

Nitrogen fixation in the western English Channel (NE Atlantic Ocean)

Andrew P. Rees^{1,*}, Jack A. Gilbert¹, Boris A. Kelly-Gerreyn²

¹Plymouth Marine Laboratory, Prospect Place, The Hoe, Plymouth PL1 3DH, UK

²National Oceanography Centre, Waterfront Campus, European Way, Southampton SO14 3ZH, UK

ABSTRACT: In temperate Atlantic waters (18.8 to 20.1°C), biological nitrogen fixation has been demonstrated by 2 independent measurements: ¹⁵N-N₂ incorporation and *nifH* identification in the DNA and expressed messenger RNA (mRNA). At 2 stations in the western English Channel, bulk waters were incubated with ¹⁵N-N₂. At the high levels of particulate nitrogen ($\leq 11.5 \mu\text{mol N l}^{-1}$), absolute fixation rates of 18.9 ± 0.01 and $20.0 \text{ nmol N l}^{-1}\text{d}^{-1}$ were determined. While a caveat must accompany the magnitude of the rates presented due to the limited number of data, the presence and activity of diazotrophic organisms in these waters is of ecological significance and may affect current attitudes to nitrogen and carbon budgets. In particular, our estimate of the rate of N fixation ($0.35 \text{ mmol N m}^{-2} \text{ d}^{-1}$) is comparable to that of denitrification rates in UK shelf seas. Molecular analysis identified a diversity of expressed *nifH* genes, and 21 different prokaryotic *nifH* transcripts were identified.

KEY WORDS: Temperate Atlantic · Nitrogen fixation · Diazotrophy · *nifH* · Western English Channel

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INTRODUCTION

The introduction of molecular biology techniques has revealed a diversity and abundance of organisms capable of nitrogen fixation (diazotrophy) in the marine environment (Zehr et al. 2001). In recent years, significant rates of nitrogen fixation have been reported (e.g. Rees et al. 2006), including by unicellular organisms whose significance was previously unrecognised (e.g. Montoya et al. 2004). While the pace of research has steadily increased, the extent of the distribution and activity of diazotrophic organisms still remains largely uncharacterised.

The focus of most research efforts in the marine environment has been on the tropical and sub-tropical seas (e.g. Voss et al. 2004, Langlois et al. 2005). There are few, if any, reports of nitrogen-fixing activity in temperate, coastal waters with persistent salinity >30. Langlois et al. (2008) and Needoba et al. (2007) have reported diazotrophic activity in temperate areas of the Atlantic and Pacific, respectively; however, these studies were both associated with waters far-off of the con-

tinental margins. Nitrogen-fixing organisms have been identified under temperate, coastal conditions elsewhere, although these have been associated with estuarine or brackish waters (e.g. Short & Zehr 2007). Extensive nitrogen fixation takes place in the brackish Baltic Sea each summer (e.g. Wasmund et al. 2005) and is also known to occur over environmental extremes including the Arctic tundra (Zielke et al. 2002), Antarctic freshwater (Davey 1982) and deep sea hydrothermal vent fluids (Mehta & Baross 2006). With the extensive range of conditions over which diazotrophs have been reported to be active, it seems unlikely that biological nitrogen fixation does not occur in temperate shelf waters, even if this might only prove significant during the limited period of summertime nutrient depletion.

The seasonal pattern of nutrient succession that takes place in waters of the western English Channel (WEC) was described by Butler et al. (1979). These waters are characterised by complete mixing of the water column during winter and thermal stratification over the summer months. Dissolved nitrogen species

*Email: apre@pml.ac.uk

are characterised by distinct minima in NO_3^- concentrations during summer, following removal by phytoplankton, and a corresponding increase in dissolved organic nitrogen (DON), which maintains the balance of the dissolved nitrogen inventory. Although inorganic nutrients become relatively depleted ($\text{NO}_3^- < 0.5 \mu\text{mol l}^{-1}$), these shelf waters remain productive and support regular algal blooms (Holligan & Harbour 1977) from spring through autumn. Although a number of algal species are able to utilise DON, the availability of this material remains largely unknown, and the manner in which these bloom-forming species obtain their nitrogen is unclear. Holligan et al. (1984) proposed that large summer blooms of *Karenia mikimotoi* (formerly *Gyrodinium aureolum*) might be sustained by nutrient supply across the thermocline.

An alternative source of nitrogen may come from N_2 fixation. Nitrogen release by the diazotrophic cyanobacterium *Trichodesmium* sp. was sufficient to support a bloom of *Karenia brevis* in the Gulf of Mexico (Mulholland et al. 2006). We hypothesised that biological nitrogen fixation may contribute to the available nitrogen pool in the WEC. We describe observations made at 2 stations (Fig. 1), one located within (Stn 2), and one outside (Stn 1) of a mixed bloom of *Emiliana huxleyi* and *K. mikimotoi* that occurred in July 2006.

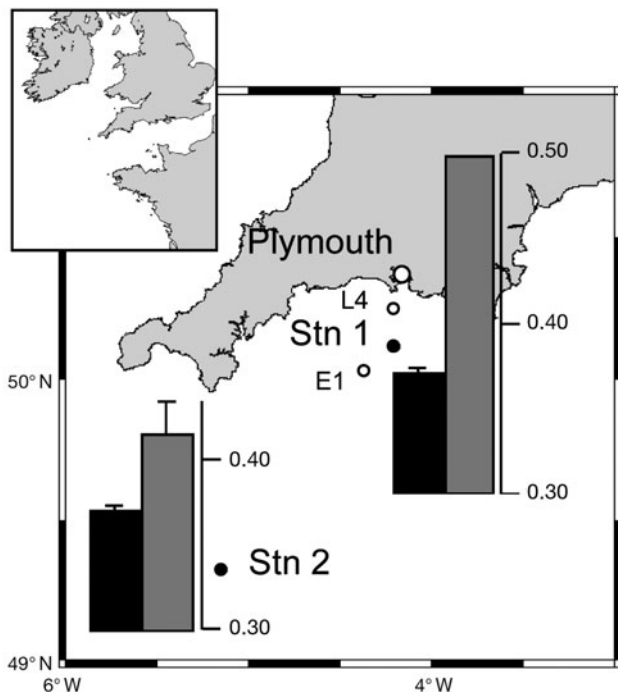


Fig. 1. Position of sampling stations (Stns 1 and 2) in the western English Channel. ^{15}N -atom% enrichment of seawater samples incubated with ^{15}N - N_2 from Stns 1 and 2 is shown (grey bars) relative to seawater samples incubated with ^{14}N - N_2 (black bars). The Plymouth Marine Laboratory long-term sampling sites L4 and E1 are also indicated

MATERIALS AND METHODS

^{15}N - N_2 fixation. Samples for the determination of nitrogen fixation rate were collected at each station from the near surface into 2 acid-cleaned 10 l carboys. Samples were maintained in the dark at ambient temperature and transported to the shore-based laboratory. Each carboy was gently agitated to homogenise the sample, which was then distributed into six 640 ml clear polycarbonate bottles (Nalgene). Each bottle was sealed, and air bubbles were excluded, using Teflon-backed butyl septa inserted into the cap. ^{15}N - N_2 (99 atom%; Isotech) was injected into 3 bottles, and local air was added to the remaining 3 bottles at 2 ml gas l^{-1} of seawater (to provide a background value of ^{15}N content, and to ensure there was no ^{15}N contamination of incubation and filtration equipment). All 6 bottles per station were placed in a laboratory incubator that was maintained at 19°C and $\sim 100 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ during the light period (8:16 h dark:light). Incubations were terminated after 24 h by filtering onto 25 mm GF/F filters (Whatman), which were stored at -20°C . Particulate nitrogen (PN) and ^{15}N atom% were measured using continuous-flow stable isotope mass spectrometry (PDZ-Europa 20-20 and GSL). Instrument precision was better than 0.27% coefficient of variation based on urea standards (Iso-Natural) analysed throughout the run (mean ± 1 SD = 0.3664 atom% \pm 0.00098, $n = 10$) in the range 0.5 to 2.0 $\mu\text{mol-N}$. Samples incubated with ^{14}N (air) additions and run in parallel to ^{15}N -amended samples showed no significant deviation from the expected atom% of 0.366.

DNA/RNA purification and *nifH* gene amplification. Total environmental DNA and RNA was isolated from samples collected at each site by pre-filtration of 10 l through a 1.6 μm GF/A (Whatman). Filtrate was collected on a 0.22 μm Sterivex filter (Millipore). Total nucleic acids were isolated using the method of Neufeld et al. (2007). RNA was isolated following extraction using the RNA-Easy mini prep kit (Qiagen) and treatment with Turbo DNA-free solution (Ambion). *nifH* amplification was performed using the method of Zehr & McReynolds (1989). Products were cloned into pGem-T Easy vector (Promega) and sequenced by the Plymouth Marine Laboratory sequencing facility (ABI BigDye 3.1 used at 1/8 reaction).

***nifH* sequence analysis.** *nifH* DNA and expressed *nifH* mRNA sequences obtained were quality checked and trimmed for vector and low quality sequence using Phred (Ewing & Green 1998) in a pregap4 sequence pipeline run in Biolinx (Natural Environment Research Council [NERC] Environmental Bioinformatics Centre, Centre for Ecology and Hydrology [CEH], UK). Nucleic acid sequences were translated and aligned against the NCBI protein database using the BLASTp

alignment tool. Phylogenetic analysis was performed on mRNA sequences using the neighbour-joining method with Poisson correction. The inferred phylogeny was tested with 500 bootstrap replicates. Sequences were submitted to GenBank under accession numbers EF470528 to EF470548.

Environmental conditions. Bacterial and cyanobacterial numbers were enumerated by analytical flow cytometry (Wilson et al. 2002), following fixation of 1.8 ml seawater with 90 μ l paraformaldehyde. Chlorophyll *a* (chl *a*) concentration was measured (Welschmeyer 1994) following filtration of 100 ml of seawater through 0.2 μ m polycarbonate filters. A Sea-Bird 19+ CTD was deployed at each station for the determination of seawater temperature and salinity.

RESULTS AND DISCUSSION

This study sampled an extensive mixed phytoplankton bloom that extended over much of the WEC and was dominated by *Emiliania huxleyi* and *Karenia mikimotoi* (D. Schroeder pers. comm.). Based on remotely sensed observations from the previous day, Stn 1 was selected to be in relatively low chlorophyll waters, whereas Stn 2 was chosen as representative of the area with highest phytoplankton biomass. Environmental conditions experienced at each station are summarised in Table 1, and placed in the context of long-term (2002 to 2006) conditions of nitrate and temperature in Fig. 2.

At both stations, a component of the microbial community fixed N_2 into cellular material (Fig. 1). Seawater samples were not pre-screened prior to incubation so that we could assess the activity of the total community. Using the equations of Montoya et al. (1996), the product of ^{15}N enrichment (Fig. 1) and the total PN observed during this study (Table 1) yielded volumetric rates of 20.0 and 18.9 $nmol N l^{-1}d^{-1}$ for Stns 1 and 2, respectively.

Clone libraries were constructed from both DNA and mRNA. These revealed 8 and 17 *nifH* clones within the DNA (not shown), and 6 and 15 *nifH* clones from within the mRNA (Fig. 3) for Stns 1 and 2, respectively.

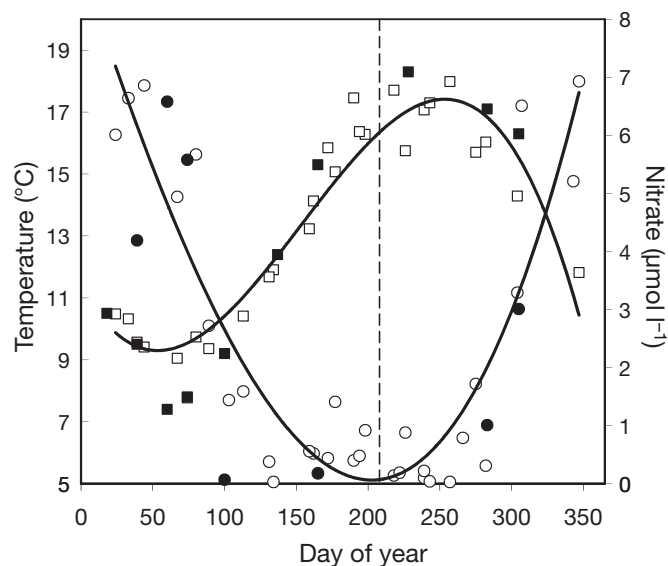


Fig. 2. Conditions of temperature (\square) and nitrate (\circ) determined at the Western Channel Observatory (www.westernchannelobservatory.org.uk/) Stn E1 between 2002 and 2006. Solid symbols indicate data for 2006. Vertical dashed line indicates the sampling day in 2006

nifH clone sequences from the DNA library for both stations were derived from *Alphaproteobacteria*, *Betaproteobacteria* and cyanobacterial groups belonging to Cluster I of Chien & Zinder (1996). The mRNA library for both stations also contained sequences from Cluster I, but also from Cluster III and a potentially novel *nifH* Cluster V (Table 2, Fig. 3). The higher diversity of sequences obtained from the mRNA library compared to the DNA library is not unprecedented, and is comparable to observations made by Man-Aharonovich et al. (2007) for coastal Mediterranean waters.

A number of the sequences observed during the present study are previously unreported under similar environmental conditions. Within Cluster I, the sequence EF470531 clustered within a *Deltaproteobacteria* group, with 83.6% amino acid identity to *Pelobacter carbinolicus*. This group is well represented in marine *nifH* studies (e.g. Langlois et al. 2005, Man-

Table 1. Station position and environmental conditions during this study of nitrogen fixation in the western English Channel, 27 July 2006. MLD: mixed layer depth, HB: heterotrophic bacteria, Cyano: cyanobacteria, PN: particulate nitrogen

	Surface temp. (°C)	Salinity	MLD (m)	Chlorophyll ($\mu g l^{-1}$)	HB (cells ml^{-1})	Cyano (cells ml^{-1})	PN ($\mu mol l^{-1}$)	N fixation ($nmol N l^{-1}d^{-1}$)
Stn 1								
50.116° N, 04.120° W	18.84	35.230	15	2.1	3.8×10^6	6.9×10^4	4.66	20.0 ^a
Stn 2								
49.322° N, 05.145° W	20.1	35.115	20	8.4	1.3×10^6	3.0×10^4	11.53	18.9 \pm 0.01
^a Mean of 2 replicates								

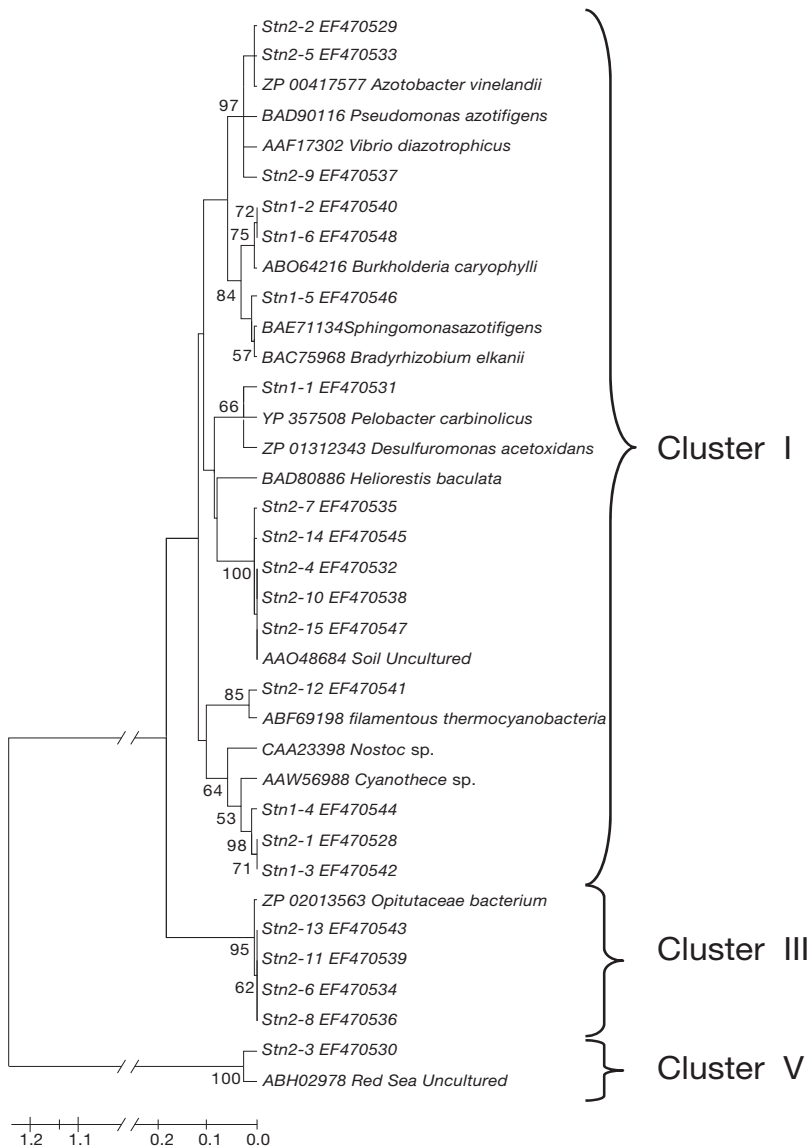


Fig. 3. Diversity of expressed *nifH* protein sequences from Stns 1 and 2. Phylogenetic relationships were inferred using the neighbour-joining method with the Poisson correction model. Bootstrap values represent 500 replications; only values >50% are shown. Clones are named based on their site of isolation and sequence number. GenBank accession numbers are provided for each sequence

Aharonovich et al. 2007), but this is the first report from a coastal temperate, marine province with a constant salinity of >30. Similarly, cyanobacterial diazotrophs were found at both sites, including the group A unicellular cyanobacteria (EF470544, EF470528 and EF470542) that have previously only been reported in temperate estuarine, sub-tropical and tropical waters (e.g. Zehr et al. 2001). Of the Cluster I mRNA sequences, 5 from Stn 2 clustered with an unknown soil bacterium (96.7% amino acid identity), were >98% identical at the protein level, but also shared >83% amino acid identity with a soil-derived firmicute,

Helioresitis baculata. The bootstrap values for this branching are <50%, and although this taxonomic affiliation is not persuasive, Man-Aharonovich et al. (2007) showed a single similar sequence from a site in the Mediterranean.

Within the mRNA library, 4 sequences from Stn 2 were found to group within the anaerobic *nifH* Cluster III (Fig. 3). All 4 sequences had >95% amino acid identity to each other and >89% amino acid identity to a Verrucomicrobium, *Opitutaceae* sp. While the occurrence of these anaerobic groups may seem anomalous in surface waters, similar sequences have been found in coastal Mediterranean, coral reef lagoon and estuarine environments (e.g. Man-Aharonovich et al. 2007). An association with particles or zooplankton might be an obvious explanation; however, the pre-filtration of samples through GF/A filters precludes this.

One sequence (EF470530) from Stn 2 formed a remote outlying cluster with a probable *nifH* sequence from an uncultured Red Sea bacterium (Fig. 3). The high bootstrap value and depth of branching for this cluster suggests that it may be a novel clade of a rare phylo-type. We call this Cluster V (based on the convention of Chien & Zinder 1996); future studies are required to describe this group.

The diversity of clones at Stn 2 appeared greater than at Stn 1 (Fig. 3, Table 2), and it is interesting to note that the community composition was also different, with *Alphaproteobacteria* and *Betaproteobacteria nifH* clusters observed at Stn 1, and *Gammaproteobacteria* sequences at Stn 2 (Fig. 3).

It has been suggested that *Alphaproteobacteria* and *Betaproteobacteria* diazotrophs may be associated with small invertebrates (e.g. Braun et al. 1999). During the present study, this is countered by the pre-filtration step which removed particles >1.6 μm . It should be noted that Zehr et al. (2003) reported several potential PCR reagent contaminants clustering with the betaproteobacterial *Burkholderia* species, so the identification of sequences EF470540 and EF470548 should be treated with caution.

These results provide the first report to confirm nitrogen fixation activity — both from $^{15}\text{N-N}_2$ incorporation

Table 2. Distribution of *nifH* types between DNA and mRNA for Stns 1 and 2

Source	No. of clones			
	Cluster I Cyanobacteria	Cluster I Bacteria	Cluster III Bacteria	Cluster V Novel
Stn 1				
DNA	4	4	0	0
mRNA	2	4	0	0
Stn 2				
DNA	12	3	0	0
mRNA	2	8	4	1

and the presence and expression of the *nifH* gene — in fully marine, mesotrophic, temperate, coastal conditions. Other workers have reported nitrogen fixation and/or the diversity and expression of *nifH* genes under temperate conditions (e.g. Affourtit et al. 2001, Needoba et al. 2007, Short & Zehr 2007), but these were in brackish or estuarine waters and off-shelf oligotrophic Pacific waters, respectively.

Intense blooms of *Karenia mikimotoi* (e.g. Holligan et al. 1984) and *Emiliania huxleyi* (e.g. Smyth et al. 2002) often occur in the WEC during periods of strong thermal stratification and nutrient depletion. During summer in the WEC, concentrations of NO_3^- (Fig. 2) routinely fall to $<0.5 \mu\text{mol l}^{-1}$; the mean (May to August) concentration at Stn E1 between 2003 and 2006 was $0.26 \pm 0.19 \mu\text{mol l}^{-1}$. While nutrient concentrations were not determined during this study, April 2006 and June 2006 values of 0.07 and $0.18 \mu\text{mol l}^{-1}$, respectively, are indicative of the concentrations expected during July. There is some contention in previously published literature regarding the levels of dissolved inorganic nitrogen (DIN) at which nitrogen fixation is viable, although this is very likely to be species dependent. While the high energy requirement of nitrogenase activity would appear to infer diazotrophy only during DIN-deplete conditions, Voss et al. (2004) observed nitrogen fixation at NO_3^- concentrations in the order of $10 \mu\text{mol l}^{-1}$. It would therefore seem that the low nutrient conditions experienced during summer in the WEC could be conducive to the growth of diazotrophic organisms, though oligotrophy would appear not to be a prerequisite for nitrogen fixation. Rather than absolute concentrations of DIN, it might be the relative concentration of DIN to other nutrient species that controls the activity of diazotrophic organisms. Ratios of DIN and dissolved inorganic phosphorus (DIP) are regularly less than the Redfield value of 16, and indicate the potential for nitrogen limitation (Butler et al. 1979, Kelly-Gerreyn et al. 2007).

Nitrogen fixation by *Trichodesmium* spp. provides a direct supply of fixed nitrogen and has been shown to support the growth of *Karenia brevis* in the Gulf of Mexico (Mulholland et al. 2006). The present study pro-

vides an initial insight into the potential for nitrogen fixation in temperate shelf seas. However, the small number of samples does not allow a full assessment of the potential contribution of nitrogen fixation to the total nitrogen budget. We can, however, use our rate estimate of $20 \text{ nmol N l}^{-1} \text{ d}^{-1}$ as a maximum and compare it to known N fluxes, to provide an initial contextual assessment. Blooms of phytoplankton consume nitrate at significant rates. The bloom of *K. brevis* in the Gulf of Mexico consumed NO_3^- at 26.5 to $44.2 \text{ nmol N l}^{-1} \text{ h}^{-1}$ (Mulholland et al. 2006), while a North Sea bloom of *Emiliania huxleyi* took up NO_3^- at 0.1 to $2.0 \text{ nmol N l}^{-1} \text{ h}^{-1}$ (Rees et al. 2002). Therefore, our maximum nitrogen fixation rate would be equivalent to $<3.1\%$ of the NO_3^- requirement during the Gulf of Mexico *K. brevis* bloom, but between 42 and 833% of the NO_3^- uptake taken up by North Sea coccolithophore bloom. Although this appears significant, both of these blooms were also supported by the supply of urea, NH_4^+ and amino acids. Consequently our nitrogen fixation rate appears less important when compared to the total fixed nitrogen uptake (NO_3^- , NH_4^+ , urea and amino acids), with relative rates of 0.02 and 1.5% for the Gulf of Mexico and North Sea, respectively.

From this preliminary investigation, there is clear evidence of nitrogen fixation in mesotrophic waters of the WEC during summer conditions of thermal stratification and nutrient depletion; this may contribute in part to the nitrogen requirement of phytoplankton during nitrogen-limited conditions. We estimate an upper limit of nitrogen fixation of $20 \text{ nmol N l}^{-1} \text{ d}^{-1}$ (equivalent to $0.35 \text{ mmol N m}^{-2} \text{ d}^{-1}$ for the surface mixed layer) which is comparable to, or greater than, rates measured in the sub-tropical Atlantic (Voss et al. 2004, A. P. Rees unpubl. data). There was considerable diversity of *nifH* transcripts and 21 diverse prokaryotic *nifH* transcripts were found: 15 were associated with an intense phytoplankton bloom and 6 were from a station outside of the bloom. The occurrence and activity of these organisms is significant from a biodiversity interest and raises a number of questions concerning the process of fixing N_2 when there are sources of fixed nitrogen readily available. While the implication of coastal, temperate diazotrophs to the global nitrogen budget remains unknown and requires further temporal and spatial investigation, our estimated upper limit nitrogen fixation rate ($0.35 \text{ mmol N m}^{-2} \text{ d}^{-1}$) is of the same order as denitrification rates in the North Sea measured both directly (0.24 to $0.32 \text{ mmol m}^{-2} \text{ d}^{-1}$; Lohse et al. 1996) and from nitrogen budgets ($0.7 \text{ mmol m}^{-2} \text{ d}^{-1}$; Hydes et al. 1999). Consequently, nitrogen fixation may effectively counter the losses of fixed N through denitrification in these waters, and thus previous nitrogen budgets of the north Atlantic watersheds that did not include nitrogen fixation (e.g. Nixon et al. 1996, Brion et al. 2004) may need to be revised.

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