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# Revisiting N<sub>2</sub> fixation in Guerrero Negro intertidal microbial mats with a functional single-cell approach

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2 **Revisiting N<sub>2</sub> fixation in Guerrero Negro intertidal microbial mats with a functional**  
3 **single-cell approach**

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33 Running title: Single-cell analysis of diazotrophs in mats

34 Subject Category: Microbial ecology and functional diversity of natural habitats

35 **Abstract**

36           Photosynthetic microbial mats are complex, stratified ecosystems in which high rates  
37 of primary production creates a demand for nitrogen, met partially by N<sub>2</sub> fixation.  
38 Dinitrogenase reductase (*nifH*) genes and transcripts from *Cyanobacteria* and heterotrophic  
39 bacteria (e.g. *Deltaproteobacteria*) are detected in these mats, yet their contribution to N<sub>2</sub>  
40 fixation is poorly understood. We used a combined approach of manipulation experiments  
41 with inhibitors, *nifH* sequencing and single-cell isotope analysis to investigate the active  
42 diazotrophic community in intertidal microbial mats at Laguna Ojo de Liebre near Guerrero  
43 Negro, Mexico. Acetylene reduction assays with specific metabolic inhibitors suggested that  
44 both sulfate reducers and *Cyanobacteria* contributed to N<sub>2</sub> fixation, while <sup>15</sup>N<sub>2</sub> tracer  
45 experiments at the bulk level only supported a contribution of *Cyanobacteria*. Cyanobacterial  
46 and *nifH* cluster III (including deltaproteobacterial sulfate reducers) sequences dominated the  
47 *nifH* gene pool, while the *nifH* transcript pool was dominated by sequences related to  
48 *Lyngbya* spp.. NanoSIMS-based single-cell isotope analysis of <sup>15</sup>N<sub>2</sub>-incubated mat samples  
49 revealed that *Cyanobacteria* were enriched in <sup>15</sup>N with the highest enrichment being detected  
50 in *Lyngbya* spp. filaments (on average 4.4 at% <sup>15</sup>N), whereas the *Deltaproteobacteria*  
51 (identified by CARD-FISH) were close to natural abundance. We investigated the potential  
52 dilution effect from CARD-FISH on the isotopic composition and concluded that the dilution  
53 bias was not substantial enough to influence our conclusions. Our combined data provides  
54 evidence that *Cyanobacteria*, especially *Lyngbya* spp., actively contributed to N<sub>2</sub> fixation in  
55 the intertidal mats, while we could not find support for N<sub>2</sub> fixation activity of targeted  
56 deltaproteobacterial sulfate reducers.

57

58 Keywords: *Cyanobacteria*, microbial mat, N<sub>2</sub> fixation, NanoSIMS, *nifH*, sulfate reducing  
59 bacteria

60

61 **Introduction**

62 Microbial mats can be generally characterized as laminated ecosystems at the  
63 millimeter scale that harbor a diverse array of biogeochemical processes (Des Marais, 2003).  
64 Two important ecophysiological processes in photosynthetic microbial mats are primary  
65 production (CO<sub>2</sub> fixation) and the conversion of N<sub>2</sub> to NH<sub>3</sub> (N<sub>2</sub> fixation). High CO<sub>2</sub> fixation  
66 activity creates a great demand for nitrogen (N), which is met by high rates of N<sub>2</sub> fixation  
67 (Herbert, 1999). Therefore, it is hypothesized that microbial mat development is dependent  
68 on the activity of N<sub>2</sub>-fixing organisms (Bergman *et al.*, 1997).

69 Microbial mats inhabiting the intertidal zone from Laguna Ojo de Liebre  
70 (Supplementary Figures 1 and 2) close to Guerrero Negro, Baja California Sur, are visually  
71 dominated by *Lyngbya* spp.. These mats experience frequent alternating periods of  
72 desiccation (and thereby aeration) and tidal flooding (Rothrock & Garcia-Pichel, 2005;  
73 Omoregie *et al.*, 2004b) and are subject to frequent physical disruption by tidal forces and  
74 wind. This environment leads to a “pioneering stage” of habitat colonization, where N<sub>2</sub>  
75 fixation is an important process that provides N for mat growth (Bebout *et al.*, 1994).

76 Although N<sub>2</sub> fixation has previously been investigated in the intertidal mats of Laguna  
77 Ojo de Liebre (Bebout *et al.*, 1993; Omoregie *et al.*, 2004a; Omoregie *et al.*, 2004b), the  
78 identity of the active N<sub>2</sub> fixers remains elusive. Historically, *Cyanobacteria* were believed to  
79 be responsible for N<sub>2</sub> fixation in microbial mats given their visually dominance and  
80 cultivation without an exogenous N source (Paerl *et al.*, 1991; Stal & Bergman, 1990; Stal &  
81 Krumbein, 1981). This was further supported by biogeochemical assays using an inhibitor of  
82 oxygenic photosynthesis (DCMU) (Bebout *et al.*, 1993; Stal *et al.*, 1984). However,  
83 molecular methods, primarily focussed on the dinitrogenase reductase (*nifH*) gene, indicated  
84 that additional microorganisms present in microbial mats have the genetic potential for N<sub>2</sub>  
85 fixation (Zehr *et al.*, 1995). Molecular surveys suggest that heterotrophic bacterial

86 diazotrophs may play an important role in microbial mat N<sub>2</sub> fixation, due to the abundance  
87 and diversity of their *nifH* genes detected in these systems (Steppe *et al.*, 1996; Zehr *et al.*,  
88 1995). In particular, sulfate reducing bacteria (SRB) are hypothesized to contribute to N<sub>2</sub>  
89 fixation in microbial mats (Steppe & Paerl, 2002). Earlier studies of the Laguna Ojo de  
90 Liebre intertidal mats, combining biogeochemical and molecular assays, were unable to  
91 detect *nifH* genes or transcripts from the dominant cyanobacterium *Lyngbya* spp. (Omoregie  
92 *et al.*, 2004a; Omoregie *et al.*, 2004b), despite the fact that these *Cyanobacteria* visually  
93 dominated the mats and several *Lyngbya* spp. possess the capability to fix N<sub>2</sub> in culture (e.g.,  
94 Paerl *et al.*, 1991). Instead, *nifH* sequences from Cluster III, which includes SRB that belong  
95 to the *Deltaproteobacteria*, dominated these *nifH* gene libraries and were also found in the  
96 transcript library (Omoregie *et al.*, 2004a; Omoregie *et al.*, 2004b). The Cluster III contains  
97 sequences from anaerobic members of the *Bacteria* and *Archaea* including spirochetes,  
98 methanogens, acetogens, SRB, green sulfur bacteria and clostridia (Zehr *et al.*, 2003).  
99 Comparative investigation of available *nifH* sequences indicates that Cluster III contains the  
100 greatest diversity of all *nifH* lineages and that its diversity is still not fully understood (Gaby  
101 & Buckley, 2011).

102         The presence and/or transcription of the *nifH* gene does not necessarily mean that an  
103 organism actively fixes N<sub>2</sub> in the environment since the nitrogenase enzyme activity can be  
104 regulated on multiple levels ranging from transcription (Chen *et al.*, 1998) to post-  
105 translational protein modification (Kim *et al.*, 1999). As such, identification of active N<sub>2</sub>  
106 fixers requires investigation on the functional level, for example through stable isotope  
107 probing (SIP) with <sup>15</sup>N<sub>2</sub> in combination with single-cell isotope analysis. The incorporation  
108 of <sup>15</sup>N into biomass can be directly imaged with secondary ion mass spectrometry (SIMS)  
109 (Cliff *et al.*, 2002; Popa *et al.*, 2007; Lechene *et al.*, 2006), typically using a Cameca  
110 NanoSIMS instrument, and has been used previously to investigate diazotrophic communities

111 across diverse environments (e.g. Halm *et al.*, 2009; Dekas *et al.*, 2009; Foster *et al.*, 2011;  
112 Ploug *et al.*, 2011; Woebken *et al.*, 2012).

113 We sought to identify the active N<sub>2</sub> fixers in intertidal mats at Laguna Ojo de Liebre,  
114 Mexico, visually dominated by *Lyngbya* spp., and ascertain with a <sup>15</sup>N<sub>2</sub>-SIP single-cell  
115 approach the active N<sub>2</sub> fixing community. We applied a combination of inhibitor amendment  
116 experiments, *nifH* gene and transcript sequencing, and <sup>15</sup>N<sub>2</sub> incubations followed by single-  
117 cell isotope measurements. As in previous studies, inhibitor experiments coupled to acetylene  
118 reduction assays (ARA) suggested that *Cyanobacteria* and SRB both play a major role in N<sub>2</sub>  
119 fixation. However, further investigations through inhibitor experiments combined with <sup>15</sup>N<sub>2</sub>  
120 incorporation experiments, molecular and NanoSIMS analysis provided strong evidence that  
121 *Cyanobacteria* (especially *Lyngbya* spp.) were the most active diazotrophs in the investigated  
122 mats.

123

## 124 **Materials and methods**

### 125 *Sampling and experimental set-up*

126 Mats with a phototrophic layer dominated by *Lyngbya* spp. (in terms of biomass, as  
127 assessed by light microscopy) were sampled from the intertidal zone at Ojo de Liebre, Baja  
128 California, Mexico (27.758 N (Lat) and -113.986 W (Long)) on 15<sup>th</sup> September 2010  
129 (Supplementary Figure 1) during low tide. The N<sub>2</sub> fixation activity of two subsamples  
130 (replicate mat pieces of ca. 20 x 30 cm) was investigated over a diel cycle at a nearby field  
131 laboratory from September 15<sup>th</sup>-16<sup>th</sup> 2010. Other mat pieces were transported to the NASA  
132 Ames Research Center, CA, USA (without water coverage) on September 16<sup>th</sup> 2010 for  
133 additional diel cycle studies including inhibition experiments, stable isotope incubations as  
134 well as nucleic acid-based investigations. For experiments at NASA Ames, mats were placed  
135 in acrylic aquaria transparent to ultraviolet radiation and covered with *in situ* water for 2 days

136 before the beginning of the diel study (starting at 12:00 pm and ending at 3:00 pm the  
137 following day). To ensure full photosynthetic activity in the mats during the N<sub>2</sub> fixation  
138 experiments, resumption of photosynthetic activity after rewetting was investigated by pulse  
139 amplitude modulation (PAM) fluorescence (Quantum Yield of photosystem II (ΦPSII).  
140 Rehydrated mats exhibited maximal photosynthetic activity (0.30-0.40) already within four  
141 hours of wetting in congruence with earlier studies (Fleming *et al.*, 2007); thus, diel cycle  
142 studies were conducted with fully active mats. Diel cycle studies were carried out under  
143 natural solar irradiance, and the water temperature was kept constant at approximately 18 °C.

144

#### 145 *Acetylene reduction assays and <sup>15</sup>N<sub>2</sub> incubations*

146 Nitrogenase activity was measured with the acetylene reduction assay (ARA) as  
147 previously described (Bebout *et al.*, 1993; Woebken *et al.*, 2012). For more details see  
148 Supplementary Information.

149 For <sup>15</sup>N<sub>2</sub> incubation experiments, mat cores (10 mm diameter, 5 mm thick,  
150 Supplementary Figure 2) were transferred to a 14 ml serum vial, covered with 1 ml of *in situ*  
151 water and capped with gas-tight rubber stoppers. The headspace was exchanged with a  
152 mixture of 78% <sup>15</sup>N<sub>2</sub> gas (>98 at% <sup>15</sup>N; Cambridge Isotope Laboratories, Andover, MA,  
153 USA), 21% O<sub>2</sub> and 0.038% CO<sub>2</sub>. Mats were incubated in triplicate in the dark for 10 hours,  
154 and subsequently, half of the mat cores were sectioned for bulk isotope analysis in two depth  
155 intervals (0 to 2 mm and 2 to 4 mm). The other half of the sectioned cores were preserved for  
156 NanoSIMS analysis by fixation in 4% paraformaldehyde (PFA) as previously described  
157 (Amann *et al.*, 1990). Mat cores incubated in air (without <sup>15</sup>N<sub>2</sub>) were treated equally and  
158 served as controls. Bulk sample <sup>15</sup>N/<sup>14</sup>N isotope ratios were determined by isotope-ratio mass  
159 spectrometry (IRMS) (ANCA-IRMS, PDZE Europa Limited, Crewe, England) at the  
160 University of California, Berkeley, corrected relative to National Institute of Standards and

161 Technology (NIST, USA) standards and are expressed as  $^{15}\text{N}/(^{14}\text{N}+^{15}\text{N})$  isotope fractions,  
162 given in at% (means  $\pm$  standard error (SE)).

163 All inhibition amendment experiments were conducted at the NASA Ames Research  
164 Center. For photosynthesis inhibition experiments, DCMU (3-(3,4-dichlorophenyl)-1,1-  
165 dimethylurea) was added to intact mat slabs before sunrise on the first day of the diel cycle  
166 study with a final concentration of 20  $\mu\text{M}$  to ensure complete inhibition of photosystem II  
167 (PSII) (Oremland & Capone, 1988). For ARA or  $^{15}\text{N}_2$  incubation experiments, mat cores  
168 were subsampled from these mat slabs and incubated as described above (but with *in situ*  
169 water containing DCMU). Mat cores from mat slabs without DCMU treatment served as  
170 controls and were incubated in seawater without DCMU. For inhibition experiments of  
171 sulfate reduction, sodium molybdate ( $\text{Na}_2\text{MoO}_4$ , a structural analog of sulfate, 30 mM final  
172 concentration) (Oremland & Capone, 1988) was added to intact mat slabs submerged in *in*  
173 *situ* seawater or artificial seawater in the early morning of the first day of the diel cycle study.  
174 Mat slabs for control measurements were incubated in *in situ* seawater or artificial seawater  
175 without molybdate. Two different diel experiments were conducted: (A) Mat samples in  
176 seawater (control) versus mat samples in molybdate-amended seawater; and (B) mat samples  
177 in artificial seawater containing 23 mM sulfate (control) versus mat samples in artificial  
178 seawater without sulfate and with added molybdate. Incubations for ARA or  $^{15}\text{N}_2$   
179 experiments were conducted as described above.

180

#### 181 *Nucleic acid extraction from mat cores*

182 All diel cycle experiments were accompanied by mat sampling for molecular analysis.  
183 At multiple time points during a diel experiments, four mat cores of 1 cm diameter were flash  
184 frozen in liquid nitrogen and stored at  $-80\text{ }^\circ\text{C}$  until further processing. Detailed information  
185 about DNA and RNA extractions can be found in the Supplementary Information. Since  $\text{N}_2$

186 fixation was observed only during the night, all sequence data were derived from nighttime  
187 samples. Briefly, biomass of the uppermost 2 mm of three to four pooled mat cores was  
188 homogenized using a rotor-stator homogenizer (Omni International, Kennesaw, GA, USA).  
189 RNA and DNA of this homogenized biomass were co-extracted by combining phenol-  
190 chloroform extraction with parts of the RNeasyMini and QIAamp DNA Mini Kit (Qiagen,  
191 Valencia, CA, USA), respectively, as previously described (Woebken *et al.*, 2012). Extracted  
192 RNA was treated with DNase using the TURBO DNA-free kit (Ambion, Austin, TX, USA)  
193 according to the manufacturer's protocol. RNA was reverse transcribed into single-stranded  
194 cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA,  
195 USA).

196

197 *Amplification, sequencing and sequence analysis of 16S rRNA and nifH genes and*  
198 *transcripts*

199 Detailed information about the construction of 454 pyrotag amplicon and clone  
200 libraries of the 16S rRNA and *nifH* gene/transcript clone libraries can be found in the  
201 Supplementary Information. In summary, 454 pyrotag amplicon libraries of the 16S rRNA  
202 V6-V8 region were constructed from two biological replicate mats. Sequencing resulted in  
203 20,616 and 15,524 reads from both DNA templates and 20,138 and 22,246 reads from both  
204 cDNA templates. The replicate libraries were processed individually (sorting, trimming,  
205 removing reads of low quality and classification) using the RDP pipeline with standard  
206 settings (<http://pyro.cme.msu.edu>) (Supplementary Table 1). Reads were taxonomically  
207 assigned using the RDP Classifier (Wang *et al.*, 2007). 16S rRNA and 16S rRNA gene clone  
208 libraries of two biological replicates were constructed from single-stranded cDNA and DNA.  
209 In total, 520 sequences (of 600 to 700 bp length) were derived from DNA samples (D3= 256,  
210 and D5= 264 sequences) and 317 sequences from cDNA samples (C3= 151, and C5= 166

211 sequences). The *nifH* genes were PCR amplified from DNA and cDNA with a nested PCR  
212 protocol (Zehr & Turner, 2001) as described previously (Woebken *et al.*, 2012). We retrieved  
213 a total of 341 sequences from DNA, 535 sequences from cDNA and 187 from cDNA of the  
214 molybdate inhibition experiment. Sequence analyses of the clone libraries listed above are  
215 described in the Supplementary Information. Mat samples treated with DCMU during the  
216 diel cycle study failed to produce any detectable PCR product from cDNA with *nifH*-  
217 targeting primers (tested in two replicate RNA extractions). To test for potential inhibition of  
218 cDNA synthesis or PCR caused by residual amount of DCMU, we conducted general 16S  
219 rRNA PCR with the cDNA template and *nifH*-specific PCRs with co-extracted DNA  
220 samples. These reactions resulted in PCR products, indicating that residual amounts of  
221 DCMU did not inhibit cDNA synthesis or PCR, and therefore were not the cause for the  
222 failed PCR amplifications of *nifH* transcripts from DCMU-treated mat cDNA. We conclude  
223 that the level of *nifH* transcripts in DCMU treated samples was below the detection limit for  
224 successful PCR amplification.

225

#### 226 *Nucleotide Accession Numbers*

227 16S rRNA gene and transcript sequences obtained in this study are deposited under  
228 GenBank accession numbers xxxxx to xxxxx. Sequences of *nifH* genes and transcripts are  
229 deposited under GenBank accession numbers xxxxx to xxxxx.

230

#### 231 *Single-cell isotope analysis by NanoSIMS*

232 *Mat sample preparation.* The upper 2 mm of PFA-fixed mat samples from the <sup>15</sup>N<sub>2</sub>  
233 incubation experiment described above and negative control mat cores were prepared for  
234 NanoSIMS analysis as previously described (Woebken *et al.*, 2012). Briefly, samples were  
235 transferred with tweezers onto silicon wafers (Ted Pella, Redding, CA, USA), teased apart,

236 attached by drying and subsequently washed in ultrapure water (MQ, Millipore). In  
237 experiments where catalyzed reporter deposition- fluorescence *in situ* hybridization (CARD-  
238 FISH) was combined with NanoSIMS analysis, wafers were coated with VectaBond (Vector  
239 Laboratories, Burlingame, CA, USA) prior to sample deposition. Samples were gold coated  
240 after light and fluorescence imaging and before scanning electron microscopy (SEM; FEI  
241 Inspect F, FEI, Hillsboro, OR, USA) and NanoSIMS imaging to mitigate sample charging  
242 and ease imaging. Filamentous *Cyanobacteria* (*Lyngbya* spp.-related and small filamentous  
243 *Cyanobacteria*) were identified based on their red autofluorescence when illuminated with  
244 green light by epifluorescence microscopy (Excitation Filter: BP 545/40, Dichromatic  
245 Mirror: 565, Suppression Filter: BP 610/75) and based on their morphology, as imaged by  
246 SEM. *Deltaproteobacteria* were stained by CARD-FISH as previously described (Pernthaler  
247 *et al.*, 2002; Woebken *et al.*, 2012) using probes DELTA495 a-c (Loy *et al.*, 2002; Lückner *et*  
248 *al.*, 2007). Stained cells were identified and localized by epifluorescence microscopy. All  
249 targeted cells were localized and imaged by reflected light microscopy and SEM to ensure  
250 that the target cells were free of overlying cells or other material so that  $^{15}\text{N}/^{14}\text{N}$  ratios could  
251 unambiguously be attributed to the target cells.

252

253 *Sample preparation for assessment of CARD-FISH effects on single-cell isotope*  
254 *composition.* The CARD-FISH procedure can have an effect on the isotopic composition  
255 ( $^{13}\text{C}/^{12}\text{C}$  and  $^{15}\text{N}/^{14}\text{N}$ ) of cells, since cells will be exposed to reagents that contain C and N  
256 (such as lysozyme, DNA probes, dextrane sulfate, blocking reagent) during lysozyme  
257 treatment, hybridization and amplification. Deposition of tyramides in the signal-  
258 amplification step further introduces C and N in the cells. In order to evaluate the potential  
259 dilution effect of the CARD-FISH procedure, we applied CARD-FISH to isotopically labeled  
260 *Escherichia coli* (DSM 498) and *Bacillus subtilis* (DSM 8439) cells, examples of Gram-

261 negative and -positive cells, respectively. Both strains were grown in defined media  
262 containing appropriate amounts of  $^{13}\text{C}$ -glucose and  $^{15}\text{N}$ -ammonium chloride to obtain two  
263 sets of co-labeled cells enriched to either 6 atom%  $^{13}\text{C}$ /6 atom%  $^{15}\text{N}$  or 99 atom%  $^{13}\text{C}$ /99  
264 atom%  $^{15}\text{N}$ . Cells were fixed using paraformaldehyde (Amann *et al.*, 1990) for *E. coli* and  
265 ethanol (Roller *et al.*, 1994) for *B. subtilis*. CARD-FISH was conducted as previously  
266 described (Pernthaler *et al.*, 2002; Hoshino *et al.*, 2008) with probes EUB338 (Amann *et al.*,  
267 1990) and nonEUB338 (Wallner *et al.*, 1993). More detailed information on the growth of  
268 cells, CARD-FISH and sample preparation for NanoSIMS is provided in the Supplementary  
269 Information.

270

271 *General information on NanoSIMS analysis.* Prior to data acquisition, analysis areas  
272 were pre-sputtered utilizing a high intensity  $\text{Cs}^+$  primary ion beam to ensure that the analyzed  
273 volumes were located within the cells. Data were acquired as images by scanning a  $\sim 100$  nm  
274 diameter primary ion beam over areas between  $10 \times 10$  and  $50 \times 50 \mu\text{m}^2$ . Images were  
275 recorded as multilayer-stacks, each consisting of 5 to 15 individual cycles (i.e. layers).  
276 Secondary electrons were detected simultaneously to secondary ions to facilitate target cell  
277 identification. Individual images were corrected for detector dead-time and image drift from  
278 layer to layer prior to stack accumulation. Regions of interest (ROIs), referring to individual  
279 cells, were manually defined based on the  $\text{CN}^-$  secondary ion maps and cross-checked by the  
280 topographical/morphological appearance in secondary electron images. The isotopic  
281 composition for each ROI was determined by averaging over the individual images of the  
282 multilayer stack. Isotopic compositions are expressed as the abundance of the tracer relative  
283 the total tracer element (e.g.,  $a_{\text{N}} = ^{15}\text{N}/(^{14}\text{N}+^{15}\text{N})$  and  $a_{\text{C}} = ^{13}\text{C}/(^{12}\text{C}+^{13}\text{C})$ ) in atom %.

284

285           *NanoSIMS analysis of mat samples.* SIMS analysis of the mat samples was performed  
286 at the Lawrence Livermore National Laboratory (LLNL) using a NanoSIMS 50 (Cameca,  
287 Gennevilliers Cedex, France) as previously described (Wobken *et al.*, 2012). Data were  
288 processed to generate quantitative isotope ratio images from ion images using custom  
289 software (LIMAGE, L. Nittler, Carnegie Institution of Washington). Target cells were  
290 identified by comparing the NanoSIMS  $^{12}\text{C}^{14}\text{N}^-$  secondary ion and secondary electron  
291 images, the respective SEM image, and for *Deltaproteobacteria* the CARD-FISH image. For  
292 small filamentous *Cyanobacteria*, typically 3–10 connected cells within a filament were  
293 analyzed collectively, thus yielding the average isotopic composition per filament. Due to the  
294 large size of the *Lyngbya* spp.-related *Cyanobacteria*, isotope enrichment measurements were  
295 done by accelerated sputtering utilizing high primary ion beam currents (~1 nA, 2  $\mu\text{m}$  beam  
296 size). Negative-control samples of *Deltaproteobacteria* and *Lyngbya* spp.-related  
297 *Cyanobacteria* with natural isotopic composition were analyzed for reference. NanoSIMS  
298 data refer to the arithmetic mean of all measurements per cell type,  $\pm$  standard error (SE).

299

300           *NanoSIMS analysis of E. coli and B. subtilis cells.* NanoSIMS measurements of *E.*  
301 *coli* and *B. subtilis* cells were carried out on a NanoSIMS 50L at the Large-Instrument  
302 Facility for Advanced Isotope Research, University of Vienna.  $^{13}\text{C}$  abundance was inferred  
303 from the signal intensities obtained from detection of  $^{12}\text{C}^-$  and  $^{13}\text{C}^-$  secondary ions. In order to  
304 optimize the counting statistics in the determination of  $^{15}\text{N}$  abundance,  $^{13}\text{C}^{14}\text{N}^-$  and  $^{13}\text{C}^{15}\text{N}^-$   
305 secondary ions were detected for highly enriched cells and  $^{12}\text{C}^{14}\text{N}^-$  and  $^{12}\text{C}^{15}\text{N}^-$  secondary  
306 ions for samples containing cells with  $^{15}\text{N}$  and  $^{13}\text{C}$  enrichment levels of  $\leq 6$  at%. Each sample  
307 was analyzed 1 to 4 times on distinct measurement areas, with an average number of 30 cells  
308 per measurement. Image data were evaluated using the WinImage software package provided

309 by Cameca. Displayed data refer to the arithmetic mean  $\pm$  standard deviation (SD) over the  
310 analyzed cells per treatment.

311 The dilution of the tracer enrichment (e.g., at%  $^{15}\text{N}$ ) in the sampled cells that is the  
312 result of sample preparation, including CARD-FISH treatment, was calculated based on a  
313 two end member mixing model (e.g., Faure 1987) modified to only account for the addition  
314 of the tracer element, not all added material (see the Supplementary Information for  
315 derivation and discussion). This dilution is defined as  $F_{X,add}$ , the fraction of the tracer element  
316  $X$  (e.g., nitrogen) added to the cells by sample preparation:

317

$$318 \quad F_{X,add} = (a_f - a_i) / (a_{add} - a_i), \quad \text{Eq. 1}$$

319

320 where  $a_i$  and  $a_f$  are the isotopic compositions of the cells before and after sample preparation,  
321 respectively, and  $a_{add}$  is the isotopic composition of the material added to the cells, which we  
322 assume to be natural (e.g.,  $\sim 1.1$  at%  $^{13}\text{C}$  and  $\sim 0.37$  at%  $^{15}\text{N}$ ). Values for  $F_{X,add}$  were estimated  
323 from the isotopic compositions of labeled *E. coli* and *B. subtilis* cells determined before and  
324 after sample preparation including CARD-FISH. With  $F_{X,add}$  estimated with the test  
325 experiments, the initial isotopic composition before sample preparation,  $a_i$ , can be estimated  
326 for unknowns by rearranging Eq. 1 to yield:

327

$$328 \quad a_i = (a_f - a_{add} F_{X,add}) / (1 - F_{X,add}). \quad \text{Eq. 2}$$

329

### 330 *Statistical analysis*

331 Data retrieved in the ARA, IRMS data of vertical sections and inhibition experiments  
332 as well as NanoSIMS data were analyzed for significant differences using student's T test or  
333 analysis of variance (ANOVA) with an alpha error of 0.05 and the Tukey-Kramer Honestly

334 Significant Difference (HSD) as a multiple means comparison test (JMP Version 7, SAS  
335 Institute Inc., Cary, NC, USA). Normal distribution was tested with the Shapiro-Wilk W test,  
336 and in cases where the data did not meet the standard of homogeneity of variance, the Welch  
337 ANOVA was used to confirm the initial result. Since  $^{15}\text{N}$  enrichment levels were very low in  
338 *Deltaproteobacteria* measured by NanoSIMS, the natural abundance values for  
339 *Deltaproteobacteria* were used to test for significant enrichment based on a 95% confidence  
340 interval.

341

## 342 **Results**

### 343 *Bulk level $\text{N}_2$ fixation analysis*

344 The activity in ARAs was significantly higher during the night (average  $\pm$  standard deviation:  
345  $131 \pm 67 \mu\text{mol C}_2\text{H}_4 \text{ m}^{-2} \text{ h}^{-1}$ ) than during the daytime (average  $\pm$  standard deviation:  $5 \pm 7$   
346  $\text{C}_2\text{H}_4 \text{ m}^{-2} \text{ h}^{-1}$ ,  $p < 0.0001$ , Figure 1A). The potential contribution of oxygenic photosynthesis  
347 (Photosystem II, or PSII) to  $\text{N}_2$  fixation was investigated by inhibiting PSII with DCMU. In  
348 experiments where DCMU was added before sunrise on the first day of a diel experiment,  
349 ARA rates were significantly reduced compared to un-amended control incubations  
350 ( $p < 0.0001$ , Figure 1B). The potential contribution of SRB to  $\text{N}_2$  fixation was investigated by  
351 adding the sulfate reduction inhibitor molybdate. There was no significant difference in ARA  
352 in assays conducted in seawater versus artificial seawater in molybdate addition experiments  
353 ( $p = 0.3386$ ). Samples exposed to molybdate had lower ARA rates compared to un-amended  
354 incubations ( $p < 0.0001$ , Figure 1C).

355  $\text{N}_2$  fixation activity (i.e. net  $^{15}\text{N}$  incorporation) in the intertidal mats was directly  
356 assessed with  $^{15}\text{N}_2$  incubation experiments (for 10 h in the dark) and subsequent IRMS  
357 analysis of both upper and lower mat layers (0 to 2 mm and 2 to 4 mm, respectively). The 0  
358 to 2 mm layer was significantly enriched in  $^{15}\text{N}$  relative to the deeper layer (Figure 2; average

359  $\pm$  standard error:  $0.486 \pm 0.023$  at% vs.  $0.373 \pm 0.001$  at%  $^{15}\text{N}$ ,  $p < 0.001$ ). Mat cores  
360 incubated in air without  $^{15}\text{N}_2$  served as control samples for natural abundance and had values  
361 of (average  $\pm$  standard error)  $0.371 \pm 0.001$  (0 to 2 mm) and  $0.373 \pm 0.001$  (2 to 4 mm) at%  
362  $^{15}\text{N}$ . Based on these results we focused additional molecular and single-cell analyses on the  
363 upper layer. In  $^{15}\text{N}_2$  incubation experiments of the 0 to 2 mm layer with and without  
364 inhibitors, mats incubated with DCMU had significantly lower  $^{15}\text{N}$  incorporation relative to  
365 control incubations without DCMU (Figure 2; average  $\pm$  standard error:  $0.380 \pm 0.007$  at%  
366 vs.  $0.452 \pm 0.021$  at%  $^{15}\text{N}$ ,  $p < 0.05$ ). In molybdate incubation experiments, incubations where  
367 molybdate was added had a slightly lower  $^{15}\text{N}$  enrichment than un-amended mats (average  $\pm$   
368 standard error:  $0.429 \pm 0.013$  at% vs.  $0.466 \pm 0.015$  at%  $^{15}\text{N}$ ,  $p = 0.063$ ), but the difference  
369 was not significant at the  $p < 0.05$  level.

370

#### 371 *Microbial diversity based on 16S rRNA and 16S rRNA gene analysis*

372 *Lyngbya* spp. and other filamentous *Cyanobacteria* dominated the biomass of the  
373 phototrophic layer of the microbial mats from Laguna Ojo de Liebre based on light  
374 micrographs (Supplementary Figure 3). However, 16S rRNA (cDNA based) and 16S rRNA  
375 gene (DNA based) sequencing indicated that the microbial community of the upper 2 mm  
376 was composed of diverse bacterial phyla (Figure 3 and Supplementary Table 2). DNA  
377 derived Sanger sequences were affiliated with 9 different phyla based on the RDP classifier.  
378 The majority of DNA sequences were classified as *Proteobacteria* (30-36%) and  
379 *Bacteroidetes* (17-33%), followed by *Chloroflexi* (6-12%), *Cyanobacteria* (5-9.5%) and  
380 *Verrucomicrobia* (1-9.5%). In contrast, the majority of 16S rRNA sequences (cDNA based)  
381 derived by Sanger sequencing grouped with *Cyanobacteria* (57-62%), followed by  
382 *Proteobacteria*, *Chloroflexi* and *Bacteroidetes* (6-20%). Based on phylogenetic analyses, all  
383 16S rRNA cyanobacterial sequences were related to filamentous *Cyanobacteria*

384 (Supplementary Figure 4), and 24.4% of these cyanobacterial 16S rRNA sequences were  
385 related to *Lyngbya* spp., with up to 98.7% sequence identity to *Lyngbya aestuarii* PCC 7419  
386 and *Lyngbya* sp. PCC 8106, or 97.9% identity to *Lyngbya majuscula* CCAP.

387 SSU amplicon pyrosequencing was used to investigate the 16S rRNA and 16S rRNA  
388 gene diversity with greater coverage, and revealed reads clustering in 15 phyla (Figure 3 and  
389 Supplementary Table 2). Similar to the Sanger-based sequences (Figure 3), most of the  
390 amplicons recovered from DNA were assigned to *Proteobacteria* (20-34%) and *Chloroflexi*  
391 (13-28%), *Cyanobacteria* (12-13%) and *Bacteroidetes* (11%). As with the Sanger sequences,  
392 the majority of reads originating from cDNA clustered with *Cyanobacteria* (70-77%),  
393 followed by *Proteobacteria*, *Bacteroidetes* and *Chloroflexi*. Calculation of the Chao1  
394 estimator and the Shannon index revealed greater diversity in DNA-based reads than in  
395 cDNA-based reads (Supplementary Table 1).

396

#### 397 *Community analysis of potential diazotrophs (nifH gene and transcript analysis)*

398 The diversity of bacteria with the genetic capability to fix N<sub>2</sub> was specifically  
399 analyzed by sequencing *nifH* genes and transcripts from the upper 2 mm of the intertidal  
400 mats. Deduced amino acid sequences formed 108 OTUs based on a cutoff of 97% sequence  
401 identity. Phylogenetic analysis of the deduced *nifH* sequences derived from extracted DNA  
402 revealed that the majority (66.9%) grouped with Cluster III (based on Zehr *et al.*, 2003; with  
403 uncultured microorganisms, *Clostridia* and *Deltaproteobacteria*), and the rest with Cluster I  
404 (with *Cyanobacteria*, *Alpha-*, *Gamma-* and *Betaproteobacteria*) (Figure 4). To focus on the  
405 community that was expressing the *nifH* gene, we also analyzed sequences derived from  
406 extracted RNA after reverse transcription into cDNA. The majority of these sequences  
407 (80.8%) were related to *Cyanobacteria* forming three major groups (*Lyngbya* spp.-cluster,  
408 cyanobacterial cluster 1 and 2, Figure 4 and Supplementary Figure 5), with *Lyngbya* spp.

409 related sequences being the most abundant. The other two abundant groups of cyanobacterial  
410 sequences clustered with sequences of filamentous *Cyanobacteria* such as *Phormidium* and  
411 *Leptolyngbya*, and sequences from other microbial mats, freshwater, sponge or sediments  
412 samples. A minor proportion of the cyanobacterial 16S rRNA sequences (0.25%) were  
413 related to a cyanobacterium that is a dominant N<sub>2</sub> fixer in mats of Northern CA (ESFC-1;  
414 Wobken *et al.*, 2012; Everroad *et al.*, 2013). Seventeen percent of all cDNA-based  
415 sequences grouped with Cluster III (1.7% of all sequences with deltaproteobacterial clusters  
416 containing known SRB and 15.3% with unclassified Cluster III sequences).

417 *NifH* clone libraries (based on cDNA) of samples from the molybdate addition  
418 experiments were almost completely comprised of cyanobacterial sequences (97.3%), and  
419 only a minor portion of the sequences clustered with Cluster III (2.1%) or  
420 *Deltaproteobacteria* within this cluster (0.5%). Mat samples treated with DCMU during the  
421 diel cycle study failed to produce any detectable PCR product from cDNA with *nifH*-  
422 targeting primers (tested in two replicate RNA extractions, see Supplementary Information  
423 for more details).

424

425 *Single-cell isotope analysis of diazotrophic microbial community members and testing the*  
426 *effect of CARD-FISH on the isotopic enrichment of cells*

427 Based on results of the inhibitor addition experiments and sequencing of expressed  
428 *nifH* genes, we focused our functional single-cell isotope analyses on *Cyanobacteria* and  
429 *Deltaproteobacteria*. The latter group was targeted to test as broadly inclusive as possible the  
430 activity of SRB within the *Deltaproteobacteria* for their <sup>15</sup>N<sub>2</sub> fixation activity. All  
431 *Cyanobacteria* (identified based on their red autofluorescence) were filamentous, and  
432 *Lyngbya* spp. filaments were easily distinguishable from other filamentous *Cyanobacteria*  
433 based on their morphology (Supplementary Figure 3). The highest <sup>15</sup>N enrichments in these

434 mats were measured in *Lyngbya* spp. filaments (maximum of 14.54 at%), with an average ( $\pm$   
435 standard error)  $^{15}\text{N}$  tracer content of  $4.40 \pm 0.57$  at% (Figure 5, Supplementary Table 3). The  
436 enrichment in *Lyngbya* spp. filaments was significantly higher than in any other analyzed  
437 cells ( $p < 0.0001$  compared to small filamentous *Cyanobacteria*;  $p < 0.0001$  compared to  
438 *Deltaproteobacteria*; and  $p = 0.01$  compared to unidentified single cells). Smaller filamentous  
439 *Cyanobacteria* had an average enrichment of  $0.60 \pm 0.03$  at%  $^{15}\text{N}$  with a maximum of 1.32  
440 at%. The  $^{15}\text{N}$  enrichment of CARD-FISH-stained *Deltaproteobacteria* cells from  $^{15}\text{N}_2$   
441 labeled mats was not significantly different than those in control mat samples ((average  $\pm$   
442 standard error)  $0.38 \pm 0.00$  at%  $^{15}\text{N}$  versus (average  $\pm$  standard error)  $0.37 \pm 0.00$  at%  $^{15}\text{N}$ ),  
443  $p = 0.170$ ). The highest  $^{15}\text{N}$  enrichment measured in an individual *Deltaproteobacteria* cell  
444 was 0.41 at%.

445 In contrast to *Cyanobacteria* that were identified by their characteristic  
446 autofluorescence, *Deltaproteobacteria* cells were identified by CARD-FISH. This procedure  
447 has the potential to dilute labeling through the introduction of C and N (mainly  $^{12}\text{C}$  and  $^{14}\text{N}$ )  
448 via probe and tyramide binding (and possibly also via buffer reagents and enzymes used for  
449 cell wall permeabilization that cannot be completely removed during the washing steps). This  
450 deposition in CARD-FISH identified cells may dilute the amount of the rarer heavy isotope  
451 incorporated during the labeling experiment, and possibly could have masked  $^{15}\text{N}$   
452 incorporation by the *Deltaproteobacteria*. To evaluate the magnitude of this dilution effect  
453 and to test for its relevance in this and other NanoSIMS studies we conducted control  
454 experiments with  $^{13}\text{C}$  and  $^{15}\text{N}$ -labeled cells (Figure 6). The enrichment of fixed cells un-  
455 treated by CARD-FISH was 98.56 at%  $^{15}\text{N}$  and 95.31 at%  $^{13}\text{C}$  in *E. coli*; versus 97.52 at%  
456  $^{15}\text{N}$  and 92.11 at%  $^{13}\text{C}$  in *B. subtilis* cells. After hybridization with probe EUB338  
457 (Supplementary Figure 6), 70.84 at%  $^{15}\text{N}$  and 62.54 at%  $^{13}\text{C}$ , and 72.52 at%  $^{15}\text{N}$  and 57.03  
458 at%  $^{13}\text{C}$  were measured for *E. coli* and *B. subtilis* cells, respectively.  $^{15}\text{N}$  and  $^{13}\text{C}$  enrichments

459 of cells stained with a negative control probe (Supplementary Figure 6) were 81.50 at% and  
460 74.65 at%, respectively, in *E. coli*, and 89.74 at% and 81.33 at%, respectively, in *B. subtilis*.  
461 Similar trends were detected in cells labeled to only approximately 6 at% <sup>15</sup>N and <sup>13</sup>C, which  
462 more closely reflects enrichment levels measured in isotope labeled environmental samples  
463 (e.g. Dekas *et al.*, 2009; Woebken *et al.*, 2012; Ploug *et al.*, 2010) (Figure 6). These data  
464 indicate that CARD-FISH analyses with the nonsense probe can result in an apparent dilution  
465 ( $F_{X,add}$ ) of up to 18% for nitrogen, and up to 22% for carbon. In CARD-FISH experiments  
466 with the EUB338 probe, these values increased up to 28% for nitrogen and 38% for carbon.

467

## 468 **Discussion**

469 The microbial diversity of the uppermost 2 mm of the investigated intertidal mats was  
470 dominated by members of the *Proteobacteria*, *Bacteroidetes*, *Chloroflexi* and *Cyanobacteria*  
471 at the 16S rRNA gene level (Figure 3). This resembles the diversity of previously  
472 investigated photosynthetic mats from salt ponds at Guerrero Negro (Ley *et al.*, 2006) and  
473 from Elkhorn Slough in Northern CA (Burow *et al.*, 2012; Burow *et al.*, 2013). However, the  
474 active microbial community (as inferred by 16S rRNA-based community analyses) was  
475 strongly dominated by *Cyanobacteria* in Laguna Ojo de Liebre intertidal mats, as was  
476 previously observed in the Elkhorn Slough libraries (Burow *et al.*, 2012; Burow *et al.*, 2013).

477 *NifH* sequencing has previously been applied to intertidal mats from Laguna Ojo de  
478 Liebre to gain insights into the microbial community with the genetic potential for N<sub>2</sub>  
479 fixation. *NifH* gene and transcript analyses suggested that both *Cyanobacteria* and  
480 *Deltaproteobacteria* (including SRB) were the major contributors for N<sub>2</sub> fixation (Omereg  
481 *et al.*, 2004a; Omereg *et al.*, 2004b; Moisander *et al.*, 2006). A microarray study on the  
482 expressed *nifH* sequences of these mats reported that the transcript abundance of  
483 cyanobacterial and deltaproteobacterial (or Cluster III) groups was comparable, suggesting

484 both groups significantly contributed to N<sub>2</sub> fixation (Moisander *et al.*, 2006). However, in the  
485 present study, we observed that the sequences retrieved from DNA and cDNA showed very  
486 different patterns. While sequences of Cluster III (including known SRB) dominated the  
487 DNA sequence pool, cyanobacterial sequences dominated by far the pool of expressed *nifH*  
488 genes, while the proportion of Cluster III sequences was strongly decreased. Interestingly, the  
489 detected *nifH* sequences in Cluster I and Cluster III were not detected in previous studies  
490 (Omoregie *et al.*, 2004a; Omoregie *et al.*, 2004b). For instance, the cyanobacterial  
491 community in our study that expressed cyanobacterial *nifH* sequences were strongly  
492 dominated by sequences related to *Lyngbya majuscula*, which were not detected previously  
493 (Omoregie *et al.*, 2004a; Omoregie *et al.*, 2004b; Moisander *et al.*, 2006). This lack of  
494 congruence in the sequence data could reflect temporal (2001 to 2010) differences in the  
495 microbial community (Omoregie *et al.*, 2004b; Omoregie *et al.*, 2004a; Moisander *et al.*,  
496 2006), as these mats are characterized by a “pioneering life style” (Bebout *et al.*, 1993) and  
497 thereby will most likely be dynamic in their microbial community. Another explanation for  
498 the differences in the detected microbial communities could be depth of sequencing effort or  
499 bias in the nucleic acid extraction methods. Although the same primer set was used in these  
500 studies (Zehr & Turner, 2001), the nucleic acid extraction protocols differ. We included a  
501 pre-homogenization step prior to nucleic acid extraction, which could have increased lysis  
502 efficiency.

503         The application of inhibitors in diel cycle studies can suggest the contribution of  
504 certain functional groups to N<sub>2</sub> fixation activity, an approach that has been used extensively  
505 in the past (e.g. Pinckney & Paerl, 1997; Griffiths & Gallon, 1987; Bebout *et al.*, 1993; Joye  
506 & Paerl 1994; Stal *et al.*, 1984; Steppe & Paerl 2002). The addition of the *Cyanobacteria*  
507 inhibitor DCMU (Oremland & Capone, 1988) during the daytime photoperiod strongly and  
508 significantly decreased N<sub>2</sub> fixation the subsequent night, based on ARAs (Figure 1B) and

509  $^{15}\text{N}_2$  incubations experiments (Figure 2). This pattern was previously observed in non-  
510 heterocystous cyanobacterial mats (Bebout *et al.*, 1993; Griffiths & Gallon 1987), and also in  
511 *Lyngbya* spp. cultures (Bebout *et al.*, 1993). The fact that DCMU addition in this study did  
512 not lead to increased  $\text{N}_2$  fixation during the day as reported previously (Stal *et al.*, 1984)  
513 suggests that  $\text{N}_2$  fixation during the day was not inhibited by high  $\text{O}_2$  concentrations. DCMU  
514 interrupts the photosynthetic electron flow by inhibiting the  $\text{O}_2$ -evolving PS II, which  
515 depletes the reductant formation required for  $\text{N}_2$  fixation and ultimately leads to the inhibition  
516 of cyanobacterial  $\text{N}_2$  fixation (Oremland & Capone 1988). Reserves of reduced organic  
517 compounds (e.g. glycogen) can serve as reductant until the supply is depleted. However,  
518 DCMU also inhibits  $\text{CO}_2$  fixation (Bebout *et al.*, 1993; Paerl *et al.*, 1996; Pinckney & Paerl,  
519 1997), most likely again due to the decrease in reductants, which in turn leads to a short  
520 supply of organic storage compounds that can be used for  $\text{N}_2$  fixation. This combined  
521 shortage in reductant can explain the observed decrease in  $\text{N}_2$  fixation rates upon DCMU  
522 addition and suggests that *Cyanobacteria* contributed to  $\text{N}_2$  fixation activity.

523 This observation was supported by the detection of expressed cyanobacterial *nifH*  
524 genes in untreated control mats that showed  $\text{N}_2$  fixation (Figure 4). *NifH* transcripts related to  
525 *Lyngbya* spp. dominated the transcript pool, indicating that in these mats *Lyngbya* spp. were  
526 actively expressing *nifH* and potentially fixing  $\text{N}_2$ . Additionally, in DCMU-treated mats, we  
527 were unable to amplify *nifH* transcripts, which is in congruence with a strong inhibition of  $\text{N}_2$   
528 fixation activity in the DCMU addition experiment as observed in ARA and IRMS  
529 experiments. Together, inhibitor experiments and sequence data suggested that  
530 *Cyanobacteria* were actively fixing  $\text{N}_2$  in intertidal mats sampled from Laguna Ojo de  
531 Liebre, especially members of *Lyngbya* spp.. However, care must be taken to infer diazotroph  
532 activity from detected *nifH* transcripts or relative *nifH* sequence abundance. First, nitrogenase  
533 enzyme activity can be regulated after transcription until the post-translational level (Kim *et*

534 *al.*, 1999). Furthermore, the potential for PCR biases such as differences in the amplification  
535 efficiencies of templates or amplification inhibition of the most abundant templates (Suzuki  
536 & Giovannoni, 1996; Polz & Cavanaugh, 1998) makes it difficult to infer the activity of  
537 certain groups based on their *nifH* transcript abundance. Therefore, direct N<sub>2</sub> fixation  
538 measurements coupled to the identification of cells are needed to clearly identify active  
539 diazotrophs in environmental samples. Incubation experiments with <sup>15</sup>N<sub>2</sub> and single-cell  
540 isotope analysis through NanoSIMS allowed us to investigate the active N<sub>2</sub>-fixing  
541 community at the functional level by measuring the incorporation of <sup>15</sup>N into cell biomass.  
542 This analysis revealed <sup>15</sup>N enrichment in filamentous *Cyanobacteria* of different  
543 morphotypes (Figure 5), which corresponded to multiple detected clusters of cyanobacterial  
544 *nifH* sequences (Supplementary Figure 5). Consistent with the result of the *nifH* transcript  
545 analysis, within the cyanobacteria, *Lyngbya* spp. had by far the highest enrichments in <sup>15</sup>N,  
546 demonstrating that *Lyngbya* spp. were the most active cyanobacterial diazotrophs in this mat.

547         Previous investigations of the mats suggested that SRB were contributing to N<sub>2</sub>  
548 fixation (Omoregie *et al.*, 2004a; Omoregie *et al.*, 2004b; Moisander *et al.*, 2006), a finding  
549 that we aimed to investigate with a function-based approach. In experiments conducted in  
550 parallel to the N<sub>2</sub> fixation experiments at NASA Ames, we measured sulfate reduction  
551 activity (as sulfide production) to ensure that the sampled mats contained active SRB, and  
552 that this process was not inhibited due to sampling and transport to the laboratory. On the  
553 intertidal flats at Laguna Ojo de Liebre, the mats experience naturally frequent alternating  
554 periods of desiccation (leading to aeration) and tidal flooding (Javor and Castenholz, 1984;  
555 Omoregie *et al.*, 2004b; Rothrock & Garcia-Pichel, 2005). It appears that SRB in these mats  
556 are tolerant against oxygen exposure, and that they can survive long oxic periods and  
557 maintain their capacity for sulfate reduction, a phenomenon in SRB previously investigated  
558 (Canfield and Des Marais 1991; Risatti *et al.*, 1994; Minz *et al.* 1999; Baumgartner *et al.*,

559 2006; Fike et al, 2008; Muyzer and Stams 2008). Based on the measured sulfate reduction in  
560 the parallel experiments (*Jackson et al.*, in review), we conclude that the SRB in the mats  
561 were physiologically active. Detection of expressed *dsrA* genes (a key functional gene for  
562 sulfate reduction) in the mats supported our conclusion, and sequencing revealed that the vast  
563 majority of the SRB that expressed *dsrA* (98%) belonged to previously known clusters within  
564 the *Deltaproteobacteria* (within the *Desulfobacterales* and *Desulfovibrionales*) (*Jackson et*  
565 *al.*, in review). These data also support our focus on deltaproteobacterial SRB in our single-  
566 cell isotope measurements. We are aware that there is the potential for non-  
567 deltaproteobacterial diazotrophs, e.g. related to unidentified groups (designated “unclassified  
568 cluster”, Figure 4) in Cluster III. Unfortunately, due to lack of isolates in these clusters, these  
569 bacteria are unidentified at the 16S rRNA level and thus cannot be targeted by a FISH-  
570 NanoSIMS approach. However, the above-mentioned data of expressed *dsrA* in this study  
571 support our focus on the known SRB within the *Deltaproteobacteria*.

572 Molybdate is frequently used in experiments to investigate the contributions of SRB  
573 to N<sub>2</sub> fixation. It serves as a structural analog of sulfate and blocks the sulfate activation step  
574 that is catalyzed by ATP sulfurylase, thereby depleting ATP pools in SRB and ultimately  
575 causing death (Oremland & Capone, 1988). Adding molybdate to the mats resulted in a  
576 reduction of ARA rates up to 85% compared to un-amended samples (Figure 1C). This  
577 finding is congruent with a previous study of an intertidal photosynthetic mat in which  
578 molybdate inhibited nighttime nitrogenase activity measured by ARAs by as much as 64%  
579 (Steppe & Paerl, 2002). However, it was previously recognized that reduced N<sub>2</sub> fixation rates  
580 in response to molybdate additions could result from many direct and indirect consequences,  
581 such as altered environmental conditions due to the inhibition of sulfide production (Steppe  
582 & Paerl, 2002). Results based on this “specific inhibitor” should be interpreted with caution;  
583 a conclusion further supported by our study. In mats from Laguna Ojo de Liebre, the effect of

584 molybdate on overall N<sub>2</sub> fixation rates was only significant in ARAs, while <sup>15</sup>N-incorporation  
585 experiments revealed a much less pronounced (and not significant) effect of molybdate  
586 addition (Figure 2). This observed difference in the effect based on the applied assays could  
587 be caused by an enhanced consumption of ethylene, the measured product in ARAs, in  
588 molybdate-treated mats relative to un-amended controls. Ethylene can be metabolized  
589 aerobically (de Bont, 1976), and anaerobically (Koene-Cottaar & Schraa, 1998) by  
590 microorganisms. Strong evidence exists that methanogens can reduce ethylene in anaerobic  
591 environments such as sediment and peat-soil (Oremland, 1981; Elsgaard L, 2013). In systems  
592 containing sulfate, SRB would usually outcompete methanogens for H<sub>2</sub> (Lovely *et al.*, 1982).  
593 However, it was shown that the inhibition of SRB through molybdate addition results in  
594 increased methane production that is comparable to sediments lacking sulfate (Lovely *et al.*,  
595 1982). Thereby, the inhibition of SRB in molybdate addition experiments of mat samples  
596 could have created an advantage for methanogens, so that active methanogens consumed the  
597 formed ethylene. Consequently, enhanced ethylene consumption in the molybdate treatments  
598 could mistakenly be interpreted as a large contribution of SRB to N<sub>2</sub> fixation.

599         In this study, molybdate inhibitor experiments coupled to <sup>15</sup>N<sub>2</sub> incubation and IRMS  
600 did not indicate a contribution of SRB to N<sub>2</sub> fixation, while earlier studies suggested that  
601 *Deltaproteobacteria* in the Cluster III, and more specifically SRB within the  
602 *Deltaproteobacteria*, were potentially important diazotrophs in photosynthetic mats (Steppe  
603 & Pearl, 2002; Omoregie *et al.*, 2004a; Omoregie *et al.*, 2004b). Sequencing of *nifH* genes  
604 and transcripts in our study indicates that sequences of Cluster III (including known SRB)  
605 dominated the DNA sequence pool, while cyanobacterial sequences dominated vastly the  
606 pool of expressed *nifH* genes, and the proportion of Cluster III sequences was strongly  
607 decreased. While considering the potential for the previous mentioned PCR bias and thereby  
608 only carefully interpreting the data quantitatively, these observations suggest that

609 cyanobacteria were more actively expressing *nifH* genes than SRB within Cluster III. These  
610 data are supported by our NanoSIMS analyses, in which *Deltaproteobacteria* were not  
611 significantly enriched in  $^{15}\text{N}$  relative to controls (average of 0.3766 at%  $^{15}\text{N}$ ,  $p=0.170$ ).

612         However, since these cells were stained by CARD-FISH in contrast to the analyzed  
613 *Cyanobacteria*, it is theoretically possible that introduction of  $^{14}\text{N}$ -containing compounds  
614 during the staining procedure led to an underestimation of the actual  $^{15}\text{N}$  enrichment in these  
615 cells. In tests analyzing the effect of CARD-FISH on the  $^{15}\text{N}$  and  $^{13}\text{C}$  isotope content (we  
616 included  $^{13}\text{C}$  to more generally analyze this effect) of two isotopically labeled reference  
617 species, we indeed detected (via NanoSIMS measurements) a significantly reduced  $^{15}\text{N}$  and  
618  $^{13}\text{C}$  isotope content in these cells (Figure 6,  $p<0.001$ ). To test whether the deltaproteobacterial  
619  $^{15}\text{N}$  isotope enrichment measured by NanoSIMS were strongly influenced by CARD-FISH,  
620 we used the data from the reference culture experiments (28% dilution as a worst case  
621 scenario for N) to back-calculate the  $^{15}\text{N}$  isotope enrichment the deltaproteobacterial cells  
622 could have had prior to the CARD-FISH procedure. Based on these calculations, when the  
623 CARD-FISH  $^{15}\text{N}$  dilution is accounted for, the deltaproteobacterial  $^{15}\text{N}$  isotope fraction  
624 values increase only slightly, and their corrected values are still not significantly enriched  
625 above natural abundance values (average of 0.38 at%  $^{15}\text{N}$ ,  $p=0.131$ ). We also considered the  
626 individual measured values (as opposed to the population mean), and found that based on the  
627 uncorrected values, 20.4% of the cells are significantly enriched in  $^{15}\text{N}$  based on a 95%  
628 confidence interval. This number increases to 31.5% if the dilution through CARD-FISH is  
629 taken into account. Based on these data we suggest that the CARD-FISH protocol has an  
630 effect on the  $^{13}\text{C}$  and  $^{15}\text{N}$  isotopic composition. This staining technique and similarly the  
631 halogen *in situ* CARD approaches (Musat *et al.*, 2008; Behrens *et al.*, 2008) used for  
632 halogen-based identification of microbes in NanoSIMS analyses can change the fraction of a  
633 population that is considered enriched if values are close to natural abundance (as was the

634 case for *Deltaproteobacteria* in this study) (see extended discussion of this point in the  
635 Supplementary Information). However, the  $^{15}\text{N}$  enrichment values of investigated  
636 *Deltaproteobacteria* in this study changed very little when CARD-FISH dilution was  
637 accounted for, supporting our previous conclusion that the targeted *Deltaproteobacteria* were  
638 not significantly enriched in  $^{15}\text{N}$ .

639

#### 640 **Summary**

641 Based on a combined approach of inhibitor experiments, *nifH* gene and transcript  
642 sequencing and  $^{15}\text{N}_2$  incubations coupled to single-cell isotope analysis, we did not find  
643 support that the analyzed deltaproteobacterial SRB contributed to  $\text{N}_2$  fixation in intertidal  
644 mats from Laguna Ojo de Liebre. Instead, the combined data indicate that *Cyanobacteria*  
645 related to *Lyngbya* spp. were the most active  $\text{N}_2$ -fixers in the mats at the investigated time.

646

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660

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662

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664

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## 666 **References**

667 Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA. (1990). Combination  
668 of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed  
669 microbial populations. *Appl Environ Microbiol* 56:1919–1925.

670 Baumgartner LK, Reid RP, Dupraz C, Decho AW, Buckley DH, Spear JR, Przekop KM,  
671 Visscher PT. (2006). Sulfate reducing bacteria in microbial mats: Changing paradigms, new  
672 discoveries. *Sediment Geol* 185:131-145.

673 Bebout B, Fitzpatrick M, Paerl H. (1993). Identification of the sources of energy for nitrogen  
674 fixation and physiological characterization of nitrogen-fixing members of a marine microbial  
675 mat community. *Appl Environ Microbiol* 59:1495–1503.

676 Bebout B, Paerl H, Bauer J, Canfield DE, Des Marais DJ. (1994). Nitrogen cycling in  
677 microbial mat communities: the quantitative importance of N-fixation and other sources of N  
678 for primary productivity. In: Stal LJ and Caumette P (eds.). *Microbial Mats*. Springer-Verlag:  
679 Berlin pp 265–272.

680 Behrens S, Lösekann T, Pett-Ridge J, Weber PK, Ng W-O, Stevenson BS, *et al.* (2008).  
681 Linking microbial phylogeny to metabolic activity at the single-cell level by using enhanced  
682 element labeling-catalyzed reporter deposition fluorescence in situ hybridization (EL-FISH)  
683 and NanoSIMS. *Appl Environ Microbiol*. 74:3143–3150.

684 Bergman B, Gallon J, Rai A, Stal L. (1997). N<sub>2</sub> fixation by non-heterocystous cyanobacteria.  
685 *FEMS Microbiol Rev* 19:139–185.

686 Burow L, Woebken D, Marshall I, Lindquist E, Bebout B, Prufert-Bebout L, *et al.* (2013).  
687 Anoxic carbon flux in photosynthetic microbial mats as revealed by metatranscriptomics.  
688 *ISME J* 7: 817-829.

689 Burow LC, Woebken D, Bebout BM, McMurdie PJ, Singer SW, Pett-Ridge J, *et al.* (2012).  
690 Hydrogen production in photosynthetic microbial mats in the Elkhorn Slough estuary,  
691 Monterey Bay. *ISME J* 6:863–874.

692 Canfield DE, Des Marais DJ. (1991). Aerobic sulfate reduction in microbial mats. *Science*

- 693 251:1471–1473.
- 694 Chen YB, Dominic B, Mellon MT, Zehr JP. (1998). Circadian rhythm of nitrogenase gene  
695 expression in the diazotrophic filamentous nonheterocystous cyanobacterium *Trichodesmium*  
696 sp. strain IMS 101. *J Bacteriol* 180:3598–3605.
- 697 Cliff JB, Gaspar DJ, Bottomley PJ, Myrold DD. (2002). Exploration of inorganic C and N  
698 assimilation by soil microbes with time-of-flight secondary ion mass spectrometry. *Appl*  
699 *Environ Microbiol* 68:4067–4073.
- 700 de Bont JAM. (1976). Oxidation of ethylene by soil bacteria. *A Van Leeuw J Microb* 42:59-  
701 71.
- 702 Dekas AE, Poretsky RS, Orphan VJ. (2009). Deep-sea archaea fix and share nitrogen in  
703 methane-consuming microbial consortia. *Science* 326:422–426.
- 704 Des Marais DJ. (2003). Biogeochemistry of hypersaline microbial mats illustrates the  
705 dynamics of modern microbial ecosystems and the early evolution of the biosphere. *Biol Bull*  
706 204:160–167.
- 707 Elsgaard L. (2013). Reductive transformation and inhibitory effect of ethylene under  
708 methanogenic conditions in peat-soil. *Soil Biol Biochem* 60: 19-22.
- 709 Everroad RC, Woebken D, Singer SW, Burow LC, Kyrpides N, Woyke T, *et al.* (2013).  
710 Draft genome sequence of an *Oscillatorian* cyanobacterium, Strain ESFC-1. *Genome*  
711 *Announc* 1:e00527-13.
- 712 Faure G. (1986). *Principles of Isotope Geology*. John Wiley & Sons, Inc.: New York, pp 141-  
713 151.
- 714 Fike DA, Gammon CL, Ziebis W, Orphan VJ. (2008). Micron-scale mapping of sulfur  
715 cycling across the oxycline of a cyanobacterial mat: a paired nanoSIMS and CARD-FISH  
716 approach. *ISME J* 2:749–759.
- 717 Fleming E, Bebout B., Castenholz RW. (2007). Effects of salinity and light on the  
718 resumption of photosynthesis in rehydrated cyanobacterial mats from Baja California Sur,  
719 Mexico. *J Phycol* 43: 15-24.
- 720 Foster RA, Kuypers MMM, Vagner T, Paerl RW, Musat N, Zehr JP. (2011). Nitrogen  
721 fixation and transfer in open ocean diatom-cyanobacterial symbioses. *ISME J* 5:1484-1493.
- 722 Gaby JC, Buckley DH. (2011). A global census of nitrogenase diversity. *Environ Microbiol*  
723 13:1790–1799.
- 724 Griffiths M, Gallon J. (1987). The diurnal pattern of dinitrogen fixation by cyanobacteria *in*  
725 *situ*. *New Phytol* 107:649–657.
- 726 Halm H, Musat N, Lam P, Langlois R, Musat F, Peduzzi S, *et al.* (2009). Co-occurrence of  
727 denitrification and nitrogen fixation in a meromictic lake, Lake Cadagno (Switzerland).  
728 *Environ Microbiol* 11:1945–1958.
- 729 Herbert RA. (1999). Nitrogen cycling in coastal marine ecosystems. *FEMS Microbiol Rev*

- 730 23:563–590.
- 731 Hoshino T, Yilmaz LS, Noguera DR, Daims H, Wagner M. (2008). Quantification of target  
732 molecules needed to detect microorganisms by fluorescence *in situ* hybridization (FISH) and  
733 catalyzed reporter deposition-FISH. *Appl Environ Microbiol* 74:5068–5077.
- 734 Jackson ZL, Burow LC, Woebken D, Everroad RC, Kubo MD, Spormann AM, Weber PK,  
735 Pett-Ridge J, Bebout BM, Hoehler TM. (In review). Fermentation couples Chloroflexi and  
736 sulfate-reducing bacteria to Cyanobacteria in hypersaline microbial mats
- 737 Joey S, Paerl H. (1994). Nitrogen cycling in microbial mats: rates and patterns of  
738 denitrification and nitrogen fixation. *Mar Biol* 119:285–295.
- 739 Kim K, Zhang Y, Roberts GP. (1999). Correlation of activity regulation and substrate  
740 recognition of the ADP-ribosyltransferase that regulates nitrogenase activity in  
741 *Rhodospirillum rubrum*. *J Bacteriol* 181:1698–1702.
- 742 Koene-Cottaar FHM, Schraa G. (1998). Anaerobic reduction of ethene to ethane in an  
743 enrichment culture. *FEMS Microbiol Ecol* 25: 251–256.
- 744 Lechene C, Hillion F, McMahon G, Benson D, Kleinfeld AM, Kampf JP, *et al.* (2006). High-  
745 resolution quantitative imaging of mammalian and bacterial cells using stable isotope mass  
746 spectrometry. *J Biol* 5:20.
- 747 Ley RE, Harris JK, Wilcox J, Spear JR, Miller SR, Bebout BM, *et al.* (2006). Unexpected  
748 diversity and complexity of the Guerrero Negro hypersaline microbial mat. *Appl Environ*  
749 *Microbiol* 72:3685–3695.
- 750 Lovley DR, Dwyer DF, Klug MJ. (1982) Kinetic analysis of competition between sulfate  
751 reducers and methanogens for hydrogen in sediments. *Appl Environ Microbiol* 43:1373-  
752 1379.
- 753 Loy A, Lehner A, Lee N, Adamczyk J, Meier H, Ernst J, *et al.* (2002). Oligonucleotide  
754 microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing  
755 prokaryotes in the environment. *Appl Environ Microbiol* 68: 5064–5081.
- 756 Lückner S, Steger D, Kjeldsen KU, MacGregor BJ, Wagner M, Loy A. (2007). Improved 16S  
757 rRNA-targeted probe set for analysis of sulfate-reducing bacteria by fluorescence *in situ*  
758 hybridization. *J Microbiol Meth* 69:523–528.
- 759 Minz D, Fishbain S, Green SJ, Muyzer G, Cohen Y, Rittmann BE, Stahl DA. (1999).  
760 Unexpected population distribution in a microbial mat community: Sulfate-reducing bacteria  
761 localized to the highly oxic chemocline in contrast to a eukaryotic preference for anoxia.  
762 *Appl Environ Microbiol* 65:4659–4665.
- 763 Moisaner PH, Shiue L, Steward GF, Jenkins BD, Bebout BM, Zehr JP. (2006). Application  
764 of a *nifH* oligonucleotide microarray for profiling diversity of N<sub>2</sub>-fixing microorganisms in  
765 marine microbial mats. *Environ Microbiol* 8:1721–1735.
- 766 Musat N, Halm H, Winterholler B, Hoppe P, Peduzzi S, Hillion F, *et al.* (2008). A single-cell  
767 view on the ecophysiology of anaerobic phototrophic bacteria. *Proc Natl Acad Sci USA*  
768 105:17861–17866.

- 769 Muyzer G, Stams AJM. (2008). The ecology and biotechnology of sulphate-reducing  
770 bacteria. *Nat Rev Microbiol* 6:441–454.
- 771 Omoregie E, Crumbliss L, Bebout B, Zehr J. (2004a). Determination of nitrogen-fixing  
772 phylotypes in *Lyngbya* sp and *Microcoleus chthonoplastes* cyanobacterial mats from  
773 Guerrero Negro, Baja California, Mexico. *Appl Environ Microbiol* 70:2119–2128.
- 774 Omoregie EO, Crumbliss LL, Bebout BM, Zehr JP. (2004b). Comparison of diazotroph  
775 community structure in *Lyngbya* sp. and *Microcoleus chthonoplastes* dominated microbial  
776 mats from Guerrero Negro, Baja, Mexico. *FEMS Microbiol Ecol* 47:305–308.
- 777 Oremland RS. (1981). Microbial formation of ethane in anoxic estuarine sediments. *Appl*  
778 *Environ Microbiol* 42:122-129.
- 779 Oremland RS, Capone DG. (1988). Use of specific inhibitors in biogeochemistry and  
780 microbial ecology. *Adv Microb Ecol* 10:285–383.
- 781 Paerl H, Prufert L, Ambrose W. (1991). Contemporaneous N<sub>2</sub> fixation and oxygenic  
782 photosynthesis in the nonheterocystous mat-forming cyanobacterium *Lyngbya aestuarii*.  
783 *Appl Environ Microbiol* 57:3086–3092.
- 784 Paerl HW, Fitzpatrick M, Bebout BM. (1996). Seasonal nitrogen fixation dynamics in a  
785 marine microbial mat: Potential roles of cyanobacteria and microheterotrophs. *Limnol*  
786 *Oceanogr* 41:419–427.
- 787 Pernthaler A, Pernthaler J, Amann R. (2002). Fluorescence *in situ* hybridization and  
788 catalyzed reporter deposition for the identification of marine bacteria. *Appl Environ*  
789 *Microbiol* 68:3094–3101.
- 790 Pinckney JL, Paerl HW. (1997). Anoxygenic photosynthesis and nitrogen fixation by a  
791 microbial mat community in a bahamian hypersaline lagoon. *Appl Environ Microbiol*  
792 63:420–426.
- 793 Ploug H, Musat N, Adam B, Moraru CL, Lavik G, Vagner T, *et al.* (2010). Carbon and  
794 nitrogen fluxes associated with the cyanobacterium *Aphanizomenon* sp. in the Baltic Sea.  
795 *ISME J* 4:1215–1223.
- 796 Ploug H, Adam B, Musat N, Kalvelage T, Lavik G, Wolf-Gladrow D, *et al.* (2011). Carbon,  
797 nitrogen and O<sub>2</sub> fluxes associated with the cyanobacterium *Nodularia spumigena* in the  
798 Baltic Sea. *ISME J* 5:1549–1558.
- 799 Polz MF, Cavanaugh CM. (1998). Bias in template-to-product ratios in multitemplate PCR.  
800 *Appl Environ Microbiol* 64:3724–3730.
- 801 Popa R, Weber PK, Pett-Ridge J, Finzi JA, Fallon SJ, Hutcheon ID, *et al.* (2007). Carbon and  
802 nitrogen fixation and metabolite exchange in and between individual cells of *Anabaena*  
803 *oscillarioides*. *ISME J* 1:354–360.
- 804 Risatti JB, Capman WC, Stahl DA. (1994). Community structure of a microbial mat: the  
805 phylogenetic dimension. *Proc Natl Acad Sci USA* 91:10173–10177.
- 806 Roller C, Wagner M, Amann R, Ludwig W, Schleifer KH. (1994). *In situ* probing of gram-

807 positive bacteria with high DNA G+C content using 23S rRNA-targeted oligonucleotides.  
808 Microbiology 140:2849–2858.

809 Rothrock MJ Jr, Garcia-Pichel F. (2005). Microbial diversity of benthic mats along a tidal  
810 desiccation gradient. Environ Microbiol 7:593–601.

811 Stal LJ and Bermann B. (1990). Immunological characterization of nitrogenase in the  
812 filamentous non-heterocystous cyanobacterium *Oscillatoria limosa*. Planta 182:287-291.

813 Stal LJ and Krumbein WE (1981). Aerobic nitrogen fixation in pure cultures of a benthic  
814 marine *Oscillatoria* (cyanobacteria). FEMS Microbiol Lett., 11:295-298.

815 Stal L, Grossberger S, Krumbein W. (1984). Nitrogen-fixation associated with the  
816 cyanobacterial mat of a marine laminated microbial ecosystem. Mar Biol 82:217–224.

817 Steppe T, Olson J, Paerl H, Litaker R, Belnap J. (1996). Consortial N<sub>2</sub> fixation: A strategy for  
818 meeting nitrogen requirements of marine and terrestrial cyanobacterial mats. FEMS  
819 Microbiol Ecol 21:149–156.

820 Steppe T, Paerl H. (2002). Potential N<sub>2</sub> fixation by sulfate-reducing bacteria in a marine  
821 intertidal microbial mat. Aquat Microb Ecol 28:1–12.

822 Suzuki MT, Giovannoni SJ. (1996). Bias caused by template annealing in the amplification  
823 of mixtures of 16S rRNA genes by PCR. Appl Environ Microbiol 62:625–630.

824 Wallner G, Amann R, Beisker W. (1993). Optimizing fluorescent *in situ* hybridization with  
825 rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms.  
826 Cytometry 14:136–143.

827 Wang Q, Garrity GM, Tiedje JM, Cole JR. (2007). Naive Bayesian classifier for rapid  
828 assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol  
829 73:5261–5267.

830 Woebken D, Burow LC, Prufert-Bebout L, Bebout BM, Hoehler TM, Pett-Ridge J, *et al.*  
831 (2012). Identification of a novel cyanobacterial group as active diazotrophs in a coastal  
832 microbial mat using NanoSIMS analysis. ISME J 6:1427–1439.

833 Zehr J, Mellon M, Braun S, Litaker W, Steppe T, Paerl H. (1995). Diversity of heterotrophic  
834 nitrogen fixation genes in a marine cyanobacterial mat. Appl Environ Microbiol 61:2527–  
835 2532.

836 Zehr JP, Turner PJ. (2001). Nitrogen fixation: Nitrogenase genes and gene expression. In:  
837 Paul JH (ed.). Methods in Microbiology. Academic Press: New York pp 271–286.

838 Zehr J, Jenkins B, Short S. (2003). Nitrogenase gene diversity and microbial community  
839 structure: a cross-system comparison. Environ Microbiol 5:539–554.

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844 **Titles and legends to figures**

845

846 Figure 1: Acetylene reduction assay as a proxy for N<sub>2</sub> fixation in intertidal microbial mats  
847 from Laguna Ojo de Liebre, Baja California, Mexico. A: The diel cycle experiment was  
848 conducted in Guerrero Negro, Mexico, before the mats were transported to California, USA,  
849 for detailed analysis. Two replicate diel cycle experiments are shown in the graphs. Each  
850 time point measurement in each diel cycle experiment was conducted in triplicate (values in  
851 graphs depict the average of the 3 replicate measurements per time point per experiment  
852 including the standard deviation as error bars). 1B: Effect of DCMU on acetylene reduction  
853 rates in intertidal mats from Laguna Ojo de Liebre. 1C: Effect of molybdate on acetylene  
854 reduction rates.

855

856 Figure 2: <sup>15</sup>N enrichment (at%) of mat cores that were incubated with <sup>15</sup>N<sub>2</sub> for 10 h in the  
857 dark measured by IRMS. Average values of three biological replicates per treatment are  
858 depicted with standard errors (for “0 to 2 mm depth” n=10). Asterisks indicate significantly  
859 different paired treatments at p<0.05. Natural abundance of 0.37 at% is indicated by a grey  
860 horizontal line.

861

862 Figure 3: Microbial community analysis based on 16S rRNA gene and transcript sequencing  
863 of the upper 2 mm of intertidal mats at Laguna Ojo de Liebre. Phyla depicted are those that  
864 contain ≥ 0.1% of sequences detected by either Sanger sequencing (dark blue and dark red  
865 bars) or 454 amplicon sequencing (bars in light blue light and red). Each sample type shows  
866 the average value of 2 biological replicates. Both approaches illustrate a diverse community  
867 based on DNA analysis, with most of the sequences grouping within *Proteobacteria*,

868 *Bacteroidetes*, *Chloroflexi* and *Cyanobacteria*. Sequences based on cDNA are strongly  
869 dominated by *Cyanobacteria* (up to 74% of the sequences), followed by *Proteobacteria*,  
870 *Bacteroidetes* and *Chloroflexi*.

871

872 Figure 4: Taxonomic classification of *nifH* gene and transcript sequences in the upper 2 mm  
873 layer of intertidal microbial mats from Laguna Ojo de Liebre. Sequences derived from DNA  
874 samples are depicted in blue (n = 341), from cDNA in red (n = 535), and from cDNA of  
875 molybdate treated samples in light red (n = 187). PCR amplification of cDNA from the  
876 DCMU treatment yielded no products.

877

878 Figure 5: Single-cell isotope measurements by NanoSIMS of  $^{15}\text{N}_2$ -incubated mat samples  
879 from Laguna Ojo de Liebre. A: Elemental composition images ( $^{12}\text{C}^{14}\text{N}$  and  $^{15}\text{N}$  at%) of  
880 *Lyngbya* spp. filaments. However, as described in Methods and Material section, image  
881 analysis of *Lyngbya* spp. was not suitable for quantitative analysis of these large cells  
882 (instead isotope enrichment measurements were done by accelerated sputtering utilizing high  
883 primary ion beam currents (~1 nA, 2  $\mu\text{m}$  beam size)). Therefore,  $^{15}\text{N}$  isotopic composition  
884 depicted in these images will be an underestimation of the actual enrichment in  $^{15}\text{N}$ . B and C:  
885 Epifluorescence micrograph and elemental composition images ( $^{12}\text{C}^{14}\text{N}$  and  $^{15}\text{N}$  at%) of  
886 analyzed small filamentous cyanobacteria. Filamentous cyanobacteria were identified based  
887 on their autofluorescence. D: Epifluorescence micrograph depicting cells stained by DAPI  
888 and deltaproteobacterial cells stained by CARD-FISH (with probe-mix DELTA495 a-c), as  
889 well as elemental composition images ( $^{12}\text{C}^{14}\text{N}$  and  $^{15}\text{N}$  at%). E: Boxplot diagram  
890 summarizing all measurements of the three cell types. The number of cells (or individual  
891 filaments) analyzed is indicated.  $^{15}\text{N}$  enrichments are depicted in at%; natural abundance is

892 0.37 at% (indicated by a horizontal line). Lowercase letters indicate significantly different  
893 isotopic compositions in different cell types.

894 Scale bars represent 5  $\mu\text{m}$ . Please note the different scales for  $^{15}\text{N}$  at% values.

895

896 Figure 6: Single-cell isotope measurements by NanoSIMS of  $^{13}\text{C}$  and  $^{15}\text{N}$  co-labeled *E. coli*  
897 (circles) and *B. subtilis* (triangles) cells. The effect of the CARD-FISH procedure on the  
898 enrichment in  $^{13}\text{C}$  and  $^{15}\text{N}$  of  $\sim 99$  at% (large graph) and  $\sim 6$  at% (small graph) labeled cells  
899 was investigated.  $^{13}\text{C}$  and  $^{15}\text{N}$  isotope fractions are displayed for formaldehyde-fixed *E. coli*  
900 (blue circles) and ethanol-fixed *B. subtilis* cells (blue triangles), which served as references in  
901 calculations of dilution factors (see Equation 1 in text). Both species show a significant  
902 decrease in the tracer contents after CARD-FISH using a probe targeting most bacteria  
903 (EUB338, red symbols). Deposition of C- and N-atoms within the cells during the CARD-  
904 FISH procedure independent from a hybridized probe was analyzed using the negative  
905 control probe nonEUB338 (green symbols).

906 Displayed data refer to the arithmetic mean  $\pm$  the standard deviation (SD) over the analyzed  
907 cells per treatment (partly performed in replicate - as indicated by multiple identical symbols  
908 of the same color). On average 30 cells were measured in each analysis. Supplementary  
909 Figure 8 depicts the same data as in Figure 6, including identifiers for each sample, which  
910 enables cross-referencing with the data obtained from individual cells, listed in  
911 Supplementary Tables 4 to 6.