Supplementary Information

Supplementary Materials and Methods

Acetylene reduction assays

Nitrogenase activity was measured with the acetylene reduction assay (ARA) as previously described (Bebout *et al.* 1993). Mat cores (10 mm diameter, 5 mm thick, including a dark sediment layer, see Supplementary Figure 2) were sampled in triplicate from mat slabs every 3 hours and placed into serum bottles (total volume 38 ml) containing 20 ml of seawater. The serum bottles were capped with gas-tight rubber stoppers, and 5 ml of the 18-ml headspace was exchanged for acetylene that was injected through the stopper into the aqueous phase to start the incubation. Mat cores were incubated with acetylene for 3 hours. Triplicate water samples without mat cores served as negative controls. Ethylene was quantified in a Shimadzu GC-14A gas chromatograph as described previously (Bebout *et al.* 1993). Rates presented are means of three replicates ± standard deviation.

$^{15}N_2$ incubations

For $^{15}N_2$ incubation experiments, mat cores (10 mm diameter, 5 mm thick, Supplementary Figure 2) were transferred to a 14 ml serum vial, covered with 1 ml of *in situ* water and capped with gas-tight rubber stoppers. The headspace was exchanged with a mixture of 78% $^{15}N_2$ gas (>98 at% ^{15}N; Cambridge Isotope Laboratories, Andover, MA, USA), 21% O_2 and 0.038% CO_2. Mats were incubated in triplicate in the dark for 10 hours, and subsequently, half of the mat cores were sectioned for bulk isotope analysis in two depth intervals (0 to 2 mm and 2 to 4 mm). The other half of the sectioned cores were preserved for NanoSIMS analysis by fixation in 4% paraformaldehyde (PFA) as previously described (Amann *et al.*, 1990). Mat cores incubated in air (without $^{15}N_2$) were treated equally and served as controls.

DNA and RNA Extraction

RNA and DNA were co-extracted from the uppermost 2 mm of three to four pooled mat cores by combining phenol-chloroform extraction with parts of the RNeasyMini and QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA), respectively. For each core (10 mm diameter, upper 2 mm), biomass was transferred to a tube containing 0.5 ml RLT^{TM} buffer and homogenized using a rotor-stator homogenizer (Omni International, Kennesaw, GA, USA). The suspension was bead-beated (BioSpec Products, Bartlesville, OK, USA) with zirconium beads (200 μm, OPS Diagnostics, Lebanon, USA) and centrifuged at 8,000 x g for 1 minute. Supernatants from the four mat cores were pooled and split into two aliquots; one for RNA and the other for DNA extraction. Each aliquot was extracted with phenol-chloroform-isoamyl alcohol (125:24:1, pH 4.5 for RNA and 25:24:1, pH 8.0 for DNA extraction) and the aqueous phase was further purified following the manufacturers' protocols. For the RNA samples, the aqueous phase was run through the gDNA eliminator spin column (QIAGEN) to remove genomic DNA and further treated with TURBO DNase^{TM} (Applied Biosystems/Ambion, Austin, TX, USA). Isolated RNA was reverse transcribed into single-stranded cDNA using the SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA).

Amplification and sequencing of 16S rRNA and *nifH* genes and transcripts

For construction of 454 pyrotag amplicon libraries, the V6-V8 region of the 16S rRNA (from the cDNA) and 16S rRNA genes (from DNA) was PCR amplified using the universal primers 926F (Lane, 1991) and 1392R (Lane *et al.*, 1985). The reverse primer included the adaptor sequence and a five-base barcode. Sequences were generated at the

Research and Testing Laboratory (Lubbock, Texas, USA) using the GS FLX Titanium Series Reagents (454 Life Sciences, Branford, CT, USA).

16S rRNA and 16S rRNA gene clone libraries of two biological replicates were constructed from single-stranded cDNA and DNA, respectively, by amplification with the broadly inclusive bacterial primers 27F and 1391R (Lane, 1991).

The *nifH* genes were PCR amplified from DNA and cDNA with a nested PCR protocol (Zehr and Turner, 2001). The primer sites were described as being conserved throughout *nifH* genes in clusters I, II, III and IV. The first PCR was conducted with primers nifH4 and nifH3, followed by a second PCR reaction with primers nifH1 and nifH2. The final PCR products were approximately 359 bp in length. Mat samples treated with DCMU during the diel cycle study failed to produce any detectable PCR product from cDNA with *nifH*-targeting primers (tested in two replicate RNA extractions). To test for potential inhibition of cDNA synthesis or PCR caused by residual amount of DCMU, we conducted general 16S rRNA PCR with the cDNA template and *nifH*-specific PCRs with co-extracted DNA samples. These reactions resulted in PCR products, indicating that residual amounts of DCMU did not inhibit cDNA synthesis or PCR, and therefore were not the cause for the failed PCR amplifications of *nifH* transcripts from DCMU-treated mat cDNA. We conclude that the level of *nifH* transcripts in DCMU treated samples was below the detection limit for successful PCR amplification.

For both, 16S rRNA and *nifH* libraries, PCR products of five replicate PCR reactions were pooled and purified using the Qiagen Min Elute PCR Purification Kit (Qiagen). Purified PCR amplicons were cloned using the TOPO TA Cloning Kit for sequencing (Invitrogen) according to the manufacturer's protocol. Clones were screened for inserts, and positive clones were sequenced by Beckman Coulter Genomics, Inc. (Danvers, MA, USA) with the primer M13F.

Sequence analysis

The replicate 16S rRNA and 16S rRNA gene 454 pyrotag amplicon libraries were processed individually (sorting, trimming, removing reads of low quality and classification) using the RDP pipeline with standard settings (http://pyro.cme.msu.edu) (Supplementary Table 1). Reads were taxonomically assigned using the RDP Classifier (Wang *et al.*, 2007). Sequencing resulted in 20,616 and 15,524 reads from both DNA templates and 20,138 and 22,246 reads from both cDNA templates.

16S rRNA and 16S rRNA gene clone libraries of two biological replicates were constructed from single-stranded cDNA and DNA. In total, 520 sequences (of 600 to 700 bp length) were derived from DNA samples (D3= 256, and D5= 264 sequences) and 316 sequences from cDNA samples (C3= 150, and C5= 166 sequences). Partial 16S rRNA and 16S rRNA gene Sanger sequences of clone libraries were taxonomically assigned using the RDP Classifier. For phylogenetic analysis, sequences were aligned using the SILVA Incremental Aligner (SINA) (Pruesse *et al.* 2012) and imported in the ARB program (Ludwig *et al.* 2004). 16S rRNA gene and transcript sequences were sorted into two bins depending whether they represented the 3' or 5' end of the 16S rRNA (gene). The closest relatives of these sequences were retrieved in SILVA (Pruesse *et al.* 2007) (http://www.arb-silva.de/). Phylogenetic trees were calculated with nearly full-length reference sequences using maximum likelihood, maximum parsimony and neighbor joining algorithms, with and without a 50% position variability filter. The partial sequences determined in this study were added with the quick add parsimony function in ARB. Bootstrap values were calculated in Geneious 5.5.6 with the PhyML algorithm, using 100 bootstrap trees.

We retrieved a total of 313 *nifH* sequences from DNA, 522 sequences from cDNA and 181 from cDNA of the molybdate inhibition experiment. *NifH* gene/transcript sequences

were quality checked in Geneious. Deduced amino acid sequences of the *nifH* genes/transcripts along with closest related *NifH* sequences collected from NCBI and reference sequences from the Zehr laboratory *nifH* database (http://www.es.ucsc.edu/~wwwzehr/research/database) were locally aligned in MUSCLE (Edgar 2004) and imported into the ARB program. Phylogenetic trees were constructed based on deduced amino acid sequences using maximum likelihood, maximum parsimony and neighbor joining algorithms. Bootstrap values were calculated as described above. Representative chlorophyllide reductase genes were included in the *NifH* database to detect sequences possibly related to those genes in the sample, but none of the newly derived sequences grouped with those genes.

NanoSIMS analysis of Laguna Ojo de Liebre mat samples

Mat samples of the upper 2 mm were transferred with tweezers onto 5 x 5 µm silicon wafer pieces (Ted Pella, Redding, CA, USA), teased apart, attached by drying and subsequently washed in ultrapure water (MQ, Millipore). In experiments where catalyzed reporter deposition- fluorescence *in situ* hybridization (CARD-FISH) was combined with NanoSIMS analysis, wafers were coated with VectaBond (Vector Laboratories, Burlingame, CA, USA) prior to sample deposition. Samples were gold coated after light and fluorescence imaging and before scanning electron microscopy (SEM; FEI Inspect F, FEI, Hillsboro, OR, USA) and NanoSIMS imaging to mitigate sample charging and ease imaging.

Target cells were identified by comparing the NanoSIMS $^{12}C^{14}N^-$ secondary ion and secondary electron images, the respective SEM image, and for *Deltaproteobacteria* the CARD-FISH image. Prior to data acquisition, analysis areas were pre-sputtered utilizing a high intensity Cs^+ primary ion beam to ensure that the analyzed volumes were located within the cells. For small filamentous *Cyanobacteria*, typically 3–10 connected cells within a filament were analyzed collectively, thus yielding the average isotopic composition per filament. Due to the large size of the *Lyngbya* spp.-related *Cyanobacteria*, isotope enrichment measurements were done by accelerated sputtering utilizing high primary ion beam currents (~1 nA, 2 µm beam size). Negative-control samples of *Deltaproteobacteria* and *Lyngbya* spp.-related *Cyanobacteria* with natural isotopic composition were analyzed for reference. Instrumental mass fractionation was less than 3%. Internal precision for individual cell measurements was approximately 1%.

Data were acquired as images by scanning a ~100 nm diameter primary ion beam over areas between 10 x 10 and 50 x 50 μm^2. Images were recorded as multilayer-stacks, each consisting of 5 to 15 individual cycles (i.e. layers). Secondary electrons were detected simultaneously to secondary ions to facilitate target cell identification. Individual images were corrected for detector dead-time and image drift from layer to layer prior to stack accumulation. Data were processed to generate quantitative isotope ratio images from ion images using custom software (LIMAGE, L. Nittler, Carnegie Institution of Washington). Regions of interest (ROIs), referring to individual cells, were manually defined based on the $^{12}C^{14}N^-$ secondary ion maps and cross-checked by the topographical/morphological appearance in secondary electron images. The isotopic composition for each ROI was determined by averaging over the individual images of the multilayer stack. Data are presented as isotope fractions given atomic percent [at%] as calculated from isotope ratios via: isotope fraction [at%] = R / (R + 1) *100,
where R is the ratio of the relative amounts of the heavy and the light isotope, i.e. ^{15}N and ^{14}N. Data refer to the arithmetic mean of all NanoSIMS measurements per cell type, ± standard error (SE) over the analyzed cells.

Assessment of CARD-FISH effects on single-cell isotope composition

The CARD-FISH procedure can have an effect on the isotopic composition ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) of cells, since cells will be exposed to reagents that contain C and N (such as lysozyme, hybridized DNA probes labeled with peroxidase, dextrane sulfate, blocking reagent) during lysozyme treatment, hybridization and amplification that will not be completely removed during the washing step. Deposition of fluorescently-labeled tyramides in the signal-amplification step further introduces C and N in the cells. Furthermore, if the assimilated isotopes are mainly present in small molecules or storage compounds, these might be preferentially lost (compared to other cellular material) during the CARD-FISH procedure. In order to evaluate the potential dilution effect of the CARD-FISH procedure, we applied CARD-FISH to isotopically labeled *Escherichia coli* (DSM 498) and *Bacillus subtilis* (DSM 8439) cells, examples of Gram-negative and -positive cells, respectively.

Bacterial strains, growth conditions and cell fixation. Escherichia coli K12 (DSM 498) and *Bacillus subtilis* (DSM 8439) were used as model organisms in this study since they possess Gram-positive and Gram-negative cell walls, respectively. Different fixation methods are commonly applied in FISH (and CARD-FISH) of Gram-negative and Gram-positive bacteria (Roller *et al.* 1994). Therefore, we aimed at testing the CARD-FISH effect on cells with different cell wall structures in combination with their respective fixation methods, which could result in different loss (washout) of cell compounds and material deposition during the CARD-FISH procedure. Cells were grown in defined medium consisting of Na_2HPO_4 (12.8 g l^{-1}), KH_2PO_4 (3.0 g l^{-1}), NaCl (0.5 g l^{-1}), and NH_4Cl (1.0 g l^{-1}). After autoclaving, 5 ml of a filter-sterilized solution containing D-glucose (2.0 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 g l^{-1}), $CaCl_2 \cdot 2H_2O$ (0.015 g l^{-1}) and $FeSO_4 \cdot 7H_2O$ (0.01 g l^{-1}) and 1 ml of sterile selenite-tungsten solution (Widdel and Pfennig, 1981) were added. Experiments were performed with approx. 6% and 99% stable isotope labeled D-glucose and ammonium chloride as the sole sources of carbon and nitrogen in this medium by using ^{13}C D-glucose (^{13}C, 99%; Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA) and ^{15}N ammonium chloride (^{15}N, 99%; Cambridge Isotope Laboratories, Inc.). The cultures were incubated overnight at 37°C with shaking at 200 rpm in 15 ml glass tubes filled with 5 ml of media. Two transfers of cells (50 µl) to fresh media (5 ml) were carried out in order to ensure equal labeling of all cells. Finally, 5 ml of media were inoculated for a third time, and cell growth was monitored by measuring the optical density at 578 nm at every hour. Cells were harvested at an OD_{578} of 0.6 in the exponential phase, washed twice with ice-cold 1 x phosphate buffered saline (1xPBS, 8.475 g NaCl, 1.093 g Na_2HPO_4, and 0.276 g NaH_2PO_4 l^{-1} in ultrapure water; pH 7.4), and collected again by centrifugation (14000 x g, for 5 min at 4°C). *B. subtilis* cells were fixed by an ethanol-PBS mixture (50/50, 96% ethanol/1xPBS, vol/vol) for 2 h on ice. *E. coli* cells were fixed in 2% formaldehyde in 1xPBS for 3 h at 4°C, washed three times with ice-cold 1xPBS and resuspended in ethanol-PBS mixture (50/50, 96% ethanol/1xPBS, vol/vol). Fixed cells were stored at -20°C until further processing.

For NanoSIMS measurements, cells were immobilized onto boron-doped silicon wafers (7 × 7 × 0.5 mm), air-dried at 46°C for 5 min and rinsed with ultrapure water (MQ, Millipore). The CARD-FISH experiments were directly performed on these silicon wafers.

Oligonucleotide probes and CARD-FISH. For all CARD-FISH experiments the 16S rRNA-targeted oligonucleotide probe EUB338-I (Amann *et al*. 1990) targeting the test strains and many other but not all bacteria was used. As a negative control probe nonEUB338-I (reverse complementary probe to EUB338-I) was applied (Wallner *et al.* 1993). Both probes were 5'-labeled with horseradish peroxidase (HRP) (Thermo Hybaid, Interactiva Division, Ulm, Germany). CARD-FISH was performed as described previously (Pernthaler *et al.* 2002a; Hoshino *et al.* 2008). Tyramide signal amplification was conducted for 30 min at 46°C, which represents a relatively high temperature and long incubation time within the range of

incubation settings typically used in CARD-FISH studies (e.g. Ishii *et al.*, 2004; Pernthaler *et al.*, 2002b). The fluorescence of CARD-FISH hybridized cells was verified by epifluorescence microscopy (see Supplementary Figure 6).

NanoSIMS analysis of E. coli and B. subtilis cells. NanoSIMS measurements of *E. coli and B. subtilis* cells were carried out on a NanoSIMS 50L (Cameca, Gennevilliers Cedex, France) at the Large-Instrument Facility for Advanced Isotope Research, University of Vienna. ^{13}C abundance was inferred from the signal intensities obtained from detection of $^{12}C^-$ and $^{13}C^-$ secondary ions. In order to optimize the counting statistics in the determination of ^{15}N abundance, $^{13}C^{14}N^-$ and $^{13}C^{15}N^-$ secondary ions were detected for highly enriched cells and $^{12}C^{14}N^-$ and $^{12}C^{15}N^-$ secondary ions for samples containing cells with ^{15}N and ^{13}C enrichment levels of ≤ 6 at%. Each sample was analysed 1 to 4 times on distinct measurement areas, with an average number of 30 cells per measurement. Image data were evaluated using the WinImage software package provided by Cameca. Displayed data refer to the arithmetic mean ± standard deviation (SD) over the analysed cells per treatment.

Quantitative evaluation of isotope label dilution. The dilution of the tracer enrichment (e.g., at% ^{15}N) in the sampled cells that is the result of sample preparation, including CARD-FISH treatment, was calculated based on a two end member mixing model (e.g., Faure, 1987) modified to only account for the addition of the tracer element, not all added material. This dilution is defined as $F_{X,add}$, the fraction of the tracer element X (e.g., nitrogen) added to the cells by sample preparation:

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

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

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

Detailed derivation of these equations: The dilution of a tracer in a labeled cell by sample preparation can be quantified based on a two-component mixing model (e.g., Faure 1986). Here we present two derivations of this equation. First we present a short derivation starting from the fraction of the tracer element X (e.g., nitrogen) in the sampled volume that came from the labeled cells, $F_{X,i}$ relative to the fraction of X added by sample preparation, $F_{X,add}$, which is defined as the dilution factor in this study. Second we present a longer derivation based on mass balance, which results in the same relationships. Note that we formulate the mixing models based on individual elements, not total mass.

For the first derivation, we start by stating that by definition for a two component system:

$$F_{X,i} + F_{X,add} = 1. \qquad \text{(S1)}$$

The isotopic composition of these components can be expressed as the tracer abundance, a, in atom percent (at%) of the tracer isotope ${}^{A}X$ relative to the other stable isotopes of element X:

$$a_{{}^{A}X} = \frac{n_{{}^{A}X}}{n_{{}^{A}X} + n_{{}^{B}X} + n_{{}^{C}X} + \ldots} \quad \text{(S2)}$$

where n is the number of atoms of each isotope of element X in the sampled volume and the superscripts A, B, C… refer to the mass number of the respective isotope. For carbon and nitrogen, respectively, eq. S2 simplifies to:

$$a_{{}^{13}C} = \frac{n_{{}^{13}C}}{n_{{}^{13}C} + n_{{}^{12}C}} \quad \text{and} \quad a_{{}^{15}N} = \frac{n_{{}^{15}N}}{n_{{}^{15}N} + n_{{}^{14}N}}.$$

For simplicity in this first derivation, the tracer abundance is represented as a_i, a_{add}, and a_f for the isotopic composition before treatment (initial), added, and after treatment (final), respectively. Using the tracer abundance for the initial and added fractions, we can write an equation for the final tracer abundance in the sample, a_f, for element X:

$$a_i F_{X,i} + a_{add} F_{X,add} = a_f \quad \text{(S3)}$$

Substituting for $F_{X,i}$ from eq. S1 we obtain:

$$a_i (1 - F_{X,add}) + a_{add} F_{X,add} = a_f \quad \text{(S4)}$$

Collecting terms yields:

$$F_{X,add} (a_{add} - a_i) = (a_f - a_i) \quad \text{(S5)}$$

Solving for the added fraction, we have:

$$F_{X,add} = (a_f - a_i)/(a_{add} - a_i), \quad \text{(S6)}$$

which is above-mentioned Eq. 1. Using the measured tracer abundance, a_f, in a cell, this equation allows us to determine the fraction of the tracer element in the measured volume that came from the labeled cell if we use known initial and added isotopic compositions, as in our dilution tests. To calculate the unknown isotopic composition of labeled cells before sample preparation for analysis, we can solve eq. S4 for a_i:

$$a_i = (a_f - a_{add} F_{X,add}) / (1 - F_{X,add}), \quad \text{(S7)}$$

which is above-mentioned Eq. 2. For unknowns, we measure a_f, use an estimate of $F_{add,X}$, and assume that the isotopic composition of the added material is natural (e.g., a_{add} ~1.1 at% ${}^{13}C$ and ~0.37 at% ${}^{15}N$).

For people who are not familiar with mixing models, below we provide a derivation of the above equations based on the *law of mass conservation*, from which follows:
(i) For any considered element X, the total number of atoms contained in a treated cell is equal to the sum of atoms contained in the cell before treatment and the number of atoms added and/or removed by the treatment

$$n_{X,f} = n_{X,i} + n_{X,add} - n_{X,rem} \quad \text{(S8)}$$

where n_X refers to the absolute number of atoms of the respective element *X* (i.e. the sum over all isotopes.) The addenda in the subscripts are acronyms designating the cells *before* and *after* the treatment (i.e., *i* and *f*, respectively), and the *added* and *removed* compounds (i.e., *add.* and *rem.*, respectively).

(ii) The number of tracer atoms n_{A_X} contained in a treated cell is equal to the sum of tracer atoms already contained in the cell before treatment and the number of tracer atoms added and/or removed by the treatment

$$n_{A_{X,f}} = n_{A_{X,i}} + n_{A_{X,add}} - n_{A_{X,rem}} \quad \text{(S9)}$$

where the superscript *A* refers to the mass number of the isotope utilized as the tracer.

Using eq. S2 and assuming that the isotopic composition of the removed compounds is identical to the isotopic composition of the untreated labeled cells, the mass balance of the tracer can be written as:

$$n_{X,f}\, a_{\boldsymbol{A_{X,f}}} = \left(n_{X,i} - n_{X,rem}\right) a_{\boldsymbol{A_{X,i}}} + n_{X,add}\, a_{\boldsymbol{A_{X,add}}} \quad \text{(S10)}$$

Rearranging eq. (S10) and substituting for $n_{X,f}$ from eq. (S8), an equation can be formulated that describes the relative change in the elemental concentration arising from the treatment of a cell as a function of the observed isotope fraction before and after the treatment.

$$\frac{n_{X,add}}{n_{X,i} - n_{X,rem}} = \frac{a_{A_{X,i}} - a_{A_{X,f}}}{a_{A_{X,f}} - a_{A_{X,add}}} \quad \text{(S11)}$$

The amount of removed biomass cannot be determined without more data, including concentration data. However, it is worth noting that removal is potentially important. The removal of biomass can originate from wash-out of intracellular constituents, the dissolution of EtOH soluble compounds, and permeabilization and enzymatic digestion by CARD-FISH treatments such as lysozyme, proteinase K or achromopeptidase.

The effect of removal on the ratio of added to the initial tracer element X is evident by re-writing Eq. (S11) as:

$$\frac{n_{X,add}}{n_{X,i}} = \frac{a_{A_{X,i}} - a_{A_{X,f}}}{a_{A_{X,f}} - a_{A_{X,add}}} \left(1 - \frac{n_{X,rem}}{n_{X,i}}\right) \quad \text{(S12)}$$

In practice, the isotopic composition of cells obtained from labeling experiments that need identification by CARD-FISH is only determinable after treatment and therefore the question is what was the isotopic composition of the analyzed cells before sample preparation. This can be obtained by rewriting eq. S11 as:

$$a_{A_{X,i}} = a_{A_{X,f}} + \frac{n_{X,add}}{n_{X,i} - n_{X,rem}} \left(a_{A_{X,f}} - a_{A_{X,add}}\right) \quad \text{(S13)}$$

which shows that the magnitude of deviation in the isotopic composition of the cells is not only, as intuitively expected, proportional to the fraction of added atoms relative to the number of atoms retained after removal, but also linearly related to the difference between the isotopic composition of the added compounds and the cells. The latter tends to be overlooked in first order estimations where the tracer content in natural abundant compounds is considered as negligible, which can lead to overestimation of the label content of untreated cells in particular at low enrichment levels.

Note that the ratio of the number of added atoms, $n_{x,add}$, relative to the number of retained

atoms, ($n_{X,i} - n_{X,rem}$), (eq. S11) is related to the quantity we are interested in, $F_{add,X}$, by the following relationship:

$$F_{add,X} = [n_{x,add}/(n_{X,i} - n_{X,rem})]/[n_{x,add}/(n_{X,i} - n_{X,rem}) + 1] \quad \text{(S14)}$$

It can readily be shown that substituting for $n_{x,add}/(n_{X,i} - n_{X,rem})$ from eq. S11 into eq. S12 we derive eq. S6 above.

Supplementary Results and Discussion

NanoSIMS of mat microorganisms and effect of CARD-FISH on isotopic enrichment. In contrast to *Cyanobacteria* that were identified by their characteristic autofluorescence, *Deltaproteobacteria* cells were identified by CARD-FISH. This procedure has the potential to dilute labeling through the introduction of C and N (mainly ^{12}C and ^{14}N) via probe and tyramide binding (and possibly also via buffer reagents and enzymes used for cell wall permeabilization that cannot be completely removed during the washing steps). This deposition in CARD-FISH identified cells may dilute the amount of the rarer heavy isotope incorporated during the labeling experiment, and possibly could have masked ^{15}N incorporation by the *Deltaproteobacteria*. To evaluate the magnitude of this dilution effect and to test for its relevance in this and other NanoSIMS studies we conducted control experiments with ^{13}C and ^{15}N-labeled cells (Supplementary Figure 6, 7 and 8). The enrichment of fixed cells un-treated by CARD-FISH was 98.56 at% ^{15}N and 95.31 at% ^{13}C in *E. coli*; versus 97.52 at% ^{15}N and 92.11 at% ^{13}C in *B. subtilis* cells. After hybridization with probe EUB338 (Supplementary Figure 6), 70.84 at% ^{15}N and 62.54 at% ^{13}C, and 72.52 at% ^{15}N and 57.03 at% ^{13}C were measured for *E. coli* and *B. subtilis* cells, respectively. ^{15}N and ^{13}C enrichments of cells stained with a negative control probe (Supplementary Figure 6) were 81.50 at% and 74.65 at%, respectively, in *E. coli*, and 89.74 at% and 81.33 at%, respectively, in *B. subtilis*. Similar trends were detected in cells labeled to only approximately 6 at% ^{15}N and ^{13}C, which more closely reflects enrichment levels measured in isotope labeled environmental samples (e.g. Dekas *et al.*, 2009; Woebken *et al.*, 2012; Ploug *et al.*, 2010) (Supplementary Figure 7). These data indicate that CARD-FISH analyses with the nonsense probe can result in an apparent dilution ($F_{X,add}$) of up to 18% for nitrogen, and up to 22% for carbon. In CARD-FISH experiments with the EUB338 probe, these values increased up to 28% for nitrogen and 38% for carbon.

To test whether the deltaproteobacterial ^{15}N isotope enrichment measured by NanoSIMS were strongly influenced by CARD-FISH, we used the data from the reference culture experiments (28% dilution as a worst case scenario for N) to back-calculate the ^{15}N isotope enrichment the deltaproteobacterial cells could have had prior to the CARD-FISH procedure. Based on these calculations, when the CARD-FISH ^{15}N dilution is accounted for, the deltaproteobacterial ^{15}N isotope fraction values increase only slightly, and their corrected values are still not significantly enriched above natural abundance values (average of 0.38 at% ^{15}N, p=0.131). We also considered the individual measured values (as opposed to the population mean), and found that based on the uncorrected values, 20.4% of the cells are significantly enriched in ^{15}N based on a 95% confidence interval. This number increases to 31.5% if the dilution through CARD-FISH is taken into account. Based on these data we suggest that the CARD-FISH protocol has an effect on the ^{13}C and ^{15}N isotopic composition. This staining technique and similarly the halogen *in situ* CARD approaches (Musat *et al.*, 2008; Behrens *et al.*, 2008) used for halogen-based identification of microbes in NanoSIMS analyses can change the fraction of a population that is considered enriched if values are close to natural abundance (as was the case for *Deltaproteobacteria* in this study) (see

391 extended discussion of this point below). However, the ^{15}N enrichment values of investigated
392 *Deltaproteobacteria* in this study changed very little when CARD-FISH dilution was
393 accounted for, supporting our previous conclusion that the targeted *Deltaproteobacteria* were
394 not significantly enriched in ^{15}N.
395
396 *Factors of CARD-FISH influencing the isotopic composition in microbial cells.* As
397 mentioned above, loss of labeled cell material is possible due to wash-out of intracellular
398 constituents through EtOH treatments and enzymatic lysis steps. The steps in the CARD-
399 FISH protocol introducing ^{12}C and ^{14}N into the cells are most likely again enzymatic
400 treatments (such as lysozyme for permeabilization) and the hybridization buffers containing
401 blocking reagent and dextrane sulfate, as apparent by the fact that hybridization with the
402 nonsense probe (nonEUB338) reduced the ^{15}N and ^{13}C enrichment without probe binding and
403 tyramide signal amplification (Supplementary Figure 6 and 7). In probe-stained cells (e.g.
404 with probe EUB338 in our test study), probe binding and the deposition of tyramides in the
405 amplification reaction lead to further dilution and thereby decrease the isotope content in the
406 cells (Supplementary Figure 6 and 7).
407
408 *Impact of the dilution through CARD-FISH on data interpretation.* Since CARD-FISH does
409 influence the isotopic composition of microbial cells (Supplementary 7), it is of importance
410 to investigate how quantitative conclusions based on NanoSIMS data (e.g. species A is more
411 enriched and therefore was more active than species B) are effected by the introduction of C-
412 and N-atoms through CARD-FISH. The following scenarios can exist:
413 (1) One target group (group A) was identified by CARD-FISH, the other (group B) could
414 be identified by morphology and did not go through the CARD-FISH procedure (like
415 in this study the *Deltaproteobacteria* and the *Cyanobacteria*; both being investigated
416 independently). In this case, only group A is affected by the introduction of C and N,
417 but not group B.
418 (2) In the second scenario, again one group (group A) was stained by a probe whereas the
419 second population (group B) was identified solely by morphological distinct features
420 (so no CARD-FISH identification necessary) (e.g. the case in Thompson *et al.*, 2012).
421 However, in this scenario group B would be analyzed on the same wafer/filter as
422 group A and therefore went through the CARD-FISH procedure - such as the cells
423 undergoing hybridization with the nonsense probe (nonEUB338) in our test study.
424 The isotopic enrichment of cells in group A will be diluted like the cells stained with
425 probe EUB338 in our test (Supplementary Figure 7), and cells belonging to group B
426 will experience less dilution. This group will experience a dilution similar to the cells
427 that went through CARD-FISH with the nonsense probe (Supplementary Figure 7).
428 Thereby, different dilution factors would have to be considered for these different
429 groups.
430 (3) Alternatively, both groups are stained by CARD-FISH (or analysis of three groups as
431 in Musat *et al.*, 2008), but one group (e.g. group A) is much more active than the
432 other. Cells of group A will have a higher ribosome content and therefore more
433 probes can bind to rRNA and more tyramides can be deposited in the cell. Hence,
434 cells of group A could encounter a stronger dilution in their ^{13}C and ^{15}N enrichment
435 than group B. Analyzed by NanoSIMS, group A could appear less enriched in ^{13}C or
436 ^{15}N than group B (although both for example incorporated the same amount of
437 isotopically labeled substrate), and the false conclusion would be that group B was
438 more active than group A. Similar biases can be introduced in this scenario if probes
439 for different target microbes show strong differences in binding efficiency or if

differences in the cell wall composition of different target microbes leads to strong differences in permeability of the fixed cells for the HRP-labeled probes.

In this context it should be noted that the *E. coli* and *B. subtilis* reference cells in our test study had a high cellular ribosome content (Supplementary Figure 6). Consequently, the back-calculation applied to correct the ^{15}N values of the deltaproteobacterial cells for CARD-FISH dilution reflected the "worst" situation (strong dilution due to high ribosome content). Ultimately, whether the dilution caused by CARD-FISH will have a significant effect in quantitative NanoSIMS studies of microbial communities tackling questions like "Is species A more labelled than species B?" or "Which species are significantly labelled compared to the natural abundance control?" is dependent on how different the measured isotopic enrichments of the targeted groups are and on whether the isotopic enrichments of the analysed cells are close to natural abundance values. The greater the enrichment differences of both target groups and the larger the enrichment difference of target microbes to natural abundance, the more confident one can be that CARD-FISH will not obscure the results of the single-cell isotope measurements.

It is worth noting that these dilution effects are relative to the natural abundance baseline of ~0.37 at% ^{15}N. Therefore, for small enrichments, the correction is correspondingly small. For example, the small cyanobacteria in this study are enriched to 0.6 at% ^{15}N, without CARD-FISH treatment. Based on Eq.1 in the text, solving for a_f, the enrichment of the small cyanobacteria would have been reduced to a value of 0.53 at% ^{15}N by CARD-FISH, which would still be easily detected. Because NanoSIMS isotopic measurements of bacteria are typically accurate to better than 5 percent of the measured value (e.g., 0.37 at% ^{15}N +/- 0.02), enrichment above background only becomes difficult to detect when the enrichment is relatively low (e.g., <0.4 at% ^{15}N).

However, particular care should be taken in estimating quantitative uptake rates of ^{13}C and/or ^{15}N-labeled substrates based on NanoSIMS measurements of cells identified by CARD-FISH as the deposition of C and N by this staining technique will lead to an underestimation of the actual rates in dependence of the ribosome content of the target cells. In contrast, dilution of ^{13}C or ^{15}N in cells will be much less pronounced if identification of target organisms for NanoSIMS measurements is not performed by CARD-FISH, but by FISH protocols that do not include signal amplification steps and do not require extensive cell permeabilization and pre-treatment (e.g. blocking) procedures (Orphan *et al.*, 2001; Li *et al.*, 2008; Berry *et al.,* 2013). However, in many ecosystems such as microbial mats (Woebken *et al.*, 2012), sediments (Morono *et al.*, 2011) or soils, strong autofluorescence can render FISH with probes directly linked to fluorochromes (such as mono-labeled or dual-labeled probes) difficult. The same applies to FISH of cells with very low ribosome content such as marine water samples (e.g. Musat *et al.*, 2008 and Thompson *et al.*, 2012). For those samples, CARD-FISH remains the best choice for identification, and for quantitative isotope analysis one should consider the above-mentioned points.

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