45	t
				2.56	u
				2.62	v

2

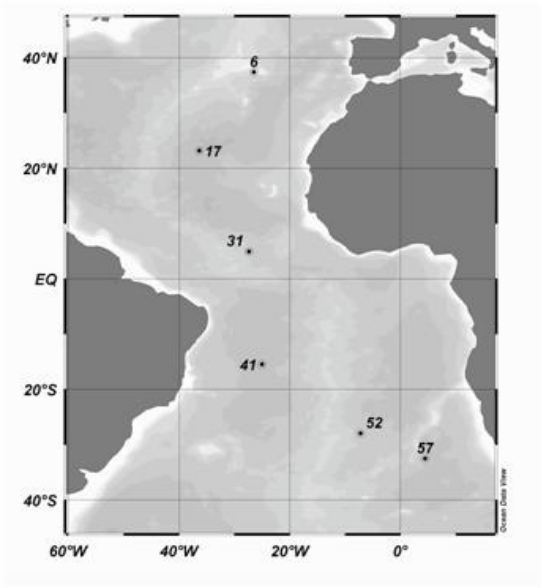

Fig. 1

1

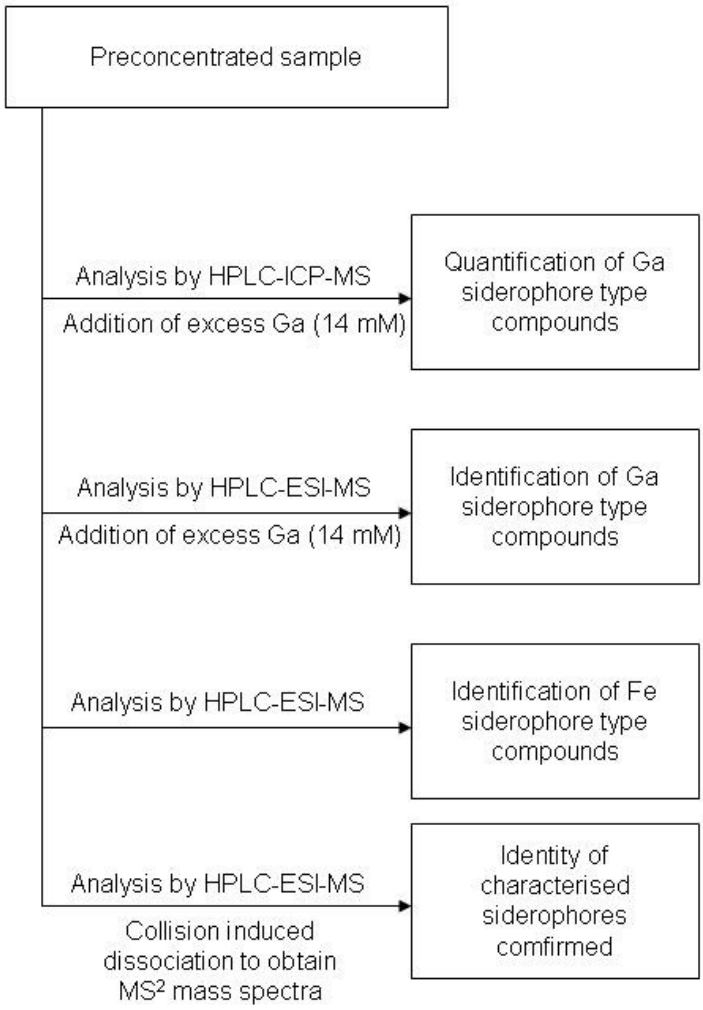
Preconcentrated sample
Analysis by HPLC-ICP-MS
Addition of excess Ga (14 mM)
Quantification of Ga siderophore type compounds
Analysis by HPLC-ESI-MS
Addition of excess Ga (14 mM)
Identification of Ga siderophore type compounds
Analysis by HPLC-ESI-MS
Identification of Fe siderophore type compounds
Analysis by HPLC-ESI-MS
Collision induced dissociation to obtain MS2 mass spectra
Identity of characterised siderophores comfirmed

Fig. 2

1

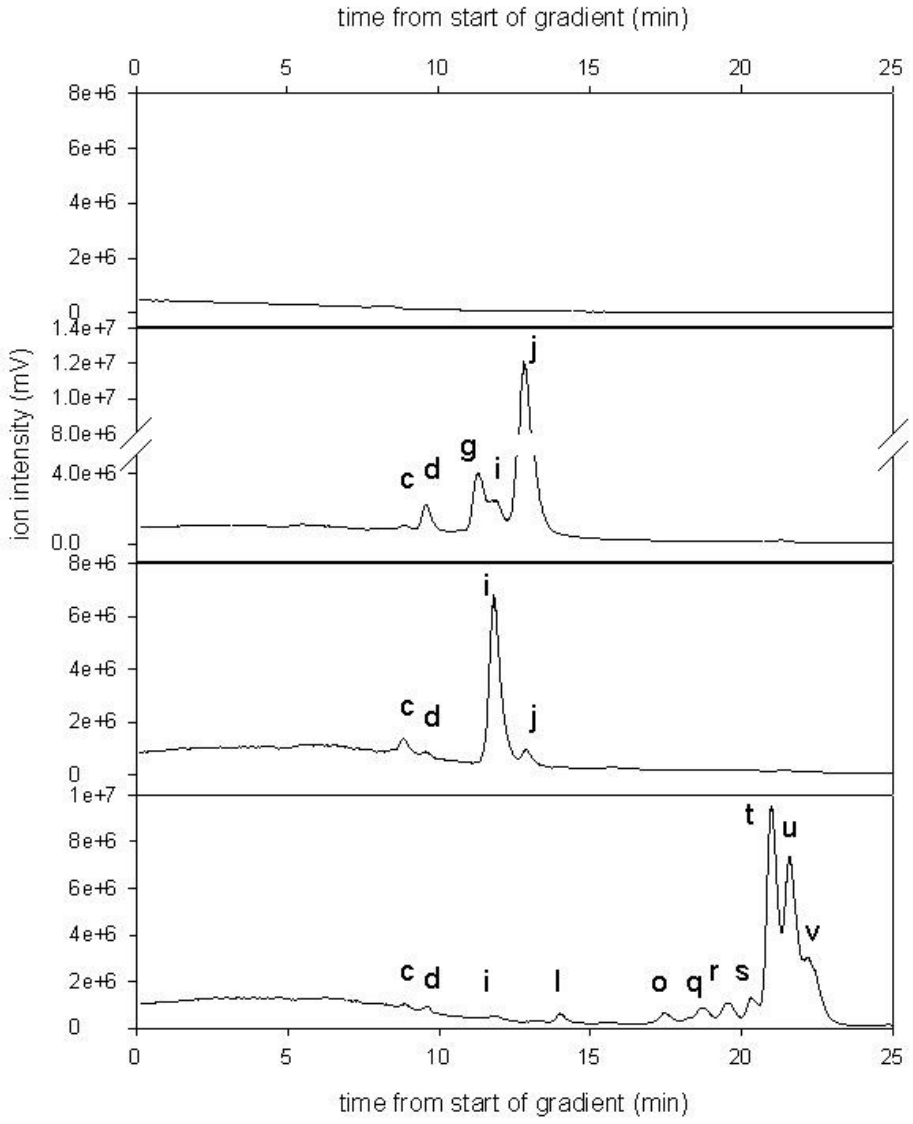
time from start of gradient (min)
ion intensity (mV)
c
d
g
i
j
t
u
v
l
o
q
r
s

Fig. 3

1

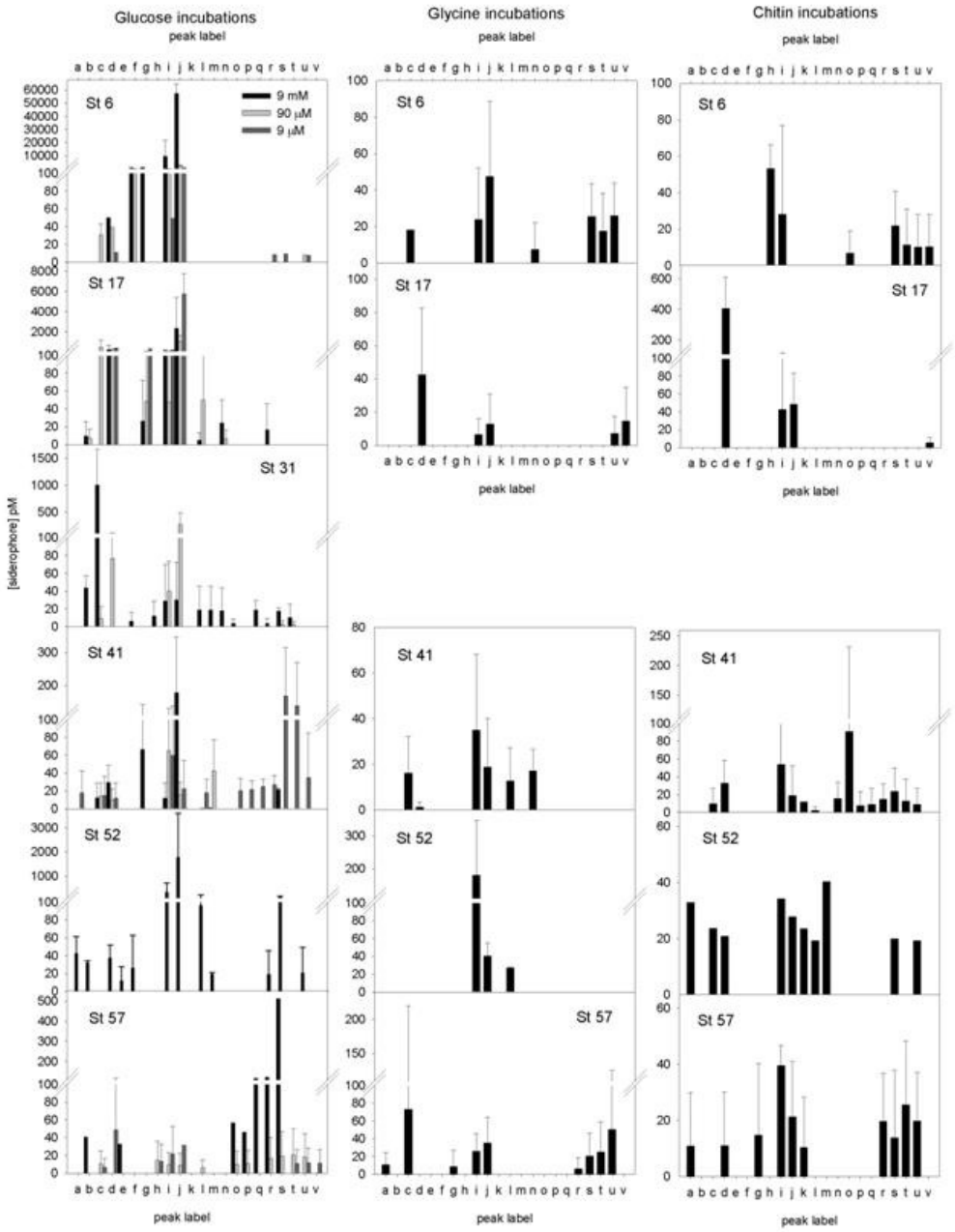
Glucose incubations
Glycine incubations
Chitin incubations
peak label
a b c d e f g h i j k l m n o p q r s t u v
9 mM
90 µM
9 µM
St 6
St 17
St 31
St 41
St 52
St 57
[siderophore] pM

Fig. 4

1

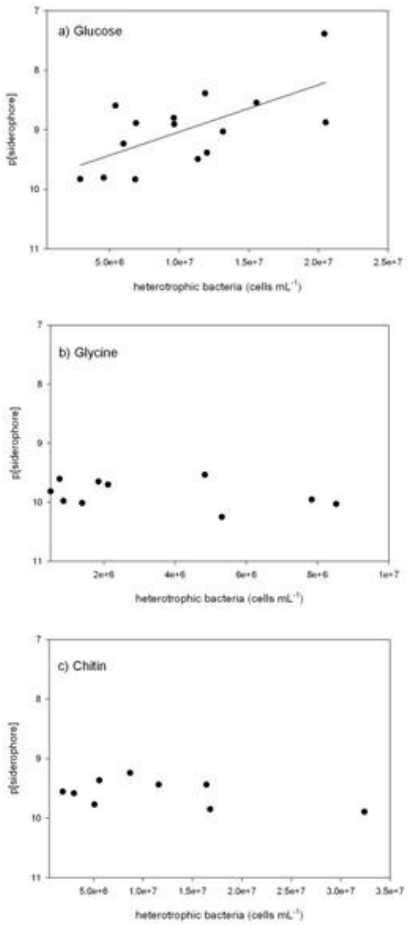
a) Glucose
b) Glycine
c) Chitin
p[siderophore]
heterotrophic bacteria (cells mL^{-1})

Fig. 5

1

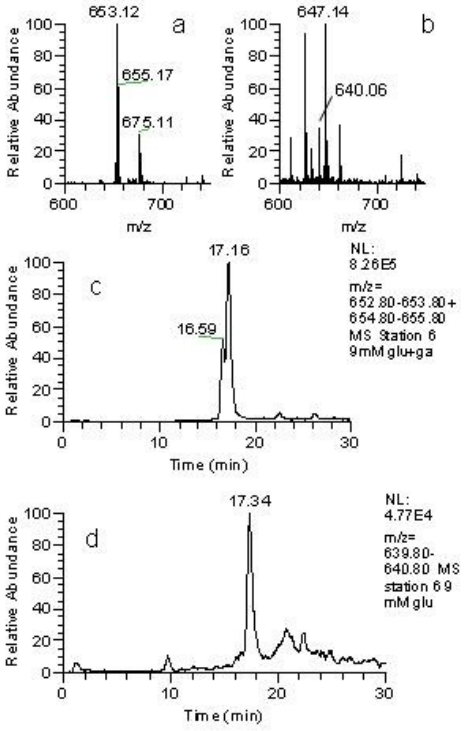
653.12
a
655.17
675.11
Relative Abundance
m/z
647.14
b
640.06
17.16
c
16.59
NL:
8.26E5
m/z=
652.80-653.80+
654.80-655.80
MS Station 6
9mM glu+ga
Time (min)
17.34
d
NL:
4.77E4
m/z=
639.80-
640.80 MS
station 6 9
mM glu

Fig. 6

1

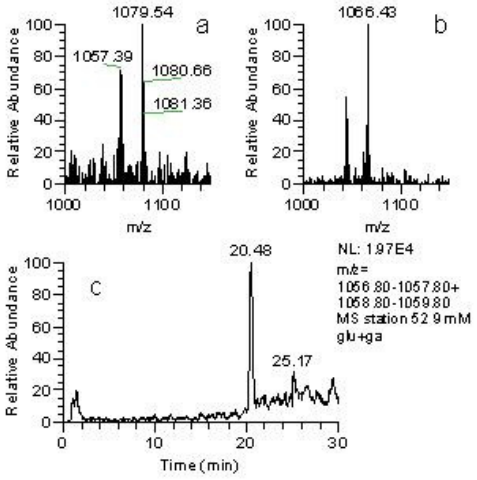
1079.54
a
1057.39
1080.66
1081.36
Relative Abundance
m/z
1066.43
b
20.48
c
25.17
Time (min)
NL: 1.97E4
m/z=
1056.80-1057.80+
1058.80-1059.80
MS station 52 9mM
glu+ga

Fig. 7

1

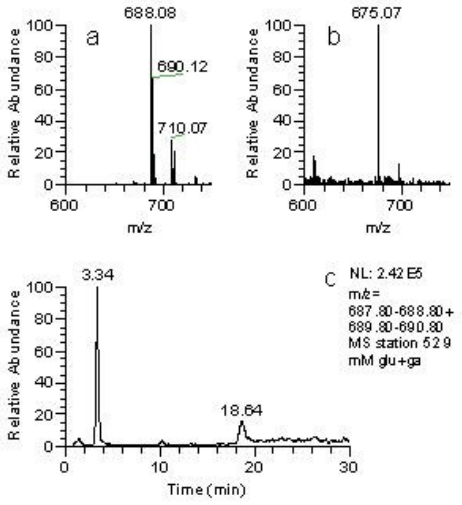
a
688.08
690.12
710.07
Relative Abundance
m/z
b
675.07
c
NL: 2.42 E5
m/z=
687.80-688.80+
689.80-690.80
MS station 529
mM glu+ga
3.34
18.64
Time (min)

Fig. 8

1