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High cyanobacterial *nif*H gene diversity in Arctic seawater and sea ice brine

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Summary

Although cyanobacterial diazotrophs are common in Arctic terrestrial and freshwater habitats, they have been assumed to be absent from Arctic marine habitats. We report here a high diversity of cyanobacterial *nif*H genes in Fram Strait and the Greenland Sea. The *nif*H gene encodes the iron protein of the nitrogenase enzyme complex, which is essential for biological N_2 fixation. Using primers specific for *nif*H genes we uncovered communities of autotrophic and heterotrophic bacteria in sea ice brine and seawater between latitudes 65 and 81°N. Cyanobacteria (*Oscillatoriales* and *Chroococcales*) with known marine planktonic and benthic distributions were distinguished, alongside a mix of metabolically versatile eubacteria (*nif*H Clusters I and III). Using primers selective for cyanobacterial *nif*H genes we identified filamentous non-heterocystous *Trichodesmium*-like and LPP (*Leptolyngbya, Phormidium* and *Plectonema*)-like *Oscillatoriales*, as well as *Cyanothece*-like *Chroococcales* in a brine sample from 81°N. The occurrence of *Trichodesmium*-like cyanobacteria was further confirmed by sequences of the *het*R gene of *Trichodesmium*. Microscopic examinations confirmed the presence of viable filamentous and unicellular cyanobacteria. Our results reveal the potential for microbial N_2 fixation in the Arctic seas. However, it is still left to determine if these genes are also metabolically active before any biogeochemical importance of diazotrophy in the polar oceans can be assessed.

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Introduction

Biological N_2 fixation is a critical component in the nitrogen biogeochemistry of the Earth (Falkowski *et al.*, 2008). About 61% of the total nitrogen added to the biosphere is produced by biological N_2 fixation (Gruber and Galloway, 2008). This equals ~ 250 Tg of 'new' nitrogen per year, of which ~ 140 Tg originates from the oceans. Only a limited set of prokaryotic organisms, collectively known as 'diazotrophs', can access the immense reserves of atmospheric N_2 and reduce it to bioavailable ammonia, a reaction catalysed by the enzyme complex nitrogenase. Marine waters are often N-limited and are therefore favourable environments for diazotrophs. Most of the N_2 fixation in the oceans is performed by cyanobacteria (Monteiro *et al.*, 2011), but in some areas heterotrophic N_2 fixation can dominate, e.g. in the ultra-oligotrophic waters of the South Pacific Gyre (Halm *et al.*, 2012).

Although cyanobacterial diazotrophs are known to be common in polar terrestrial and freshwater habitats (Olson *et al.*, 1998; Jungblut and Neilan, 2010; Harding *et al.*, 2011), it was until recently assumed that they are absent from the polar marine ecosystems. Bowman and colleagues (2012) showed for the first time the presence of cyanobacteria (although not further identified) in multiyear sea ice and seawater close to the geographic North Pole by 16S rRNA gene sequencing studies. Picocyanobacteria had previously been found in coastal Arctic waters further south, but they turned out to be of allochthonous, riverine origin (Waleron *et al.*, 2007). In current global nitrogen budgets, marine N_2 fixation is dominated by the filamentous genus *Trichodesmium*, which is thought to be limited to tropical and subtropical oceans (Tyrrell *et al.*, 2003). Planktonic unicellular diazotrophic cyanobacteria have a reported wider geographical range, but their abundance decreases markedly from temperate latitudes to the polar oceans (Langlois *et al.*, 2008; Zakhia *et al.*, 2008; Moisander *et al.*, 2010).

Heterotrophic diazotrophs are common at low water temperatures (Riemann *et al*., 2010) and have been reported from latitudes up to 71°33′N (Farnelid *et al*., 2011). Their ability to fix N_2 and their possible contribution to the global nitrogen cycle has been questioned, but evidence is now building up that their role may be substantial. For example, Halm and colleagues (2012) calculated that heterotrophic diazotrophs in the South Pacific Gyre may account for the production of 8–20% of global oceanic new nitrogen.

It is unlikely that temperature itself is a factor restricting the occurrence of diazotrophic cyanobacteria and N_2 fixation in marine Arctic habitats since diazotrophy is known to take place in terrestrial and freshwater habitats of the Arctic and Antarctic regions and to represent a major source of nitrogen in these ecosystems (Holm-Hansen, 1963; Chapin *et al*., 1991; Liengen and Olsen, 1997; Pandey *et al*., 2000). Recent metagenomic sequencing of N-limited permafrost soils in the Arctic region showed a low microbial diversity, but a high abundance of genes related to N_2 fixation (Yergeau *et al*., 2010). Adaptation of diazotrophs to cold environments has been demonstrated, e.g. the nitrogenase in Antarctic strains of *Gloeocapsa* has a 10°C lower temperature optimum than that of tropical strains (Pandey *et al*., 2000). Also, in polar freshwater habitats the optimum temperature for cyanobacterial growth is much higher than the maximum temperature they will ever experience in the field, but, nevertheless, they can dominate these systems (Tang *et al.,* 1997; Zakhia *et al*., 2008; Jungblut *et al*., 2010).

Even if they do have a crucial functional role as N_2 fixers, cyanobacterial diazotrophs may appear to be absent in molecular studies targeting the 16S rRNA gene (Taton *et al*., 2003) or in metagenomic analyses of ocean microbes (Johnston *et al*., 2005) because they are outnumbered by heterotrophic bacteria. A direct assessment of the functional gene for N_2 fixation, the *nif*H gene, is more appropriate to assess the N_2-fixation potential of microbial communities. This gene encodes the iron protein of the nitrogenase enzyme complex, which catalyses biological N_2 fixation, and may reach 10^6 copies per litre of ocean water (Tyrrell *et al*., 2003; Foster *et al*., 2009).

We hypothesized that diazotrophic cyanobacteria would occur in the Arctic seas, like in other parts of the oceans and in Arctic terrestrial and freshwater systems. In order to test this, we collected and analysed microbial communities from seawater, sea ice brine and snow samples in Fram Strait and the Greenland Sea. We targeted two genes: *nif*H (both universal for all bacteria and cyanobacteria-selective) and *het*R, the master gene for heterocyst (N_2-fixing cell) development, a gene also present in some non-heterocystous filamentous cyanobacteria, including *Trichodesmium*.

Results and discussion

Environmental conditions, biomass and microscopic observations

We collected nine samples of microbial communities from seawater, sea ice brine and snow at eight stations in the Greenland Sea and Fram Strait (Fig. 1) during a research cruise, which took place in Arctic spring (May 2002). The brine channels in the ice contained high-saline water of 88–115 psu (Table S1). Chlorophyll *a* levels indicated extremely low phototrophic biomass in the snow (0.01 $\mu g \cdot l^{-1}$), and higher biomass in the brine (0.1–0.5 $\mu g \cdot l^{-1}$) and the seawater (0.1–2.1 $\mu g \cdot l^{-1}$). These concentrations are in line with those previously found in Fram Strait of on average 1 $\mu g \cdot l^{-1}$ in multi-year sea ice and 0.5 $\mu g \cdot l^{-1}$ in seawater (Meiners *et al*., 2003). The number of viable bacterial cells in the brine (only quantified at Station D with epifluorescence light microscopy directly on-board) was $0.6 \cdot 10^9$ cells $\cdot l^{-1}$, which is similar to figures reported by Meiners and colleagues (2003) of *c*. $0.4 \cdot 10^9$ cells $\cdot l^{-1}$ in multi-year sea ice. While the particulate organic carbon: particulate nitrogen ratios in the microbial communities in seawater were close to the Redfield ratio of 6.6, they were higher in the brine (13–75; Table S1). This is typical for multi-year sea ice in the Greenland Sea, and is at least partly explained by high abundances of exopolymer particles from the pennate diatoms living in the brine (Meiners *et al*., 2003; Krembs *et al*., 2011). We verified the presence of cyanobacteria with intact unicellular and filamentous morphologies by epifluorescence light microscopy of glutaraldehyde-fixed (2.5%) material stained with acridine orange (Hobbie *et al*., 1977). Typically, the diameter of the unicellular cyanobacteria was < 1–3 μm while the filaments were < 1 μm wide and up to 50 μm long (Fig. 2).

nif*H genes reveal high diazotrophic diversity*

We did not obtain any signal of potential diazotrophs from 16S rRNA gene analyses for any of the nine samples, which may be one of the reasons why the communities described here have not been detected before. We used a battery of other molecular approaches. This included two target genes related to nitrogen fixation (*nif*H, *het*R) and two methodological approaches (DGGE and clone libraries). The bacterial *nif*H gene oligonucleotide primers PolF and PolR (Poly *et al*., 2001) following the protocol of Bauer and colleagues (2008), generated amplicons (~ 360 bp) for all samples, except for the snow from Station D and the seawater from Station G (Fig. 1). The cyanobacteria-selective *nif*H gene oligonucleotide primers CNF and CNR (Olson *et al*., 1998) following the protocol of Díez and colleagues (2007), generated amplicons (~ 359 bp) for three of the four seawater samples

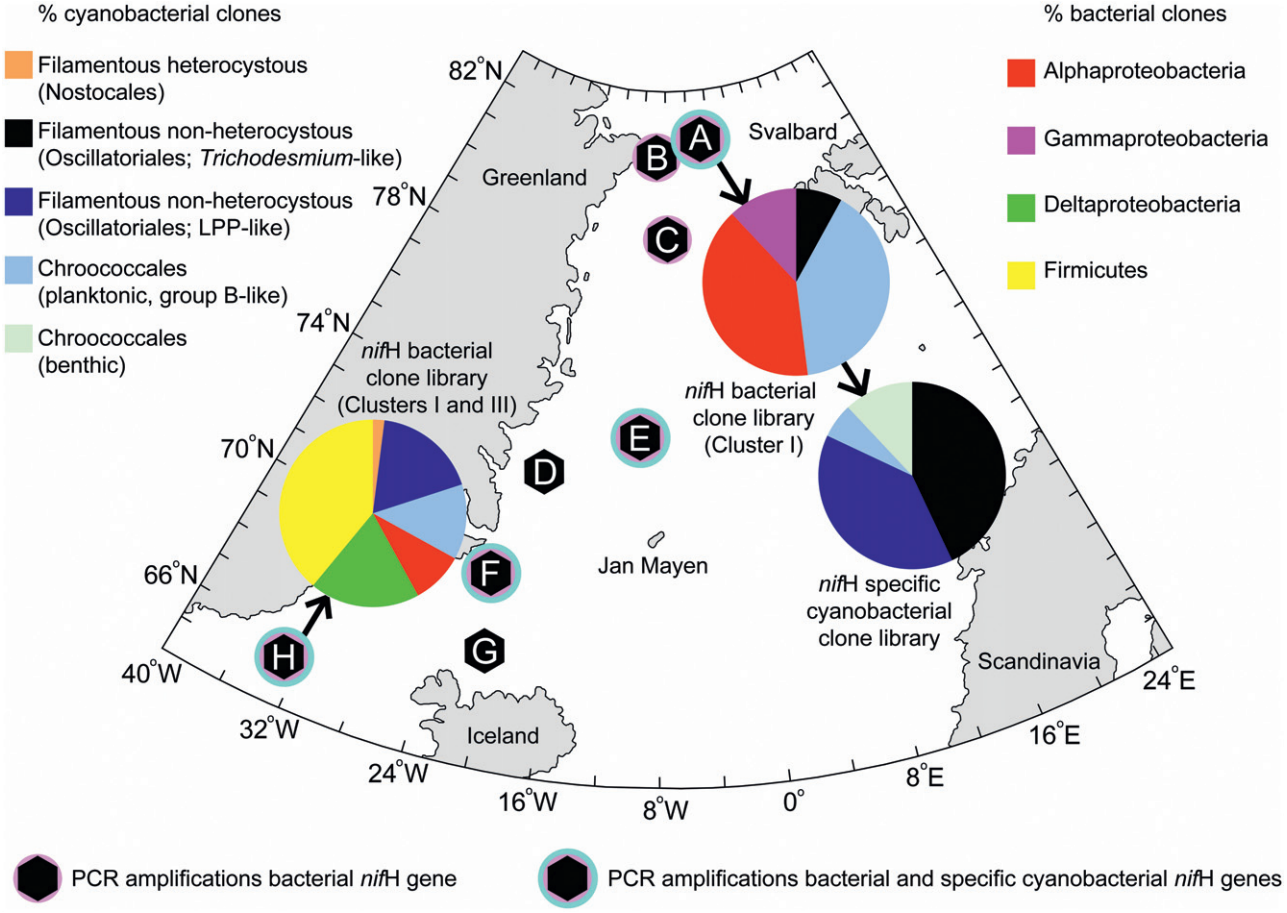

Fig. 1. The eight offshore sampling stations in Fram Strait (A–C) and the Greenland Sea (D–H) were located between 81°19′N, 4°30′W and 65°11′N, 32°38′W and sampling took place from 8 May until 29 May 2002. The five northern-most located stations (A–E) were ice-covered as opposed to the three stations (F–H) further south. Brine samples were taken at Stations A–C, a snow sample was taken at Station D and seawater samples were taken at Stations E–H. Brine water was pumped up with a foot pump from 1–2 m deep holes made in the ice with a motor-driven ice corer to which brine water was discharged from the brine channels in the ice. Seawater samples were collected in Niskin bottles attached to a rosette with a CTD profiler. Snow samples were taken with a spade. The microbial communities were collected on glass fibre filters and immediately frozen in liquid nitrogen (Table S1) for molecular and biomass analyses. Bacterial *nif*H gene PCR amplifications were obtained from Stations A–C, E, F and H, and cyanobacterial *nif*H gene PCR amplifications were obtained from Stations A, E, F and H. No *nif*H gene PCR amplifications were obtained from Stations D and G. Clone libraries were constructed for Station A (brine, two libraries) and Station H (seawater, one library). Pie charts show the % of microbial clones in the three clone libraries.

(Stations E, F, H) and for one (Station A) of the four brine samples (Fig. 1). The *Trichodesmium*-selective *het*R gene oligonucleotide primers *het*R1 (Janson *et al*., 1998) and *het*R-reverse (Orcutt *et al*., 2002), generated amplicons (~ 272 bp) for the brine sample at Stations A (the only one tested). All clones and DGGE bands obtained were purified using GFX PCR DNA and Gel Band Purification kit (GE Healthcare Bioscience), and sequenced using 6 Applied Biosystems 3730xl (Macrogen, Korea). The sequences generated in this study are deposited in GenBank (http://www.ncbi.nlm.nih.gov/) under accession numbers: DGGE-*nif*H (JN032473– JN032479); clones-*nif*H (JN032411–JN032472); clones-*het*R (JN050993–JN051001).

The large differences between the identity of the organisms found in seawater and brine (analysed simultaneously) confirm the absence of contamination in our PCR reactions and verify that our sequences originate from the Arctic samples. Despite the relatively small water volumes filtered, 300 ml at Station A (brine) and 1000 ml at Station H (seawater), 25 and 49 clones of diazotrophs were identified in the bacterial and cyanobacterial *nif*H clone libraries from the brine, and 46 clones in the bacterial *nif*H clone library from the seawater. The diazotrophs identified here are as diverse as those previously reported from marine and non-marine habitats more to the south (Fig. 3). Hence, our data expand the global distribution of marine organisms carrying the *nif*H gene and thus increase potential N_2 fixation in the Arctic marine system from 71°N (Farnelid *et al*., 2011) to 81°N (our Station A). While Farnelid and colleagues (2011) did not detect any cyanobacterial *nif*H genes in their seawater sample from

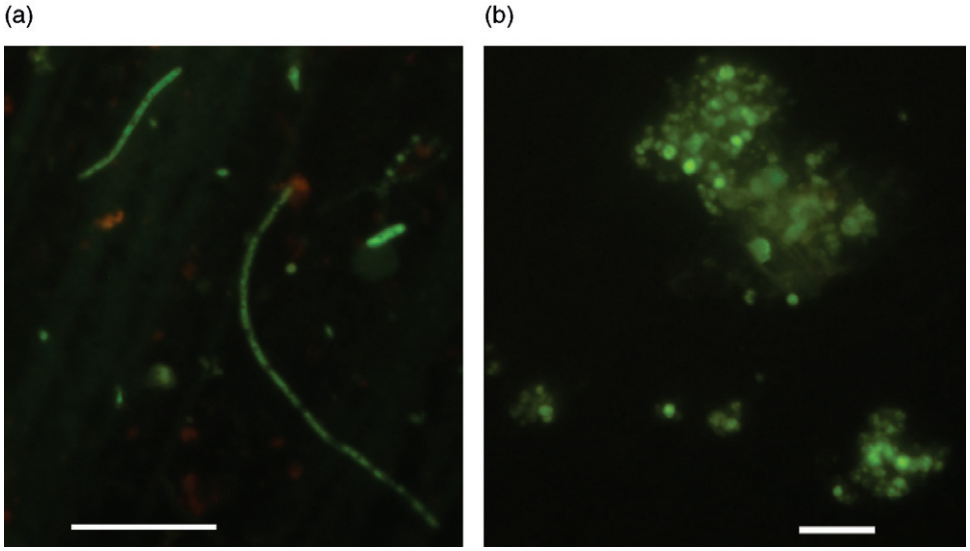

Fig. 2. Epifluorescence micrographs of glutaraldehyde-fixed samples stained with acridine orange showing cyanobacteria-like cells (A) filamentous morphotypes (B) unicellular aggregates. Scale bar = 10 μm.

71°N, we discovered diverse cyanobacterial communities in both seawater and brine.

On the whole, our *nif*H sequences were mainly related to previously reported sequences of marine diazotrophs. This suggests a marine origin for the offshore Arctic marine *nif*H genes and not an allochthonous freshwater origin as found in coastal waters by Waleron and colleagues (2007). Furthermore, our data illustrate a rich microdiversity (sequence variation at the 99% similarity level) within the brine and seawater samples (Fig. S1). Nine subclusters, with several almost identical clones, were found in each. This implies the coexistence of different ecotypes and/or a fine-tuned adaptation to environmental shifts (Fuhrman and Campbell, 1998; Acinas *et al*., 2004). Such subclusters were found within all major nitrogenase phylotypes, but mainly among cyanobacteria from Cluster I and δ-proteobacteria and *Firmicutes* from Cluster III (Fig. 3).

Taxonomic affiliations of the Arctic cyanobacteria

Relatively large proportions of the clones in the two bacterial *nif*H clone libraries were affiliated with cyanobacteria, 48% in the brine and 33% in the seawater (Fig. 1). The *nif*H sequences obtained in these two libraries, as well as those obtained in the cyanobacterial *nif*H clone library, belonged to a variety of phylotypes not reported before from Arctic latitudes, including *Chroococcales* (*Cyanothece*-like), *Nostocales* (*Nostoc*-like) and *Oscillatoriales* (*Trichodesmium*-like and LPP = *Leptolyngbya, Phormidium* and *Plectonema*-like) phylotypes (Fig. 1).

Sequences similar to those of the genus *Trichodesmium* were identified only in the brine sample from Station A (Figs 1 and 3), using both general and cyanobacteria-selective *nif*H gene primers. The occurrence of *Trichodesmium*-like cyanobacteria in this sample was further confirmed by sequences of the *Trichodesmium het*R gene. Together, these data demonstrate that *Trichodesmium*-like cyanobacteria are present in the Arctic marine environment. It is likely that they might represent a cold-adapted novel *Trichodesmium* species or a species belonging to a genus closely related to *Trichodesmium*.

LPP-like *Oscillatoriales* were identified in both brine and the seawater and proved to be related to planktonic and benthic forms of rather diverse geographical origin (Fig. 3). The cyanobacterial unicellular phylotypes related to *Cyanothece* spp. recovered from the brine, were affiliated with a different clade than those retrieved from the seawater (Fig. 3), which suggests differential origins or adaptations. Only a few phylotypes were affiliated with filamentous heterocystous *Nostocales* (Figs 1 and 3). This is in contrast to Arctic and Antarctic terrestrial and freshwater habitats where this group constitutes the dominant diazotrophs (Lennihan *et al*., 1994; Sheath and Müller, 1997; Taton *et al*., 2003). In our seawater sample from Station H this phylotype only represented 3% of the total clone library, and in the brine from Station A only two heterocystous phylotypes were recovered. These *Nostocales* can be classified as a cold clade, which also includes phylotypes from other cold systems such as the brackish Baltic Sea (Farnelid *et al*., 2009), the Damma glacier in Switzerland (Duc *et al*., 2009) and melt-water ponds of Antarctic McMurdo Ice Shelf (Olson *et al*., 1998; Jungblut and Neilan, 2010) (Fig. 3). Given the low number of phylotypes belonging to this group, it cannot be excluded that they originate from land-runoff from Greenland.

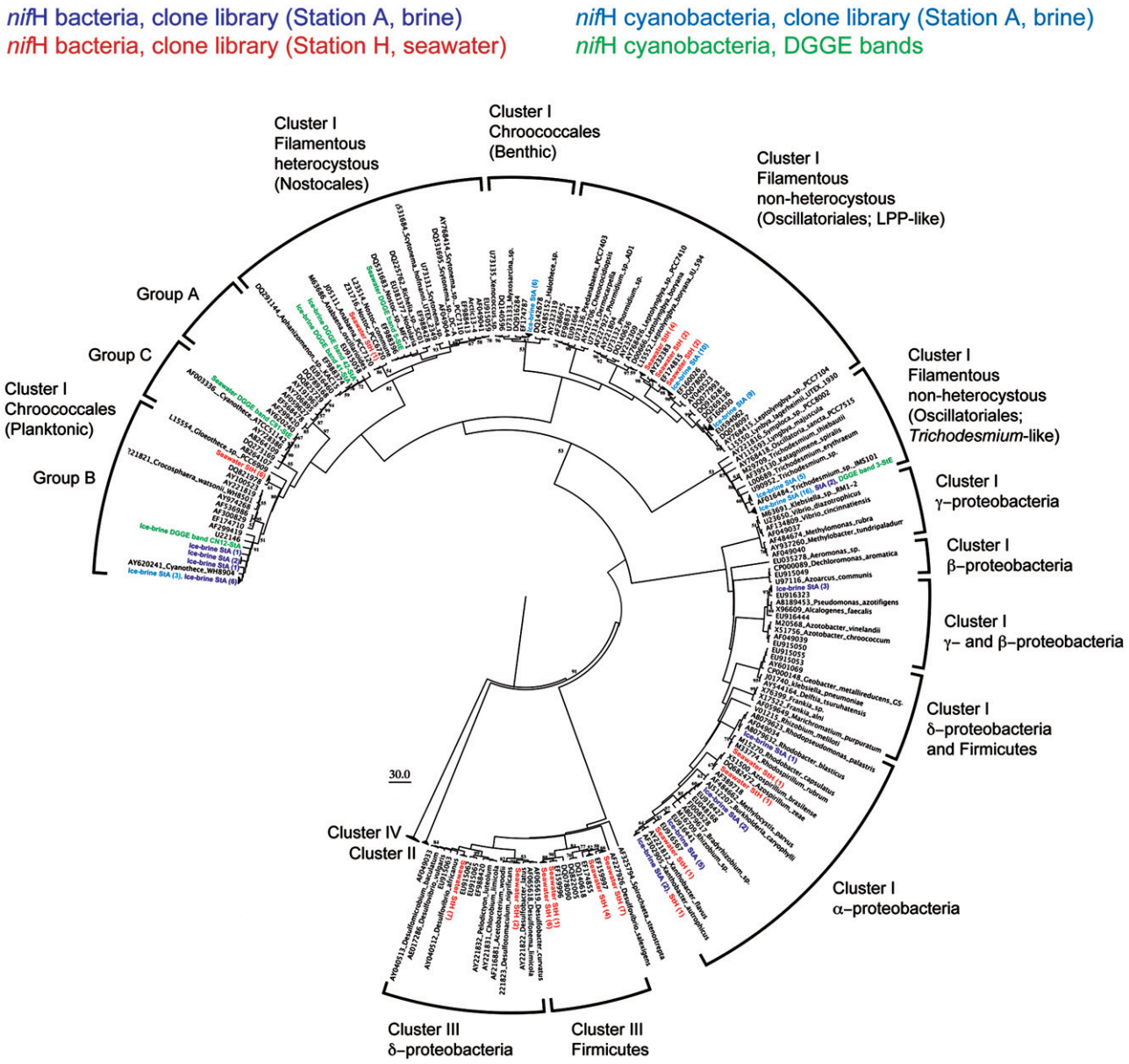

Fig. 3. Nucleotide phylogenetic relationships (maximum likelihood) within the Arctic diazotrophic microbial communities. Bacterial and cyanobacterial diazotrophs retrieved from seawater and brine (in colour) and the closest match from the database (in black), based on partial *nif*H gene sequencing. The number of clones per operational taxonomic unit (OTU) recovered in each clone library are given within brackets. A total of 250 pb were used for the phylogenetic reconstruction. The numbers associated with the internal nodes represent bootstrap values (> 50%) obtained after 1000 replicates. All *nif*H DGGE bands and clones sequences obtained in this study were used in the phylogenetic reconstruction. The sequences were aligned in Bioedit using CLUSTALW (Tom Hall, Ibis Therapeutics, Carlsbad, USA), corrected manually and subjected to BLAST searches (http://www.ncbi.nlm.nih.gov/blast; Altschul *et al*., 1997), and the closest relatives obtained from GenBank were included in the subsequent nucleotide phylogenetic analysis. Only sequences from published studies or culture collections were included and sequences included in the analysis were tested for chimera. MrAIC_Test (http://www.abc.se/~nylander/mraic/mraic.html) was used to search for the best nucleotide substitution model (GTRIG). Likelihood scores under different models were estimated using PHYML (Guindon and Gascuel, 2003).

Taxonomic affiliations of the Arctic heterotrophic bacteria

Altogether, 52% of the clones recovered from the brine and 67% of the clones recovered from the seawater were affiliated to bacteria other than cyanobacteria (Figs 1 and 3). In both habitats α-proteobacteria (e.g. a *Rhodobacter*-like purple, non-sulfur photosynthetic bacterium, *Rhizobiales*-like legume nodulating endosymbiotic bacteria and *Xantobacter*-like bacteria) belonging to Cluster I of nitrogenase occurred. γ-proteobacteria

(A*zotobacter*-like free-living diazotrophic bacteria) were present only in the brine, and members of Cluster III nitrogenase (δ-proteobacteria and *Firmicutes*) only in the seawater (Figs 1 and 3). Altogether, our *nif*H gene clone libraries revealed a diverse collection of diazotrophic bacterial phylotypes representing Clusters I and III, while Farnelid and colleagues (2011) detected Cluster III and IV bacterial phylotypes.

Outlook

The fact that a range of *nif*H gene representing microorganisms with potential diazotrophic capacity inhabits the marine Arctic ecosystem may suggest that they represent a source of new nitrogen in the area. However, it is still left to determine if these genes are also metabolically active before any biogeochemical importance of diazotrophy in the polar oceans can be assessed.

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Supporting information

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

Fig. S1. Number of operational taxonomic units (OTUs) in relation to the % similarity of the clusters.

Table S1. Sampling data, environmental factors measured in the field, biomass data and pigment composition. Microbial biomass was collected on precombusted Whatman® GF/F glass fibre filters (pore size ~ 0.7 μm) using a peristaltic pump with a filtration rate of 50–100 ml · min^{-1}. The filters were immediately frozen in liquid nitrogen and stored at –80°C. Concentrations of chlorophyll *a* (Chla) and the carotenoids echinenone (Echi) and zeaxanthin (Zea) were measured by high-performance liquid chromatography according to Andersson and colleagues (2003). Measurements of particulate organic carbon (POC) and particulate nitrogen (PN) from the filters were carried out with a CHN-900 analyser (Leco, USA).

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