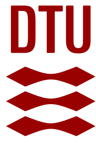
Downloaded from orbit.dtu.dk on: Nov 11, 2019



DTU Library

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

Severin, Ina; Bentzon-Tilia, Mikkel; Moisander, Pia H.; Riemann, Lasse

Published in:
F E M S Microbiology Letters

Link to article, DOI:
10.1093/femsle/fnv105

Publication date:
2015

Document Version
Peer reviewed version

Link back to DTU Orbit

Citation (APA):
Severin, I., Bentzon-Tilia, M., Moisander, P. H., & Riemann, L. (2015). Nitrogenase expression in estuarine bacterioplankton influenced by organic carbon and availability of oxygen. *F E M S Microbiology Letters*, *362*(14), [fnv105]. https://doi.org/10.1093/femsle/fnv105

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

FEMS Microbiology Letters Advance Access published July 6, 2015

1

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

4

5 Ina Severin[1,*], Mikkel Bentzon-Tilia[1], Pia H. Moisander[2], Lasse Riemann[1]

6

7 [1]Marine Biological Section, Department of Biology, University of Copenhagen, Helsingør, Denmark
8 [2]Department of Biology, University of Massachusetts Dartmouth, North Dartmouth, MA, USA

9

10 *Correspondence
11 Ina Severin, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 310,
12 12587 Berlin, Germany
13 Phone: 0049 30 64181 648
14 E-Mail: severin@igb-berlin.de

15

16 Present address
17 Ina Severin, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany.
18 Mikkel Bentzon-Tilia, Department of Systems Biology, Technical University of Denmark, Kgs.
19 Lyngby, Denmark

20

21 Keywords
22 Bacterial activity, limiting factors, nitrogen fixation

23

24 Running Title
25 Estuarine diazotrophs under DOC and oxygen manipulations

26

27

28 ABSTRACT

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

45

46

46

47 INTRODUCTION

48 Nitrogen transformations carried out by microorganisms include N-loss via denitrification and

49 anaerobic ammonium oxidation and N-gain via biological nitrogen fixation (BNF). In some N-

50 limited aquatic systems BNF may account for more than 80 % of the N input (e.g., Howarth *et al.*,

51 1988). Yet, due to the fact that cyanobacteria have long been considered the sole important N

52 fixing organisms (diazotrophs; e.g., Capone *et al.*, 1997; Zehr *et al.*, 2001; LaRoche & Breitbarth,

53 2005) and research has concentrated on areas where cyanobacteria are common, other N-limited

54 systems without cyanobacteria are believed to be free from BNF. However, it is now evident that

55 non-cyanobacterial diazotrophs are widespread and active in marine waters (e.g., Riemann *et al.*,

56 2010; Farnelid *et al.*, 2011; Moisander *et al.*, 2014). Due to their fundamentally different energy

57 acquisition, heterotrophic diazotrophs could fix N in underexplored marine regions, such as cold or

58 dark waters and coastal regions, and thereby significantly increase the known spatio-temporal

59 distribution of BNF. Limitations of heterotrophic BNF in open ocean waters would likely be low

60 concentrations of organic carbon and/or the lack of low oxygen loci for a process that is typically

61 impaired by the inhibition of nitrogenase at ambient oxygen concentrations. Heterotrophic

62 diazotrophs are indeed widespread in oligotrophic and well-oxygenated waters (e.g., Farnelid *et*

63 *al.*, 2011), but it has been argued that, at least in some oceanic regions, even abundant

64 heterotrophic diazotrophs cannot account for measured BNF due to their low cell-specific N

65 fixation (Turk-Kubo *et al.*, 2013). However, in a recent study with heterotrophic isolates from the

66 Baltic Sea we observed that higher cell-specific N fixation rates are possible in more productive

67 waters, indicating that abundances similar to those observed for certain phylotypes *in situ* could

68 account for low but measurable BNF (Bentzon-Tilia M, Severin I, Hansen LH, Riemann L,

69 unpublished).

70 Many studies of the *nifH* gene, which encodes the Fe-protein of nitrogenase, have only reported a

71 small overlap between *nifH* genes (DNA) and transcripts (mRNA) in a community (Moisander *et*

72 *al*., 2006; Zhang *et al*., 2006; Man-Aharonovich *et al*., 2007). This indicates that only a minute
73 proportion of all present diazotrophs is active at a given point in time and that their distribution may
74 not solely be dependent on selection based on diazotrophy (e.g., Short *et al*., 2004; Moisander *et*
75 *al*., 2007). Nevertheless, the wide distribution of *nifH* among planktonic *Bacteria* and *Archaea*
76 indicates a selective advantage of diazotrophy since only advantageous functional genes will be
77 fixed in the genome due to the high rate of random gene mutations (Berg & Kurland, 2002).
78 Therefore, marine *nifH* genes are conceivably expressed at least occasionally. Consequently, all
79 *nifH* genes in a given gene pool should theoretically be expressed under suitable conditions.
80
81 Here, we assumed that 'suitable' conditions for BNF by heterotrophic diazotrophs include low
82 oxygen conditions and sufficient energy (carbon), and hypothesized that these conditions would
83 increase the similarity of phylotype composition based on *nifH* DNA and complementary DNA
84 (cDNA). Hence, by experimental manipulation we sought to demonstrate that suitable conditions
85 would result in diazotrophy becoming an expressed phenotype among putative diazotrophs. We
86 subjected N-limited water from Roskilde Fjord (Denmark), where BNF is partially carried out by
87 heterotrophic diazotrophs (Bentzon-Tilia *et al*., 2014), to low oxygen/high carbon conditions and
88 assessed the present and active diazotrophic communities by high-throughput sequencing and
89 quantitative PCR targeting of the *nifH* genes and transcripts.
90
91
92 METHODS
93 *Experimental set-up*
94 Surface water (1 m) from Roskilde Fjord, Denmark (55°42.00'N, 12°04.46'E; 4.8 m total depth),
95 was sampled on June 13th, 2012 and transported to the laboratory in Helsingør where it was
96 distributed into 12 carboys (12 L volume each) and placed in a climate controlled room (approx.
97 19°C) under illumination (200 – 340 µE m^{-2} s^{-1}) according to an *in situ* day-night cycle. Treatment

98 'HCLOP' (high C, low O_2, P-addition) received a carbon mix (50 µmol L^{-1} final conc.; glucose,
99 galactose, mannitol, acetate and pyruvate) and phosphate (NaH_2PO_4, 1 µmol L^{-1} final conc.) to
100 ensure N-limited conditions, and was bubbled with an N_2/CO_2 gas mix (99.6 % N_2/0.4 % CO_2; Air
101 Liquide Danmark A/S). Treatment 'P' was amended with phosphate only to assess if N-limited
102 conditions alone affected the bacterial community (without high C and low O_2). The control carboys
103 received no nutrient amendments. 'P' and the control treatment were bubbled with filtered air.
104 Treatments were run in quadruplicates and incubated for 6 days. Temperature and oxygen
105 (oxygen meter oxi 3210, WTW Weilheim) were monitored daily. Samples for bacterial abundance
106 and production were collected daily. At the start of the experiment (*in situ*) as well as after 3 and 6
107 days, concentrations of dissolved organic carbon (DOC) and inorganic nutrients were determined.
108 After terminating the incubations (Day 6), samples for DNA and RNA were collected during the first
109 half of the light cycle. This may have resulted in the failure to detect *nifH* transcripts from
110 diazotrophs transcribing *nifH* during the night, such as heterocystous cyanobacteria. However,
111 since no *nifH* genes from these organisms were detected in the DNA samples, their absent *nifH*
112 gene expression was most likely not due to the chosen time of sampling.
113
114 *Background parameters*
115 *In situ* physicochemical data (TP: total phosphorus, PO_4 and TN: total nitrogen) were obtained
116 during a concurrent seasonal study of Roskilde Fjord (Bentzon-Tilia *et al*., 2014, Table S1). DOC
117 and dissolved organic nitrogen (DON) were measured using a TOC-V_{cph} analyzer (Shimadzu)
118 according to Cauwet (1999). TP and inorganic nitrogen concentrations (NO_2^- and NO_x after
119 reduction of NO_3^- to NO_2^-) were determined fluorometrically (Turner Trilogy Fluorometer, following
120 the protocol by Turner Designs), NH_4^+ was determined after Holmes *et al*. (1999). Concentrations
121 of NO_2^- and NO_3^- were below the detection limit (0.5 µM).
122 Bacterial abundance was determined from samples fixed with glutaraldehyde (1% final conc.)
123 using a FACSCanto™ II flowcytometer (BD Biosciences) according to Gasol & Del Giorgio (2000).

124 Bacterial production (BP) was estimated by ^{3}H-thymidine incorporation (20 nmol L^{-1} final
125 concentration, PerkinElmer; Fuhrman & Azam, 1982) using microcentrifugation (Smith & Azam,
126 1992), and 1.1 × 10^{18} cells mol^{-1} (Riemann *et al.*, 1987) and 2 × 10^{-14} g C $cell^{-1}$ (Lee & Fuhrman,
127 1987).
128
129 *Diazotrophic community composition*
130 At Day 6, 700 mL of water was pre-filtered through a 10 µm polycarbonate filter (GE Water &
131 Process Technologies) and then onto a Sterivex-PG filter (0.22 µm, EMD Millipore). Separate
132 filters were taken for DNA and RNA. Filters were immediately frozen at -80 ºC until extraction of
133 DNA and RNA using the EZNA Tissue DNA Purification kit and the Total RNA kit I (Omega Bio-
134 Tek), respectively. The RNA extraction included an extra on-column DNase step (EZNA RNase
135 free DNase). cDNA was synthesized using TaqMan reverse transcription reagents (Applied
136 Biosystems) and the nifH3 reverse primer (Zehr & McReynolds, 1998). DNase-treated, but not
137 reverse-transcribed, template was included to check for presence of genomic DNA in the RNA
138 template, but these never amplified in PCR.
139 *nifH* was amplifiable from 12 DNA and 14 cDNA templates, corresponding to quadruplicate DNA
140 samples for 'HCLOP', triplicates for 'P' and the control, and 2 negative controls (extraction control
141 and PCR control) as well as quadruplicate RNA samples for all treatments and 2 negative controls
142 (extraction control and PCR control). Amplifications were carried out using 5-10 ng of DNA or
143 cDNA template, Pure Taq Ready-To-Go PCR Beads (GE Healthcare) and a nested PCR protocol
144 (Zehr & Turner, 2001). Illumina indices (Table S2) were added to the amplicons in the second
145 PCR round. Three to four technical PCR replicates were run per sample, pooled, purified (AMPure
146 XP purification kit; Beckman Coulter Inc.), and quantified (PicoGreen; Invitrogen). The negative
147 controls did not show bands when checked on the gel, but volumes similar to those of the samples
148 were included in the following steps nonetheless. 75 ng of purified PCR product per sample were
149 pooled and subjected to one run of paired-end sequencing on the Illumina MiSeq platform at the

150 Berlin Center for Genomics in Biodiversity Research. Sequence data are deposited in MG-RAST
151 (Meyer *et al*., 2008) under accession numbers 4565187.3 – 4565230.3
152 (http://metagenomics.anl.gov/linkin.cgi?project=9299). Sequences characterizing the *in situ*
153 diazotrophic community composition were obtained from Bentzon-Tilia *et al*. (2014).
154 Demultiplexed reads were assembled in Mothur v. 1.32.0 (Schloss *et al*., 2009), trimmed to 250 nt,
155 and screened for ambiguous basecalls. Sequences that failed the quality filtering and aligned
156 poorly to the *nifH* reference database on fungenes (http://fungene.cme.msu.edu/) were excluded.
157 Chimeras were removed using UCHIME and sequences were clustered at 97 % nucleotide
158 sequence similarity. After removal of singletons, an average of 3731 sequences per sample (min =
159 916, max = 6004) was used for further analyses. The negative controls did not yield any
160 sequences. Representative sequences from the most abundant operational taxonomic units
161 (OTUs) were blasted against the NCBI nucleotide database. Based on the OTU-tables, pairwise
162 Morisita-Horn dissimilarities were estimated between all samples in R version 3.0.2 (R
163 Development Core Team, 2008) using the vegan package 2.0-10 (Oksanen *et al*., 2012). Pairwise
164 dissimilarities were averaged to determine the degree of difference between the treatments and
165 between the present and active community for a certain treatment (values between 0 and 1 show
166 identical and absolutely different diazotrophic community composition, respectively). SIMPER
167 (Similarity Percentage) was run in PAST (version 3.01, Hammer *et al*., 2001) to identify the OTUs
168 responsible for the difference in community composition.
169 In order to quantify *nifH* genes and transcripts, 3 quantitative PCR (qPCR) assays were designed
170 based on the dominant cDNA clusters (Table 1), targeting 2 gammaproteobacterial ('Vib' and
171 'Azo') and 1 epsilonproteobacterial ('Arc') cluster, accounting for 72 % of the obtained sequences.
172 Specific primers were designed using Primer3 (v 0.4.0 online resource (http://bioinfo.ut.ee/primer3-
173 0.4.0/, Table S3) and presence of hairpins and dimers was checked (NetPrimer; PREMIER
174 Biosoft). Specificity was verified *in silico* (Primer-BLAST; NCBI) and by testing amplification of non-
175 target sequences (the other 2 standards and DNA from three isolates: *Raoultella ornithinolytica*,

176 *Pseudomonas stutzeri*, and *Rhodopseudomonas palustris* (accession numbers AY972875,
177 KC140355, and KC140365, respectively). Those non-target sequences never yielded qPCR
178 products. The PCR standards were synthesized commercially (GenScript), linearized by Hind*III*
179 digestion, and used in triplicate 10-fold dilution series ranging from 10^1 to 10^7 copies. Two of the
180 standards (10^3 and 10^6 copies) were also spiked with sample template to exclude potential
181 inhibition. No changes in amplification efficiencies were detected. The 20 μL reaction mixtures
182 contained 1 × SYBR© Select Master Mix (Life Technologies Europe BV), 300 nM of each primer,
183 RT-PCR grade water, and 2 µl template. Amplification efficiencies were 70 % for one assay ('Azo')
184 and 100 % for the other assays ('Vib' and 'Arc'). Specificity was confirmed by melt curve analyses.
185
186 *Statistics*
187 Tests for normality and equality of variances of data as well as One-way ANOVAs and Tukey's
188 post hoc tests on normally distributed data were run in PAST (version 3.01, Hammer *et al.*, 2001).
189
190
191 RESULTS
192 *Background parameters*
193 Oxygen saturation in 'HCLOP' was 16 % and 10 % after 3 and 6 days, respectively, while the air-
194 bubbled carboys were fully oxygenated (Table S1). One-way ANOVAs showed no difference in
195 NH_4^+ between treatments at the same time point but a decrease over time for 'P' (Tukey's post
196 hoc, $p < 0.01$). 'HCLOP' showed an increase in DON ($p < 0.01$) and a decrease in DOC ($p < 0.01$)
197 over time. Moreover, due to the C-addition, DOC concentrations were higher in 'HCLOP' ($p < 0.01$)
198 compared to 'P' and the control.
199
200 *Bacterial abundance and activity*

201 ‘P’ and the control treatment showed similar bacterial abundances over time (Fig. 1A). ‘HCLOP’
202 showed no lag phase and reached highest abundances after 2 days. Maximum and minimum
203 abundance was higher in ‘HCLOP’ than in ‘P’ and the control treatment. ‘HCLOP’ had significantly
204 higher BP than ‘P’ and the control after 3 and 6 days ($p < 0.01$; Fig. 1B), probably due to an
205 increase in abundance caused by the addition of C. BP showed an increasing trend in ‘HCLOP’
206 and a decreasing trend in ‘P’ and the control. There was, however, no significant difference in cell-
207 specific BP between treatments after 6 days (not shown).
208
209 *Diazotrophic community composition*
210 In total more than 2700 *nifH* OTUs were obtained from all samples at the end of the experiment
211 (Day 6, details given in Table S4). The 9 most abundant OTUs (containing 455 to 25644
212 sequences each) were exclusively affiliated with proteobacterial diazotrophs (Table 1). Although
213 the same 9 OTUs were most abundant in all treatments at the end of the experiment and
214 accounted for 84 – 90 % of the sequences, their relative abundances showed pronounced
215 variation (Fig. 2). SIMPER analysis showed that the difference in abundance of two or more of
216 these OTUs caused more than 75 % of the difference in diazotrophic community composition
217 between treatments.
218 Pairwise Morisita-Horn dissimilarities (averages of treatment replicates) between treatments as
219 well as between *in situ* and any of the treatments were calculated for the present and active
220 diazotrophic community, respectively (Table 2A). This shows that i) the present and active *in situ*
221 diazotrophic community was dissimilar from any of the treatments, ii) the present diazotrophic
222 communities in ‘P’ and the control were very similar, and dissimilar to ‘HCLOP’, iii) the active
223 diazotrophic community in ‘HCLOP’ was more similar to that of ‘P’ and the control than the present
224 diazotrophic community of these treatments. Moreover, pairwise Morisita-Horn dissimilarities
225 (averages of treatment replicates) between the present and the active diazotrophic communities *in*

226 *situ* and in all treatments (Table 2B) show that more of the present diazotrophs were active in
227 'HCLOP' (lower Morisita-Horn dissimilarity).
228 None of the most abundant OTUs had close cultivated relatives (Table 1). The
229 gammaproteobacterial OTUs 1, 3, 7, 8 and 9 (representing 43 % of all sequences) showed 84-88
230 % similarity (at the nucleotide level) with *Vibrio* species. No perfect matches for these *Vibrio*-like
231 sequences were found in the database. Uncultured *nifH*-sequences found in the temperate
232 Northwest Pacific showed only slightly higher sequence similarities (92 %) to OTUs 3 and 7 than
233 those given for the most closely related identified sequence. They were more common in cDNA
234 than in DNA, and in DNA of 'HCLOP' compared to 'P' and the control. The same pattern was
235 observed for OTU 6 (1 % of all sequences), an epsilonproteobacterial phylotype most closely
236 related to *Arcobacter*. No perfect matches for this sequence were found in the database. The
237 gammaproteobacterial OTUs 2 and 5 (27 % of all sequences) clustered with the genus *Azomonas*
238 and showed 99 % similarity to *nifH* sequences found in the *Zostera marina* rhizosphere and
239 marine sediments. They were more abundant in cDNA than in DNA in all treatments. The
240 gammaproteobacterial OTU 4 (11 % of all sequences) was most closely related to *Pseudomonas*
241 *stutzeri* and was mainly found in DNA samples, especially in 'P' and the control. Identical *nifH*
242 sequences were also found in the South China Sea, the Baltic Sea, and the tropical eastern North-
243 Atlantic.
244 Quantitative PCR on the most abundant OTUs in the cDNA showed that for *Vibrio*- and
245 *Arcobacter*-like organisms, *nifH* expression (L^{-1}) was higher in 'HCLOP' than in 'P' and the control
246 (Table 3). *nifH* expression in *Vibrio*-like phylotypes was more than one order of magnitude higher
247 in 'HCLOP' than in 'P' and two orders of magnitude higher than in the control. However, *Vibrio*-like
248 *nifH* genes were also more abundant in 'HCLOP' than in 'P' and the control. Consequently, the
249 ratio of *Vibrio*-like *nifH* transcripts to copies (cDNA/DNA) was highest in 'P' compared to the
250 control and 'HCLOP'. The abundance of the *Arcobacter*-like diazotrophs was similar in 'HCLOP'
251 and the control while no *Arcobacter*-like *nifH* copies could be detected in 'P'. *Arcobacter*-like *nifH*

252 expression was similar in 'P' and the control, being one order of magnitude lower than in 'HCLOP',
253 i.e. the ratio of *nifH* transcripts to copies was approximately 4 times higher in 'HCLOP' than in the
254 control. *Azomonas*-like transcripts could only be detected in 'HCLOP'. However, due to a low
255 reaction efficiency of this assay the absence of *nifH* transcripts and copies in the other treatments
256 does not necessarily mean that none were present.
257
258
259 DISCUSSION
260 Our aim was to test whether it is possible to experimentally increase the proportion of active
261 diazotrophs of the total diazotrophic population in estuarine environments. Especially when
262 considering heterotrophic nitrogen fixation, low oxygen loci and energy are likely two limiting
263 factors (e.g., Riemann *et al*., 2010). To that end, we added a mix of labile carbon substrates and
264 reduced oxygen saturation. Although Roskilde Fjord is known to be N-limited in summer, we also
265 tested if further N-limitation could be induced by phosphate addition. This alone had, however, no
266 measurable effect on either total bacterial growth or production, or diazotrophic community
267 composition. Hence, the addition of phosphate *per se* did not interfere with our assessment of the
268 effect of low oxygen/high carbon conditions. However, phosphate addition had an effect on the
269 ratio of *nifH* transcripts/copy in *Vibrio*-like diazotrophs, as discussed below. Bacterial abundance
270 and production generally increased under low oxygen/high carbon conditions, consistent with
271 findings of carbon-limited growth of estuarine bacteria (Hoikkala *et al*., 2009; Hitchcock *et al*.,
272 2013). The diazotrophic communities developing in all treatments showed dramatically different
273 compositions than the indigenous community, presumably due to confinement. This is inherent in
274 any laboratory manipulation and limits the direct transferability of our results to the natural
275 conditions in Roskilde Fjord. Nevertheless, the high carbon/low oxygen conditions had substantial
276 consequences for the composition of diazotrophs; for example through the loss of *Pseudomonas*-
277 related diazotrophs, whereby the relative importance of *Vibrio*-, *Azomonas*- and *Arcobacter*-related

278 taxa increased. Since especially *Vibrio*- and *Azomonas*-related taxa were found among the active
279 diazotrophs, the overlap between present and active diazotrophs increased in the high carbon/low
280 oxygen treatment. While the community that was active (based on cDNA) remained relatively
281 stable, the relieved inhibition of BNF by oxygen and lack of energy resulted in active diazotrophs
282 outcompeting inactive ones, causing a more similar composition of present and active diazotrophic
283 groups (Morisita-Horn = 0.46, Table 2). Hence, the results show that although the active
284 diazotrophs commonly account for only a small proportion of the total putative diazotrophic
285 community present *in situ* (e.g., Moisander *et al*., 2006; Zhang *et al*., 2006; Man-Aharonovich *et*
286 *al*., 2007), a significant fraction of planktonic heterotrophic diazotrophs may indeed express
287 nitrogenase if exposed to conditions suitable for BNF.
288
289 It was surprising that no transcription was observed for *Pseudomonas*-related diazotrophs since
290 active transcription was found at the sampled site *in situ* (Bentzon-Tilia *et al*., 2015). We speculate
291 that very specific environmental conditions are required for nitrogenase expression in this
292 phylotype. The closest cultivated relative to the *Pseudomonas*-related sequence is a suspected
293 contaminant (Turk *et al*., 2011), yet, the low sequence similarity of 91 % and the absence of this
294 sequence in other of our samples imply that contamination is not likely to be the reason for the
295 presence of this *nifH* sequence in the DNA samples from our experiment.
296
297 Results from the quantitative analysis of *nifH* copies and transcripts show that, surprisingly, only
298 one of the three most abundant active diazotrophs, the *Arcobacter*-related diazotrophs, increased
299 gene-specific *nifH* expression (*nifH* cDNA/DNA) in the high carbon/low oxygen treatment while the
300 *nifH* cDNA/DNA ratio even decreased for *Vibrio*-related diazotrophs. It therefore seems that there
301 is no uniform response among groups of heterotrophic diazotrophs to the introduction of
302 environmental conditions assumed to be favourable for BNF. *Arcobacter*-related diazotrophs did
303 not increase abundance of *nifH* copies in HCLOP but showed an increase in *nifH* transcripts in

304 HCLOP (and in HCLOP only). Hence, this diazotroph regulated its activity through increased *nifH*
305 expression. In contrast, the *Vibrio*-related diazotrophs showed increased *nifH* expression in P and
306 HCLOP but increased abundance of *nifH* copies only in HCLOP, resulting in higher *nifH* transcript
307 per copy ratios in P compared to HCLOP. Apparently, this diazotroph does not require high
308 carbon/low oxygen conditions *per se* for nitrogenase activity. Nevertheless, the pronounced
309 increase in abundance of this diazotroph due to carbon addition did cause a higher per volume
310 expression of *nifH* and, presumably, also in higher per volume nitrogenase activity.
311 We observed some mismatch between quantitative PCR and sequencing results for the proportion
312 of *Vibrio*- and *Arcobacter*-related *nifH* copies in the control samples, i.e. sequencing results
313 suggest a higher relative abundance of *Vibrio*- than of *Arcobacter*-related *nifH* copies while the
314 opposite is evidenced from the quantitative PCR results. This might be due to preferential
315 amplification of *Vibrio*-like sequences, as observed earlier for some *nifH* phylotypes (Turk *et al.,*
316 2011), but is unlikely to be due to PCR contamination since these sequences are only distantly
317 related to know PCR reagent contaminants (e.g., Zehr *et al*., 2003; Goto *et al*., 2005).
318
319 In the present study we observed variable responses of key groups of heterotrophic diazotrophs to
320 the introduction of environmental conditions presumed to be suitable for BNF. This illustrates the
321 difficulty of predicting BNF by diverse diazotrophic assemblages in various environments, and
322 highlights the need for an improved understanding of the autecology and ecophysiology of key
323 heterotrophic diazotrophs in estuaries.
324
325
326 ACKNOWLEDGEMENTS
327 We thank Jeanett Hansen and Patrick J. Kearns for support with sampling and analyses, and two
328 anonymous reviewers for constructive comments. This work was supported by grants 09-066396

329 and 11-105450 from The Danish Council for Independent Research, Natural Sciences to L.R., and
330 1130495 from NSF OCE to P.H.M.
331
332
333 REFERENCES
334
335 Bentzon-Tilia M, Traving SJ, Mantikci M, Knudsen-Leerbeck H, Hansen JLS, Markager S,
336 Riemann L (2015) Significant N_2 fixation by heterotrophs, photoheterotrophs and heterocystous
337 cyanobacteria in two temperate estuaries. *ISME* **9**: 273-285
338
339 Berg OG, Kurland CG (2002) Evolution of microbial genomes: sequence acquisition and loss. *Mol*
340 *Biol Evol* **19**: 2265-2276
341
342 Capone DG, Zehr JP, Paerl HW, Bergman B, Carpenter EJ (1997) *Trichodesmium*, a globally
343 significant marine cyanobacterium. *Science* **176**: 1221-1229
344
345 Cauwet G (1999) Determination of dissolved organic carbon (DOC) and nitrogen (DON) by high
346 temperature combustion. *Methods of Seawater Analyses* (Grasshoff K, Kremling K, Erhard M,
347 eds), pp 407–420. Wiley-VCH
348
349 Farnelid H, Andersson AF, Bertilsson S, Abu Al-Soud W, Hansen LH, Sørensen S, Steward GF,
350 Hagström Å, Riemann L (2011) Nitrogenase gene amplicons from global marine surface waters
351 are dominated by genes of non-cyanobacteria. *PLOS ONE* **6**: doi: 10.1371/journal.pone.0019223
352

353 Fuhrman JA, Azam F (1982) Thymidine incorporation as a measure of heterotrophic
354 bacterioplankton production in marine surface waters – evaluation and field results. *Mar Biol* **66**:
355 109-120
356
357 Gasol JM, DelGiorgio PA (2000) Using flow cytometry for counting natural planktonic bacteria and
358 understanding the structure of planktonic bacteria communities. *Sci Mar* **64**: 197-224
359
360 Goto M, Ando S, Hachisuka Y, Yoneyama T (2005) Contamination of diverse *nifH* and *nifH*-like
361 DNA into commercial PCR primers. *FEMS Microbiol Lett* **246**: 33–38.
362
363 Hammer Ø, Harper DAT, Ryan PD (2001) PAST: Paleontological statistics software package for
364 education and data analysis. Palaeontologia Electronica 4: http://palaeo-
365 electronica.org/2001_1/past/issue1_01.htm
366
367 Hitchcock JN, Mitrovic SM (2013) Different resource limitation by carbon, nitrogen and phosphorus
368 between base flow and high flow conditions for estuarine bacteria and phytoplankton. *Estuar*
369 *Coast Shelf S* **135**: 106-115
370
371 Hoikkala L, Aamos H, Lignell R (2009) Changes in nutrient and carbon availability and
372 temperature as factors controlling bacterial growth in the Northern Baltic Sea. *Estuar Coast* **32**:
373 720-733
374
375 Holmes RM, Aminot A, Kérouel R, Hooker BA, Peterson BJ (1999) A simple and precise method
376 for measuring ammonium in marine and freshwater ecosystems. *Can J Fish Aquat Sci* **56**: 1801-
377 1808
378

379 Howarth RE, Marino R, Lane J, Cole LL (1988) Nitrogen-fixation in fresh-water, estuarine, and
380 marine ecosystems. 1. Rates and importance. *Limnol Oceanogr* **33**: 669-687
381
382 LaRoche J, Breitbarth E (2005) Importance of the diazotrophs as a source of new nitrogen in the
383 ocean. *J Sea Res* **53**: 67-91
384
385 Lee S, Fuhrman JA (1987) Relationships between biovolume and biomass of naturally derived
386 marine bacterioplankton. *Appl Environ Microb* **53**: 1298-1303
387
388 Man-Aharonovich D, Kress N, Bar Zeev E, Berman-Frank I, Beja O (2007) Molecular ecology of
389 *nifH* genes and transcripts in the eastern Mediterranean Sea. *Environ Microbiol* **9**: 2354-2363
390
391 Meyer F, Paarman D, D'Souza M *et al.* (2008) The metagenomic RAST server – a public resource
392 for the automatic phylogenetic and functional analysis of metagenomes. *MBC Bioinf* **9**: 386-393
393
394 Moisander PH, Morrison AE, Ward BB, Jenkins BD, Zehr JP (2007) Spatial-temporal variability in
395 diazotroph assemblages in Chesapeake Bay using an oligonucleotide *nifH* microarray. *Environ*
396 *Microbiol* **9**: 1823-1835
397
398 Moisander PH, Serros T, Paerl RW, Beinart RA, Zehr JP (2014) Gammaproteobacterial
399 diazotrophs and *nifH* gene expression in surface waters of the South Pacific Ocean. *ISME J* **8**:
400 1962-1973
401
402 Moisander PH, Shiue L, Steward GF, Jenkins BD, Bebout BM, Zehr JP (2006) Application of a
403 *nifH* oligonucleotide microarray for profiling diversity of N_2 fixing microorganisms in marine
404 microbial mats. *Environ Microbiol* **8**: 1721-1735

405

406 Moisander PH, Beinart RA, Hewson I, White AE, Johnson KS, Carlson CA, Montoya JP, Zehr JP
407 (2010) Unicellular cyanobacterial distributions broaden the oceanic N_2 fixation domain. *Science*
408 **327**: 1512-1514
409

410 Oksanen J, Guillaume BF, Kindt R *et al.* (2012) vegan: Community Ecology Package. R package
411 version 2.0-4. http://vegan.r-forge.r-project.org/.
412

413 R Development Core Team (2008) R: A language and environment for statistical computing. R
414 Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-
415 project.org.
416

417 Riemann L, Farnelid H, Steward GF (2010) Nitrogenase genes in non-cyanobacterial plankton:
418 prevalence, diversity and regulation in marine waters. *Aquat Microb Ecol* **61**: 225-237
419

420 Riemann B, Bjørnsen PK, Newell S, Fallon R (1987) Calculation of Cell Production of Coastal
421 Marine Bacteria Based on Measured Incorporation of [^{3}H]thymidine. *Limnol Oceanogr* **32**: 471-476
422

423 Schloss PD, Westcott SL, Ryabin T et al. (2009) Introducing mothur: open-source, platform-
424 independent, community-supported software for describing and comparing microbial communities.
425 *Appl Environ Microb* **75**: 7537-7541
426

427 Short SM, Jenkins BD, Zehr JP (2004). Spatial and temporal distribution of two diazotrophic
428 bacteria in the Chesapeake Bay. *Appl Environ Microb* **70**: 2186-2192
429

430 Smith DC, Azam F (1992) A simple, economical method for measuring bacterial protein synthesis
431 rates in seawater using tritiated-leucine. *Mar Microb Food Webs* **6**: 107-114
432
433 Turk KA, Rees AP, Zehr JP, Pereira N, Swift P, Shelley R, Lohan M, Woodward EMS, Gilbert J
434 (2011) Nitrogen fixation and nitrogenase (nifH) expression in tropical waters of the eastern North
435 Atlantic. *ISME J* **5**: 1201-1212
436
437 Turk-Kubo KA, Karamchandani M, Capone DG, Zehr JP (2014) The paradox of marine
438 heterotrophic nitrogen fixation: abundance of heterotrophic diazotrophs do not account for nitrogen
439 fixation rates in the Eastern Tropical South Pacific. *Environ Microbiol* **16**: 3095-3114
440
441 Zehr JP, Crumbliss LL, Church MJ, Omoregie EO, Jenkins BD (2003) Nitrogenase genes in PCR
442 and RT-PCR reagents: implications for studies of diversity of functional genes. *Bio Techniques* **35**:
443 996–1005
444
445 Zehr JP, McReynolds LA (1989) Use of degenerate oligonucleotides for amplification of the *nifH*
446 gene from the marine cyanobacterium *Trichodesmium thiebautii*. *Appl Environ Microb* **55**: 2522-
447 2526
448
449 Zehr JP, Turner PJ (2001) Nitrogen fixation: nitrogenase genes and gene expression. *Methods in*
450 *microbiology* (Paul JH, ed.), pp. 271-285. Academic Press, NY
451
452 Zehr JP, Waterbury JB, Turner PJ, Montoya JP, Omoregie E, Steward GF, Hansen A, Karl DM
453 (2001) Unicellular cyanobacteria fix N_2 in the subtropical North Pacific Ocean. *Nature* **412**: 635-
454 638
455

456 Zhang L, Hurek T, Reinhold-Hurek B (2006) A *nifH*-based oligonucleotide microarray for functional
457 diagnostics of nitrogen-fixing microorganisms. *Microb Ecol* **53**: 456-470
458 TABLES
459 **Table 1** The 9 most abundant OTUs in DNA and cDNA across all samples at the end of the
460 incubations (Day 6), with decreasing relative abundance from the top. Given are the OTU number
461 (assigned by Mothur), the most closely related cultivated diazotroph in the NCBI GenBank
462 database, the phylogenetic affiliation (class level), the nucleotide sequence similarity (%), the
463 accession number of the closest cultivated representative, the relative abundance in the entire
464 DNA and cDNA dataset (%), and the OTUs targeted by qPCR.
465

OTU	closest cultivated diazotroph on NCBI	phylogenetic affiliation	seq sim (%)	NCBI accession no	rel abundance DNA (%)	rel abundance cDNA (%)	qPCR assay
1	*Vibrio parahaemolyticus* strain 22702	Gammaproteobacteria	88	EF203422.1	18	28	'Vib'
3	*Vibrio parahaemolyticus* strain 22702		87	EF203422.1	5	24	
7	*Vibrio parahaemolyticus* strain 22702		88	EF203422.1	0	1	
8	*Vibrio porteresiae* strain MSSRF30		85	EF554362.1	2	0	
9	*Vibrio diazotrophicus*		84	AF111110.2	0	0	
2	*Azomonas macrocytogenes*	Gammaproteobacteria	85	AY644349.1	1	23	'Azo'
5	*Azomonas macrocytogenes*		84	AY644349.1	0	9	
4	*Pseudomonas stutzeri* CMT.9.A	Gammaproteobacteria	91	AF117978.1	61	0	
6	*Arcobacter nitrofigilis* clone CC1097A1	Epsilonproteobacteria	91	AY221825.1	1	1	'Arc'

466
467

467 **Table 2** Averaged pairwise Morisita-Horn dissimilarities between treatments based on DNA and

468 cDNA of each treatment (A) and between DNA and cDNA (B).

469

470 **A**

	DNA	cDNA
in situ vs HCLOP	1	1
in situ vs P	1	1
in situ vs control	1	1
HCLOP *vs* P	0.66	0.46
HCLOP *vs* control	0.69	0.45
P *vs* control	0.01	0.46

471

472 **B**

DNA *vs* cDNA	
in situ	1
HCLOP	0.46
P	0.93
control	0.96

473

474

474

475 **Table 3** *nifH* gene copies and transcripts as assessed by quantitative PCR targeting *Vibrio*- (Vib),

476 *Arcobacter*- (Arc), and *Azomonas*- (Azo) like diazotrophs, see Table 1. Standard deviations are

477 given in parentheses.

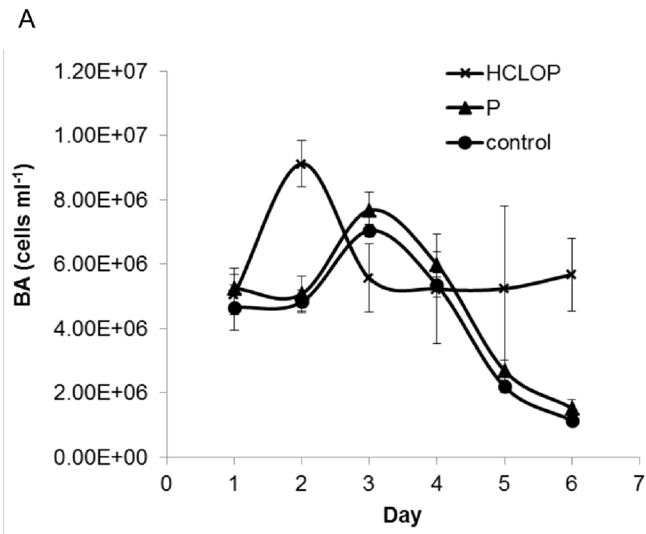
		gene copies L^{-1}	transcripts L^{-1}	transcripts per gene copy
Vib	HCLOP	7.58×10^{9} (1.05×10^{10})	8.90×10^{9} (1.66×10^{10})	1.17
	P	2.89×10^{6} (1.52×10^{6})	2.80×10^{8} (4.82×10^{8})	97.01
	control	9.62×10^{5} (8.40×10^{5})	2.07×10^{7} (3.03×10^{7})	21.48
Arc	HCLOP	3.18×10^{6}	1.88×10^{7} (2.72×10^{7})	5.92
	P		5.05×10^{6} (1.18×10^{6})	
	control	3.58×10^{6}	5.12×10^{6} (1.79×10^{6})	1.43
Azo	HCLOP	2.92×10^{8} (2.61×10^{8})		
	P			
	control			

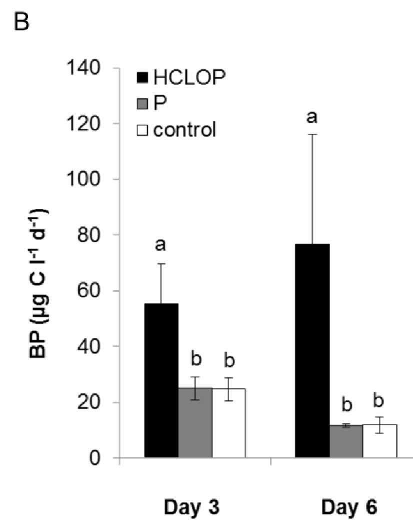
478

479

479

480 ## FIGURE LEGENDS





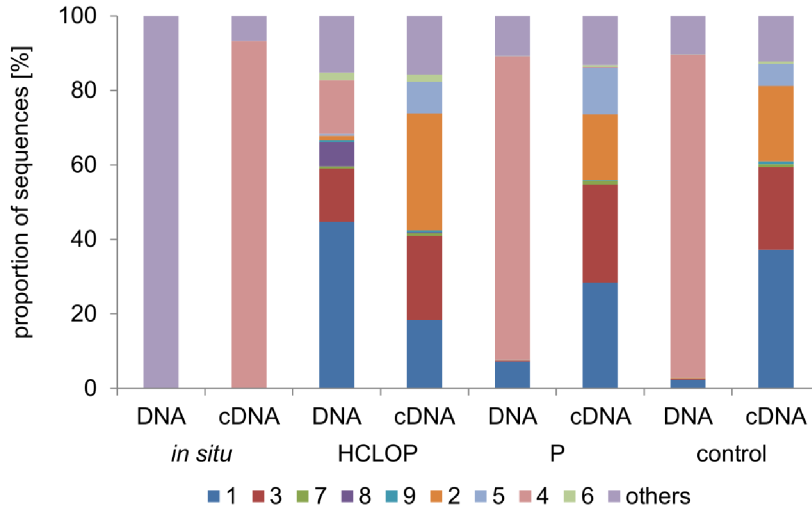
481

482 **Fig. 1** Mean bacterial abundance (A) and production (B). Error bars denote the standard deviation

483 for four treatment replicates. Bars with different letters in (B) denote significant differences

484 between treatments, assessed from Tukey's post-hoc test in one-way ANOVAs.

485



485

486 **Fig. 2** Diazotrophic community composition on Day 6 as assessed by *nifH* Illumina sequencing.

487 Bar charts represent the relative proportion of *nifH* sequences belonging to the 9 most abundant

488 OTUs, with all the rest of the OTUs pooled to 'others' (OTU number and affiliation as listed in

489 Table 1). Numbers are means of three to four treatment averages.

490

491