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Nitrogenase expression in estuarine bacterioplankton influenced by organic carbon and availability of oxygen

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ABSTRACT

The genetic capacity to fix gaseous nitrogen (N) is distributed among diverse diazotrophs belonging to the Bacteria and Archaea. However, only a subset of the putative diazotrophs present actively fix N at any given time in the environment. We experimentally tested whether the availability of carbon and inhibition by oxygen constrain N fixation by diazotrophs in coastal seawater. The goal was to test whether by alleviating these constraints an increased overlap between nitrogenase (nifH)-gene-carrying and -expressing organisms could be achieved. We incubated water from a eutrophic but N-limited fjord in Denmark under high-carbon/low-oxygen conditions and determined bacterial growth and production, diazotrophic community composition (Illumina nifH amplicon sequencing), and nifH gene abundance and expression [quantitative PCR (qPCR) and quantitative reverse transcriptase PCR (qRT-PCR)]. Bacterial abundances and production increased under high-carbon/low-oxygen conditions as did the similarity between present and active diazotrophic communities. This was caused by the loss of specific abundant yet non-active gammaproteobacterial phylotypes and increased expression by others. The prominent active gamma- and epsilonproteobacterial diazotrophs did not, however, respond to these conditions in a uniform way, highlighting the difficulty to assess how a change in environmental conditions may affect a diverse indigenous diazotrophic community.

Keywords: bacterial activity; limiting factors; nitrogen fixation

INTRODUCTION

Nitrogen transformations carried out by microorganisms include N-loss via denitrification and anaerobic ammonium oxidation and N-gain via biological nitrogen fixation (BNF). In some N-limited aquatic systems, BNF may account for more than 80% of the N input (e.g., Howarth et al. 1988). Yet, due to the fact that cyanobacteria have long been considered the sole important N-fixing organisms (diazotrophs; e.g., Capone et al. 1997; Zehr et al. 2001; LaRoche and Breitbarth 2005) and research has
concentrated on areas where cyanobacteria are common, other N-limited systems without cyanobacteria are believed to be free from BNF. However, it is now evident that non-cyanobacterial diazotrophs are widespread and active in marine waters (e.g., Riemann, Farnelid and Steward 2010; Farnelid et al. 2011; Moisander et al. 2014). Due to their fundamentally different energy acquisition, heterotrophic diazotrophs could fix N in underexplored marine regions, such as cold or dark waters and coastal regions, and thereby significantly increase the known spatio-temporal distribution of BNF. Limitations of heterotrophic BNF in open ocean waters would likely be low concentrations of organic carbon and/or the lack of low-oxygen loci for a process that is typically impaired by the inhibition of nitrogenase at ambient oxygen concentrations. Heterotrophic diazotrophs are indeed widespread in oligotrophic and well-oxygenated waters (e.g., Farnelid et al. 2011), but it has been argued that, at least in some oceanic regions, even abundant heterotrophic diazotrophs cannot account for measured BNF due to their low cell-specific N fixation (Turk-Kubo et al. 2014). However, in a recent study with heterotrophic isolates from the Baltic Sea, we observed that higher cell-specific N fixation rates are possible in more productive waters, indicating that abundances similar to those observed for certain phenotypes in situ could account for low but measurable BNF (Bentzon-Tilia et al., 2015b).

Many studies of the nifH gene, which encodes the Fe-protein of nitrogenase, have only reported a small overlap between nifH genes (DNA) and transcripts (mRNA) in a community (Moisander et al. 2006; Man-Aharonovich et al. 2007; Zhang, Hurek and Reinhold-Hurek 2007). This indicates that only a minute proportion of all present diazotrophs is active at a given point in time and that their distribution may not solely be dependent on selection based on diazotrophy (e.g., Short, Jenkins and Zehr 2004; Moisander et al. 2007). Nevertheless, the wide distribution of nifH among planktonic Bacteria and Archaea indicates a selective advantage of diazotrophy since only advantageous functional genes will be fixed in the genome due to the high rate of random gene mutations (Berg and Kurland 2002). Therefore, marine nifH genes are conceivably expressed at least occasionally. Consequently, all nifH genes in a given gene pool should theoretically be expressed under suitable conditions.

Here, we assumed that ‘suitable’ conditions for BNF by heterotrophic diazotrophs include low-oxygen conditions and sufficient energy (carbon), and hypothesized that these conditions would increase the similarity of phenotype composition based on nifH DNA and complementary DNA (cDNA). Hence, by experimental manipulation we sought to demonstrate that suitable conditions would result in diazotrophy becoming an expressed phenotype among putative diazotrophs. We subjected N-limited water from Roskilde Fjord (Denmark), where BNF is partially carried out by heterotrophic diazotrophs (Bentzon-Tilia et al. 2015a), to low-oxygen/high-carbon conditions and assessed the present and active diazotrophic communities by high-throughput sequencing and quantitative PCR (qPCR) targeting of the nifH genes and transcripts.

**METHODS**

**Experimental set-up**

Surface water (1 m) from Roskilde Fjord, Denmark (55°42.00′N, 12°04.46′E; 4.8 m total depth), was sampled on 13 June 2012 and transported to the laboratory in Helsingør where it was distributed into 12 carboys (12 L volume each) and placed in a climate-controlled room (~19°C) under illumination (200–340 μE m⁻² s⁻¹) according to an in situ day–night cycle. Treatment ‘HCLOP’ (high C, low O₂, P-addition) received a carbon mix (50 μmol L⁻¹ final conc.; glucose, galactose, mannitol, acetate and pyruvate) and phosphate (NaH₂PO₄, 1 μmol L⁻¹ final conc.) to ensure N-limited conditions, and was bubbled with an N₂/CO₂ gas mix (99.6% N₂/0.4% CO₂; Air Liquide Danmark A/S). Treatment ‘P’ was amended with phosphate only to assess if N-limited conditions alone affected the bacterial community (without high C and low O₂). The control carboys received nutrient amendments. ‘P’ and the control treatment were bubbled with filtered air. Treatments were run in quadruplicates and incubated for 6 days. Temperature and oxygen (oxygen meter ox 3210, WTW Weilheim) were monitored daily. Samples for bacterial abundance and production were collected daily. At the start of the experiment (in situ) as well as after 3 and 6 days, concentrations of dissolved organic carbon (DOC) and inorganic nutrients were determined. After terminating the incubations (day 6), samples for DNA and RNA were collected during the first half of the light cycle. This may have resulted in the failure to detect nifH transcripts from diazotrophs transcribing nifH during the night, such as non-heterocystous cyanobacteria. However, since no nifH genes from these organisms were detected in the DNA samples, their absent nifH gene expression was most likely not due to the chosen time of sampling.

**Background parameters**

In situ physicochemical data (TP: total phosphorus, PO₄³⁻; TN: total nitrogen) were obtained during a concurrent seasonal study of Roskilde Fjord (Bentzon-Tilia et al. 2015a, Table S1, Supporting Information). DOC and dissolved organic nitrogen (DON) were measured using a TOC-Vcph analyser (Shimadzu) according to Cauvet (1999). TP and inorganic nitrogen concentrations (NO₃⁻ and NO₂⁻ after reduction of NO₃⁻ to NO₂⁻) were determined fluorometrically (Turner Trilogy Fluorometer, following the protocol by Turner Designs); NH₄⁺ was determined after Holmes et al. (1999). Concentrations of NO₃⁻ and NO₂⁻ were below the detection limit (0.5 μM).

Bacterial abundance was determined from samples fixed with glutaraldehyde (1% final conc.) using a FACScan™ II flow cytometer (BD Biosciences) according to Gasol and Del Giorgio (2000). Bacterial production (BP) was estimated by [³H]-thymidine incorporation (20 nmol L⁻¹ final concentration, PerkinElmer; Fuhrman and Azam 1982) using microcentrifugation (Smith and Azam 1992), and 1.1 × 10¹⁸ cells mol⁻¹ (Riemann et al. 1987) and 2 × 10⁻¹⁴ g cell⁻¹ (Lee and Fuhrman 1987).

**Diazotrophic community composition**

At day 6, 700 mL of water was pre-filtered through a 10 μm polycarbonate filter (GE Water & Process Technologies) and then onto a Sterivex-PG filter (0.22 μm, EMD Millipore). Separate filters were taken for DNA and RNA. Filters were immediately frozen at −80°C until extraction of DNA and RNA using the EZNA Tissue DNA Purification kit and the Total RNA kit I (Omega Bio-Tek), respectively. The RNA extraction included an extra on-column DNA purification step (EZNA RNase-free DNase). cDNA was synthesized using TaqMan reverse transcription reagents (Applied Biosystems) and the nifH3 reverse primer (Zehr and McReynolds 1989). DNase-treated, but not reverse-transcribed, template was included to check for the presence of genomic DNA in the RNA template, but these never amplified in PCR.

nifH was amplifiable from 12 DNA and 14 cDNA templates, equivalent to quadruplicate DNA samples for ‘HCLOP’,
triplicates for ‘P’ and the control, and two negative controls (extraction control and PCR control) as well as quadruplicate RNA samples for all treatments and two negative controls (extraction control and PCR control). Amplifications were carried out using 5–10 ng of DNA or cDNA template, Pure Taq Ready-To-Go PCR Beads (GE Healthcare) and a nested PCR protocol (Zehr and Turner 2001). Illumina indices (Table S2, Supporting Information) were added to the amplicons in the second PCR round. Three to four technical PCR replicates were run per sample, pooled, purified (AMPure XP purification kit; Beckman Coulter Inc.) and quantified (PicoGreen; Invitrogen). The negative controls did not show bands when checked on the gel, but volumes similar to those of the samples were included in the following steps nonetheless. 75 ng of purified PCR product per sample was pooled and subjected to one run of pair-end sequencing on the Illumina MiSeq platform at the Berlin Center for Genomics in Biodiversity Research. Sequence data are deposited in MG-RAST (Meyer et al. 2008) under accession numbers 4565187.3–4565230.3 (http://metagenomics.anl.gov/linkin.cgi?project=9299). Sequences characterizing the in situ diazotrophic community composition were obtained from Bentzon-Tilia et al. (2015a).

Demultiplexed reads were assembled in Mothur v. 1.32.0 (Schloss et al. 2009), trimmed to 250 nt, and screened for ambiguous basecalls. Sequences that failed the quality filtering and aligned poorly to the nifH reference database on fungenes (http://fungene.cme.msu.edu/) were excluded. Chimeras were removed using UCHIME and sequences were clustered at 97% nucleotide sequence similarity. After removal of singlets, an average of 3731 sequences per sample (min = 916, max = 6004) was used for further analyses. The negative controls did not yield any sequences. Representative sequences from the most abundant operational taxonomic units (OTUs) were blasted against the NCBI nucleotide database. Based on the OTU tables, pairwise Morisita–Horn dissimilarities were estimated between all samples in R version 3.0.2 (R Development Core Team 2008) using the vegan package 2.0–10 (Oksanen et al. 2012). Pairwise dissimilarities were averaged to determine the degree of difference between the treatments and between the present and active community for a certain treatment (values between 0 and 1 show identical and absolutely different diazotrophic community composition, respectively). SIMPER (Similarity Percentage) was run in PAST (version 3.01, Hammer, Harper and Ryan 2001) to identify the OTUs responsible for the difference in community composition.

In order to quantify nifH genes and transcripts, three qPCR assays were designed based on the dominant cDNA clusters (Table 1), targeting two gammaproteobacterial (‘Vib’ and ‘Azo’) and one epsilonproteobacterial (‘Arc’) cluster, accounting for 72% of the obtained sequences. Specific primers were designed using Primer3 (v 0.4.0 online resource; http://bioinfo.ut.ee/primer3-0.4.0/, Table S3, Supporting Information), and the presence of hairpins and dimers was checked (NetPrimer; PREMIER Biosoft). Specificity was verified in silico (Primer-BLAST; NCBI) and by testing amplification of non-target sequences [the other two standards and DNA from three isolates: Raoultella ornithinolytica, Pseudomonas stutzeri and Rhodopseudomonas palustris (accession numbers AY972875, KC140355 and KC140365, respectively)]. The non-target sequences never yielded qPCR products.

The PCR standards were synthesized commercially (GenScript), linearized by HindIII digestion and used in triplicate 10-fold dilution series ranging from 10^3 to 10^7 copies. Two of the standards (10^3 and 10^6 copies) were also spiked with sample template to exclude potential inhibition. No changes in amplification efficiencies were detected. The 20 μL reaction mixtures contained 1 × SYBR® Select Master Mix (Life Technologies Europe BV), 300 nM of each primer, RT-PCR grade water and 2 μL template. Amplification efficiencies were 70% for one assay (‘Azo’) and 100% for the other assays (‘Vib’ and ‘Arc’). Specificity was confirmed by melting curve analyses.

Statistics

Tests for normality and equality of variances of data as well as one-way ANOVAs and Tukey’s post-hoc tests on normally distributed data were run in PAST (version 3.01, Hammer, Harper and Ryan 2001).

RESULTS

Background parameters

Oxygen saturation in ‘HCLOP’ was 16% and 10% after 3 and 6 days, respectively, while the air-bubbled carboys were fully oxygenated (Table S1, Supporting Information). One-way ANOVAs showed no difference in NH4+ between treatments at the same time point but a decrease over time for ‘P’ (Tukey’s post hoc, P < 0.01). ‘HCLOP’ showed an increase in DON (P < 0.01) and a decrease in DOC (P < 0.01) over time. Moreover, due to the C-addition, DOC concentrations were higher in ‘HCLOP’ (P < 0.01) compared to ‘P’ and the control.

Bacterial abundance and activity

‘P’ and the control treatment showed similar bacterial abundances over time (Fig. 1A). ‘HCLOP’ showed no lag phase

Table 1. The nine most abundant OTUs in DNA and cDNA across all samples at the end of the incubations (day 6), with decreasing relative abundance from the top. Given are the OTU number (assigned by Mothur), the most closely related cultivated diazotroph in the NCBI GenBank database, the phylogenetic affiliation (class level), the nucleotide sequence similarity (%), the accession number of the closest cultivated representative, the relative abundance in the entire DNA and cDNA dataset (%), and the OTUs targeted by qPCR.

<table>
<thead>
<tr>
<th>OTU</th>
<th>closest cultivated diazotroph on NCBI</th>
<th>phylogenetic affiliation</th>
<th>seq sim (%)</th>
<th>NCBI accession no</th>
<th>rel abundance DNA (%)</th>
<th>rel abundance cDNA (%)</th>
<th>qPCR assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vibrio parahaemolyticus strain 22702</td>
<td>Gammaproteobacteria</td>
<td>88</td>
<td>EF203422.1</td>
<td>18</td>
<td>28</td>
<td>‘Vib’</td>
</tr>
<tr>
<td>2</td>
<td>Vibrio parahaemolyticus strain 22702</td>
<td>Gammaproteobacteria</td>
<td>87</td>
<td>EF203422.1</td>
<td>5</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Vibrio parahaemolyticus strain 22702</td>
<td>Gammaproteobacteria</td>
<td>88</td>
<td>EF203422.1</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Vibrio portorosae strain MSSRF30</td>
<td>Gammaproteobacteria</td>
<td>85</td>
<td>EF554362.1</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Vibrio diazotrophicus</td>
<td></td>
<td>84</td>
<td>AF111110.2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Azomonas macrorygenotes</td>
<td>Gammaproteobacteria</td>
<td>85</td>
<td>AY544349.1</td>
<td>1</td>
<td>23</td>
<td>‘Azo’</td>
</tr>
<tr>
<td>7</td>
<td>Azomonas macrorygenotes</td>
<td>Gammaproteobacteria</td>
<td>84</td>
<td>AY544349.1</td>
<td>0</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Arcobacter nitrofigilis clone CC1097A1</td>
<td>Epsilonproteobacteria</td>
<td>91</td>
<td>AF117978.1</td>
<td>61</td>
<td>0</td>
<td>‘Arc’</td>
</tr>
<tr>
<td>9</td>
<td>Pseudomonas stutzeri CMT.9.A</td>
<td>Gammaproteobacteria</td>
<td>91</td>
<td>AY221825.1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
reached highest abundances after 2 days. Maximum and minimum abundances were higher in 'HCLOP' than in 'P' and the control treatment. 'HCLOP' had significantly higher BP than 'P' and the control after 3 and 6 days (P < 0.01; Fig. 1B), probably due to an increase in abundance caused by the addition of C. BP showed an increasing trend in 'HCLOP' and a decreasing trend in 'P' and the control. There was, however, no significant difference in cell-specific BP between treatments after 6 days (not shown).

**Diazotrophic community composition**

In total, more than 2700 nifH OTUs were obtained from all samples at the end of the experiment (day 6, details given in Table S4, Supporting Information). The nine most abundant OTUs (containing 455 to 25 644 sequences each) were exclusively affiliated with proteobacterial diazotrophs (Table 1). Although the same nine OTUs were most abundant in all treatments at the end of the experiment and accounted for 84–90% of the sequences, their relative abundances showed pronounced variation (Fig. 2). SIMPER analysis showed that the difference in abundance of two or more of these OTUs caused more than 75% of the difference in diazotrophic community composition between treatments. Pairwise Morisita–Horn dissimilarities (averages of treatment replicates) between treatments as well as between in situ and any of the treatments were calculated for the present and active diazotrophic community, respectively (Table 2A). This shows...
that (i) the present and active in situ diazotrophic community was dissimilar from any of the treatments, (ii) the present diazotrophic communities in 'P' and the control were very similar, and dissimilar to 'HCLOP', (iii) the active diazotrophic community in 'HCLOP' was more similar to that of 'P' and the control than the present diazotrophic community of these treatments. Moreover, pairwise Morisita–Horn dissimilarities (averages of treatment replicates) between the present and the active diazotrophic communities in situ and in all treatments (Table 2B) show that more of the present diazotrophs were active in 'HCLOP' (lower Morisita–Horn dissimilarity).

None of the most abundant OTUs had close cultivated relatives (Table 1). The gammaproteobacterial OTUs 1, 3, 7, 8, and 9 (representing 43% of all sequences) showed 84–88% similarity (at the nucleotide level) with Vibrio species. No perfect matches for these Vibrio-like sequences were found in the database. Uncultured \(nifH\) sequences found in the temperate Pacific Northwest showed only slightly higher sequence similarities (92%) to OTUs 3 and 7 than those given for the most closely related identified sequence. They were more common in cDNA than in DNA, and in DNA of 'HCLOP' compared to 'P' and the control. The same pattern was observed for OTU 6 (1% of all sequences), an epsilon-proteobacterial phylotype most closely related to Arcobacter. No perfect matches for this sequence were found in the database. The gammaproteobacterial OTUs 2 and 5 (27% of all sequences) clustered with the genus Azomonas and showed 99% similarity to \(nifH\) sequences found in the Zostera marina rhizosphere and marine sediments. They were more abundant in cDNA than in DNA in all treatments. The gammaproteobacterial OTU 4 (11% of all sequences) was most closely related to Pseudomonas stutzeri and was mainly found in DNA samples, especially in 'P' and the control. Identical \(nifH\) sequences were also found in the South China Sea, the Baltic Sea and the tropical eastern North Atlantic.

qPCR on the most abundant OTUs in the cDNA showed that for Vibrio- and Arcobacter-like organisms, \(nifH\) expression (L\(^{-1}\)) was higher in ‘HCLOP’ than in ‘P’ and the control (Table 3). \(nifH\) expression in Vibrio-like phylotypes was more than one order of magnitude higher in ‘HCLOP’ than in ‘P’ and two orders of magnitude higher than in the control. However, Vibrio-like \(nifH\) genes were also more abundant in ‘HCLOP’ than in ‘P’ and the control. Consequently, the ratio of Vibrio-like \(nifH\) transcripts to copies (cDNA/DNA) was highest in ‘P’ compared to the control and ‘HCLOP’. The abundance of the Arcobacter-like diazotrophs was similar in ‘HCLOP’ and the control, while no Arcobacter-like \(nifH\) copies could be detected in ‘P’. Arcobacter-like \(nifH\) expression was similar in ‘P’ and the control, being one order of magnitude lower than in ‘HCLOP’, i.e. the ratio of \(nifH\) transcripts to copies was approximately four times higher in ‘HCLOP’ than in the control. Azomonas-like transcripts could only be detected in ‘HCLOP’. However, due to a low reaction efficiency of this assay, the absence of \(nifH\) transcripts and copies in the other treatments does not necessarily mean that none were present.

**DISCUSSION**

Our aim was to test whether it is possible to experimentally increase the proportion of active diazotrophs of the total diazotrophic population in estuarine environments. Especially when considering heterotrophic nitrogen fixation, low-oxygen loci and energy are likely two limiting factors (e.g., Riemann, Farnelid and Steward 2010). To that end, we added a mix of labile carbon substrates and reduced oxygen saturation. Although Roskilde Fjord is known to be N-limited in summer, we also tested if further N-limitation could be induced by phosphate addition. This alone had, however, no measurable effect on either total bacterial growth or production, or diazotrophic community composition. Hence, the addition of phosphate per se did not interfere with our assessment of the effect of low-oxygen/high-carbon conditions. However, phosphate addition had an effect on the ratio of \(nifH\) transcripts/copy in Vibrio-like diazotrophs, as discussed below. Bacterial abundance and production generally increased under low-oxygen/high-carbon

**Table 2.** Averaged pairwise Morisita–Horn dissimilarities between treatments based on DNA and cDNA of each treatment (A) and between DNA and cDNA (B).

<table>
<thead>
<tr>
<th></th>
<th>DNA</th>
<th>cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>in situ vs HCLOP</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>in situ vs P</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>in situ vs control</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>HCLOP vs P</td>
<td>0.66</td>
<td>0.46</td>
</tr>
<tr>
<td>HCLOP vs control</td>
<td>0.69</td>
<td>0.45</td>
</tr>
<tr>
<td>P vs control</td>
<td>0.01</td>
<td>0.46</td>
</tr>
<tr>
<td>B</td>
<td>DNA vs cDNA</td>
<td></td>
</tr>
<tr>
<td>in situ</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HCLOP</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.96</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.** \(nifH\) gene copies and transcripts as assessed by qPCR targeting Vibrio- (Vib), Arcobacter- (Arc) and Azomonas- (Azo) like diazotrophs, see Table 1. Standard deviations are given in parentheses.

<table>
<thead>
<tr>
<th>Gene copies L(^{-1})</th>
<th>Transcripts L(^{-1})</th>
<th>Transcripts per gene copy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vib</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCLOP</td>
<td>7.58 × 10(^6) (1.05 × 10(^8))</td>
<td>8.90 × 10(^6) (1.66 × 10(^8))</td>
</tr>
<tr>
<td>P</td>
<td>2.89 × 10(^6) (1.52 × 10(^8))</td>
<td>2.80 × 10(^6) (4.82 × 10(^8))</td>
</tr>
<tr>
<td>Control</td>
<td>9.62 × 10(^6) (8.40 × 10(^8))</td>
<td>2.07 × 10(^6) (3.03 × 10(^8))</td>
</tr>
<tr>
<td>Arc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCLOP</td>
<td>3.18 × 10(^6)</td>
<td>1.88 × 10(^7) (2.72 × 10(^7))</td>
</tr>
<tr>
<td>P</td>
<td>5.05 × 10(^6) (1.18 × 10(^7))</td>
<td>5.12 × 10(^6) (1.79 × 10(^7))</td>
</tr>
<tr>
<td>Control</td>
<td>3.58 × 10(^6)</td>
<td></td>
</tr>
<tr>
<td>Azo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCLOP</td>
<td>2.92 × 10(^6) (2.61 × 10(^8))</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
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</tbody>
</table>
conditions, consistent with findings of carbon-limited growth of estuarine bacteria (Hoikkala, Aamos and Lignell 2009; Hitchcock and Mitrovic 2013). The diazotrophic communities developing in all treatments showed dramatically different compositions than the indigenous community, presumably due to confinement. This is inherent in any laboratory manipulation and limits the direct transferability of our results to the natural conditions in Roskilde Fjord. Nevertheless, the high-carbon/low-oxygen conditions had substantial consequences for the composition of diazotrophs, for example through the loss of Pseudomonas-related diazotrophs, whereby the relative importance of Vibrio-, Azomonas- and Arcobacter-related taxa increased. Since especially Vibrio- and Azomonas-related taxa were found among the active diazotrophs, the overlap between present and active diazotrophs increased in the high-carbon/low-oxygen treatment. While the community that was active (based on cDNA) remained relatively stable, the relieved inhibition of BNF by oxygen and lack of energy resulted in active diazotrophs out-competing inactive ones, causing a more similar composition of present and active diazotrophic groups (Morisita–Horn = 0.46, Table 2). Hence, the results show that although the active diazotrophs commonly account for only a small proportion of the total putative diazotrophic community present in situ (e.g., Moisander et al. 2006; Man-Aharonovich et al. 2007; Zhang, Hurek and Reinhold-Hurek 2007), a significant fraction of planktonic heterotrophic diazotrophs may indeed express nitrogenase if exposed to conditions suitable for BNF.

It was surprising that no transcription was observed for Pseudomonas-related diazotrophs since active transcription was found at the sampled site in situ (Bentzon-Tilia et al. 2015a). We speculate that very specific environmental conditions are required for nitrogenase expression in this phyotype. The closest cultivated relative to the Pseudomonas-related sequence is a suspected contaminant (Turk et al. 2011), yet the low sequence similarity of 91% and the absence of this sequence in other of our samples imply that contamination is not likely to be the reason for the presence of this nifH sequence in the DNA samples from our experiment.

Results from the quantitative analysis of nifH copies and transcripts show that, surprisingly, only one of the three most abundant active diazotrophs, the Arcobacter-related diazotrophs, increased gene-specific nifH expression (nifH cDNA/DNA) in the high-carbon/low-oxygen treatment while the nifH cDNA/DNA ratio even decreased for Vibrio-related diazotrophs. It therefore seems that there is no uniform response among groups of heterotrophic diazotrophs to the introduction of environmental conditions assumed to be favourable for BNF. Arcobacter-related diazotrophs did not increase abundance of nifH copies in HCLOP but showed an increase in nifH transcripts in HCLOP (and in HCLOP only). Hence, this diazotroph regulated its activity through increased nifH expression. In contrast, the Vibrio-related diazotrophs showed increased nifH expression in P and HCLOP but increased abundance of nifH copies only in HCLOP, resulting in higher nifH transcript per copy ratios in P compared to HCLOP. Apparently, this diazotroph does not require high-carbon/low-oxygen conditions per se for nitrogenase activity. Nevertheless, the pronounced increase in abundance of this diazotroph due to carbon addition did cause a higher per volume expression of nifH and, presumably, also higher per volume nitrogenase activity.

We observed some mismatch between qPCR and sequencing results for the proportion of Vibrio- and Arcobacter-related nifH copies in the control samples, i.e. sequencing results suggest a higher relative abundance of Vibrio- than of Arcobacter-related nifH copies while the opposite is evidenced from the qPCR results. This might be due to preferential amplification of Vibrio-like sequences, as observed earlier for some nifH phyotypes (Turk et al. 2011), but is unlikely to be due to PCR contamination since these sequences are only distantly related to known PCR reagent contaminants (e.g., Zehr et al. 2003; Goto et al. 2005).

In the present study, we observed variable responses of key groups of heterotrophic diazotrophs to the introduction of environmental conditions presumed to be suitable for BNF. This illustrates the difficulty of predicting BNF by diverse diazotrophic assemblages in various environments, and highlights the need for an improved understanding of the autecology and ecophysiology of key heterotrophic diazotrophs in estuaries.

SUPPLEMENTARY DATA
Supplementary data are available at FEMSLE online.

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