LETTER

doi:10.1038/nature11338

Doubling of marine dinitrogen-fixation rates based on direct measurements

Tobias Großkopf[1]*, Wiebke Mohr[1]*†, Tina Baustian[1], Harald Schunck[1], Diana Gill[1], Marcel M. M. Kuypers[2], Gaute Lavik[2], Ruth A. Schmitz[3], Douglas W. R. Wallace[4] & Julie LaRoche[1]†

Biological dinitrogen fixation provides the largest input of nitrogen to the oceans, therefore exerting important control on the ocean's nitrogen inventory and primary productivity[1–3]. Nitrogen-isotope data from ocean sediments suggest that the marine-nitrogen inventory has been balanced for the past 3,000 years (ref. 4). Producing a balanced marine-nitrogen budget based on direct measurements has proved difficult, however, with nitrogen loss exceeding the gain from dinitrogen fixation by approximately 200 Tg N yr^{-1} (refs 5, 6). Here we present data from the Atlantic Ocean and show that the most widely used method of measuring oceanic N_2-fixation rates[7] underestimates the contribution of N_2-fixing microorganisms (diazotrophs) relative to a newly developed method[8]. Using molecular techniques to quantify the abundance of specific clades of diazotrophs in parallel with rates of $^{15}N_2$ incorporation into particulate organic matter, we suggest that the difference between N_2-fixation rates measured with the established method[7] and those measured with the new method[8] can be related to the composition of the diazotrophic community. Our data show that in areas dominated by *Trichodesmium*, the established method underestimates N_2-fixation rates by an average of 62%. We also find that the newly developed method yields N_2-fixation rates more than six times higher than those from the established method when unicellular, symbiotic cyanobacteria and γ-proteobacteria dominate the diazotrophic community. On the basis of average areal rates measured over the Atlantic Ocean, we calculated basin-wide N_2-fixation rates of 14 ± 1 Tg N yr^{-1} and 24 ± 1 Tg N yr^{-1} for the established and new methods, respectively. If our findings can be extrapolated to other ocean basins, this suggests that the global marine N_2-fixation rate derived from direct measurements may increase from 103 ± 8 Tg N yr^{-1} to 177 ± 8 Tg N yr^{-1}, and that the contribution of N_2 fixers other than *Trichodesmium* is much more significant than was previously thought.

The oceans support roughly half of Earth's biological carbon fixation[9]. Carbon itself is rarely considered to be limiting in the ocean; instead, elements such as nitrogen, phosphorus and iron control primary productivity[2]. Of these elements, nitrogen is special because it is always present in large amounts as dissolved N_2, but this form is available only to the diazotrophs, a restricted group of prokaryotes[1]. Biological N_2 fixation is the largest source of fixed-nitrogen input into the ocean and it has been proposed that this controls marine primary productivity on geological timescales[10]. Whereas phytoplankton seem to be limited in growth over large areas by the availability of fixed N (for example, nitrate, nitrite or ammonium), the productivity of diazotrophs is controlled by other environmental factors, such as the availability of Fe and P (ref. 11). Thus, increased desertification owing to changes in land use may promote marine N_2 fixation through increased aeolian input of iron[12], whereas the increased use of fertilizers and riverine run-off or increased atmospheric N deposition may have the opposite effect[13,14]. An understanding of marine N_2 fixation and its response to anthropogenic forcing is crucial for assessing the future of oceanic primary productivity. However, recent attempts to produce a balanced N budget have usually left a major gap on the input side[5,6]. This creates a paradox, because stable isotopes recorded in ocean sediments suggest that the marine N inventory has been balanced for the past 3,000 years (ref. 4). The paradox can be reconciled in three ways[6]. One hypothesis is that current, transient N-loss rates are exceeding N_2 fixation, but that over timescales of several thousand years, variable losses and gains act to maintain a steady-state fixed-N inventory[15,16]. Two alternate hypotheses invoke the possibilities that either the N-loss or the N-gain terms are in error. Several lines of evidence point out that even the most conservative estimates of oceanic N losses are higher than current estimates of N input based on field measurements of N_2 fixation. This suggests that contemporary N_2-fixation rates may have been underestimated grossly[5,6].

In the past decade, molecular techniques have led to the discovery of a variety of previously unrecognized diazotrophs that express their nitrogenase genes and fix N_2, thereby showing that there are gaps in our knowledge of marine N_2 fixation[17–21]. The discovery of N_2 fixation in mesopelagic waters in the Pacific[22,23] and the possibility of significant involvement of heterotrophic bacteria in global marine N_2 fixation[24,25] reinforce the idea that we are missing a large fraction of global marine N input by N_2 fixation. Biogeochemical modelling and geochemical-tracer techniques have been used to estimate regional and global magnitudes of marine N_2 fixation, but these indirect approaches rely on assumptions that require verification by direct biological rate measurements of N_2 fixation.

A recent laboratory study showed that the established method for measuring oceanic N_2 fixation (accounting for around 90% of the published rates[26]) leads to an underestimate, owing to slow and incomplete equilibration between the water sample and the $^{15}N_2$ tracer added as a gas bubble[8]. If this finding holds for measurements of environmental N_2-fixation rates, an upward revision of the largest source of combined N into the ocean—that is, N_2-fixation—would be inevitable. We investigated the magnitude of the underestimation of N_2-fixation rates in open-ocean settings. In autumn 2009, on two research cruises to the Atlantic Ocean between 25° N and 45° S, we compared the established $^{15}N_2$ tracer method for measuring N_2 fixation[7] (hereafter called the bubble-addition method) with a recently developed method[8] in which the $^{15}N_2$ tracer is added as a dissolved gas (the dissolution method). Our sampling covered a wide variety of oceanic conditions with sea surface temperatures ranging from 10 °C to more than 28 °C (Fig. 1). At each station, we conducted parallel incubations with the dissolution and the bubble-addition methods, using dual labelling with $NaH^{13}CO_3$ and $^{15}N_2$ gas in the same incubation bottles to measure

[1]Helmholtz Centre for Ocean Research Kiel (GEOMAR), Düsternbrooker Weg 20, 24105 Kiel, Germany. [2]Max Planck Institute for Marine Microbiology, Celsiusstraße 1, 28359 Bremen, Germany. [3]Institute for General Microbiology, Christian-Albrechts University Kiel, Am Botanischen Garten 1–9, 24118 Kiel, Germany. [4]Oceanography Department, Dalhousie University, 1355 Oxford Street, PO Box 15000, Halifax, Nova Scotia, B3H 4R2, Canada. †Present addresses: Harvard University, Department of Earth and Planetary Sciences, 20 Oxford Street, Cambridge, Massachusetts 02138, USA (W.M.); Department of Biology, Dalhousie University, 1355 Oxford Street, PO Box 1500, Halifax, Nova Scotia B3H 4R2, Canada (J.L.R.).
*These authors contributed equally to this work.

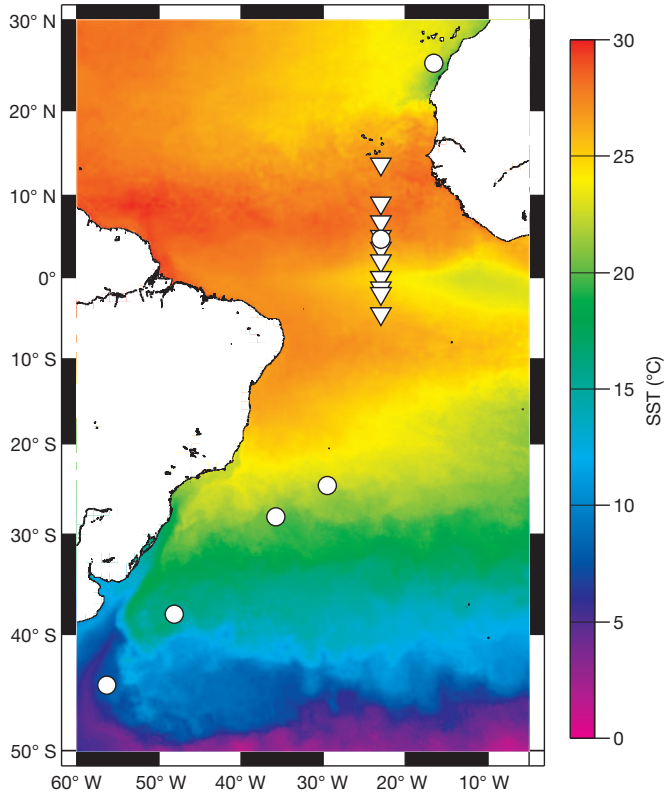

Figure 1 | Sampling sites and sea surface temperature. Map of average night-time sea surface temperature (SST) in °C in autumn 2009 in the Atlantic Ocean (http://oceancolor.gsfc.nasa.gov/), overlaid with stations sampled on the RV *Meteor* cruise 80–81 (triangles) and the RV *Polarstern* cruise ANT-XXVI/1 (circles). Triangles also correspond to 11 vertical profile stations used in Fig. 3. The equatorial station was sampled twice during the course of the cruise, so two triangles are superimposed at this location.

dissolved inorganic carbon (DIC) assimilation and N_2 fixation simultaneously with both methods (see Supplementary Information for experimental details).

The ^{13}C-labelled DIC was added to check for systematic differences in biological activity attributable to the differences in methods, and the results showed that carbon-fixation rates were in good agreement between the two experimental methods (Fig. 2b). By contrast, there was a large difference and poor overall correlation between the N_2-fixation rates calculated using the two methods (Fig. 2a). Average depth-integrated rates of N_2 fixation over the whole Atlantic Ocean (25° N to 45° S) differed by a factor of 1.7 (91 ± 4 and 54 ± 2 μmol N m^{-2} d^{-1} for the dissolution and bubble-addition methods, respectively; mean and propagated s.e.m., $n = 17$; Wilcoxon matched pairs test $P < 0.01$). Furthermore, a geographical pattern emerged among the differences in the rates measured with the two methods. In the northern part of the 23° W section (15° N to 5° S), the depth-integrated N_2-fixation rates derived from four vertical profiles were, on average, 62% higher with the dissolution method than with the bubble-addition method (194 ± 12 and 120 ± 5 μmol N m^{-2} d^{-1}, respectively; means and propagated s.e.m., $n = 4$). In the equatorial Atlantic Ocean (4.5° N to 5° S), a region previously not considered important for N_2 fixation[27], the depth-integrated N_2-fixation rates were on average 570% higher with the dissolution method (55 ± 7 μmol N m^{-2} d^{-1}) than with the bubble-addition method (8 ± 1 μmol N m^{-2} d^{-1}; means and propagated s.e.m., $n = 7$) (Fig. 3). Moreover, in the South Atlantic Gyre (38° S) and in the Falkland Current (44° S), where water temperatures were as low as 16° C and 10° C, respectively, considerable N_2 fixation was detected with the dissolution method (0.44 ± 0.1 and 0.54 ± 0.1 μmol N m^{-3} d^{-1}; means and s.e.m., $n = 3$). The rates detected with the bubble-addition method were substantially lower (0.10 ± 0.01 and 0.18 ± 0.03 μmol N m^{-3} d^{-1} for 38° S and 44° S, respectively; means and s.e.m., $n = 3$).

We used TaqMan assays based on the presence of the nitrogenase reductase (*nifH*) gene to quantify the relative abundance of the diazotrophic phylotypes found in the tropical Atlantic Ocean[20,21]. We identified two geographically separated areas, dominated by distinct diazotrophic phylotypes (Fig. 4 and Supplementary Fig. 2). The most abundant diazotroph was *Trichodesmium*, peaking at 9° N with 5 × 10^5 *Trichodesmium nifH* copies per litre. We measured the highest N_2-fixation rates inside a *Trichodesmium* bloom at 13.75° N with 360 ± 8.5 μmol N m^{-2} d^{-1} and 219 ± 8.2 μmol N m^{-2} d^{-1} measured with the dissolution and bubble-addition methods, respectively (means and s.e.m., $n = 3$). Although *Trichodesmium* was present throughout the whole area (Fig. 4), its abundance declined rapidly south of 5° N. Diatom-associated heterocystous diazotrophs, unicellular cyanobacteria and γ-proteobacteria showed a peak of abundance within the *Trichodesmium* bloom and a second peak in the equatorial Atlantic region, where they outnumbered *Trichodesmium*. We characterize the tropical North Atlantic (5° N to 15° N) as a region of *Trichodesmium* dominance, where underestimation of N_2-fixation rates by the bubble-addition method was less severe (but still significant). By contrast, we found the largest underestimation (570%) with the bubble-addition method in the equatorial Atlantic (4.5° N to 5° S), which was dominated by diazotrophs other than *Trichodesmium* (Supplementary Figs 4 and 5). The combined results indicate that the magnitude of underestimation in N_2-fixation rates measured with the bubble-addition method relative to the

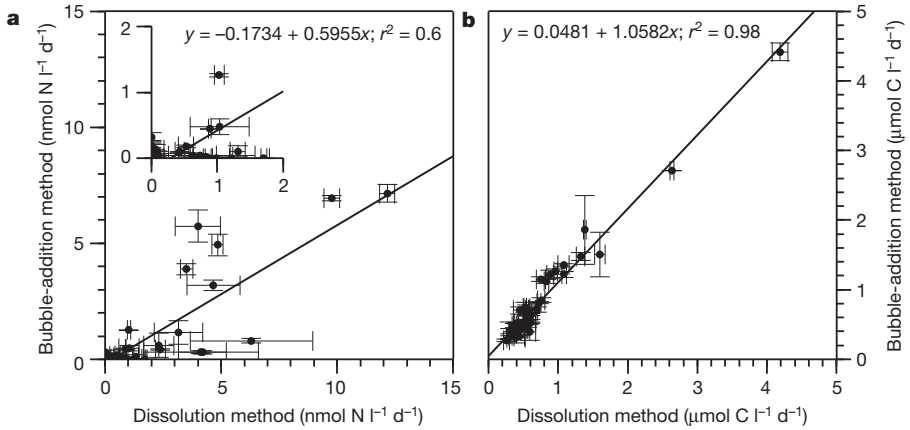

Figure 2 | Comparison between bubble-addition and dissolution methods. **a**, N_2-fixation rates for all stations and depths. Inset shows zoom on the 0–2-nmol l^{-1} d^{-1} range. **b**, Carbon-fixation rates for all stations and depths. Error bars indicate standard errors of triplicate incubations (s.e.m., $n = 3$).

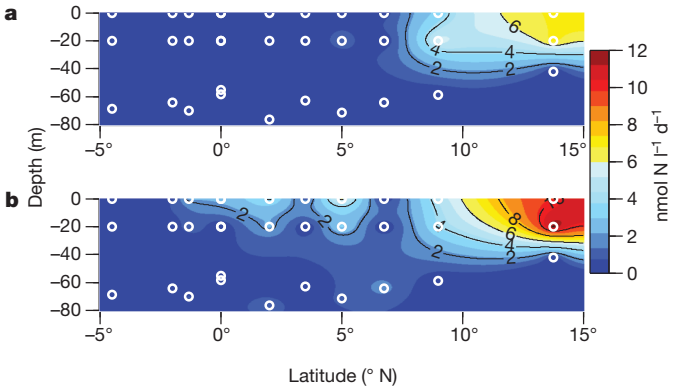

Figure 3 | Mixed-layer inventory of N_2-fixation rates in the tropical and equatorial Atlantic Ocean. Stations correspond to triangles in Fig. 1. **a**, N_2-fixation rates measured with the bubble-addition method. **b**, N_2-fixation rates measured with the dissolution method.

dissolution method was related to the composition of the diazotrophic community (Supplementary Fig. 2).

The species composition of the diazotrophic community can affect the level of underestimation for several reasons. In the bubble-addition method, the equilibration time for a $^{15}N_2$ gas bubble is longer than the typical incubation time of 24 h, despite manual shaking at the onset of the incubation[8] (Supplementary Fig. 6). This means that the bubble and its immediate surroundings will remain highly enriched in $^{15}N_2$, whereas water at the bottom of the incubation bottle will be less enriched (see Supplementary Information). As a result, buoyant diazotrophs (such as *Trichodesmium*) may be exposed to a higher fraction of the added $^{15}N_2$ label, whereas diazotrophs that sink (such as *Richelia*, when in symbiotic relationship with diatoms[28]) and accumulate at the bottom of the bottle may be exposed to a lower fraction of the $^{15}N_2$. Underestimation bias will also be greater when

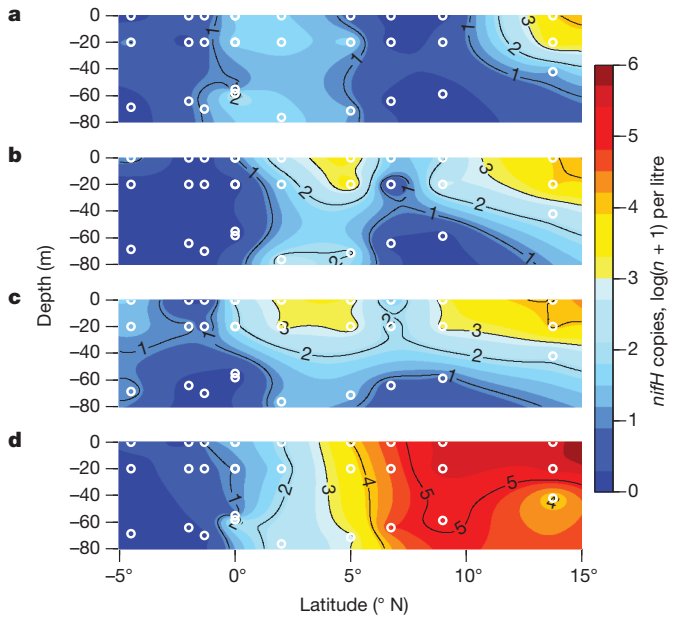

Figure 4 | Relative abundance of various phylotypes of diazotrophic bacteria from the same stations as the N_2-fixation rate measurements, estimated with TaqMan *nifH* gene assays. **a**, Unicellular cyanobacteria (Group A, Group B and Group C cyanobacteria). **b**, Diatom-associated heterocystous cyanobacteria. **c**, Diazotrophic γ-proteobacteria. **d**, *Trichodesmium*. There was no DNA sampling at the 3.5° N station. Note that the *nifH* gene copies (*n*) per litre of diatom-associated heterocystous cyanobacteria (such as *Richelia*) detects all cells of the symbionts, not just the heterocysts in which N_2 fixation is actively taking place.

the start of incubation coincides with the peak of N_2-fixation activity of the dominant diazotrophs (such as *Crocosphaera* for night-time incubations and *Trichodesmium* for daytime ones). These biases are avoided with the dissolution method because the $^{15}N_2$ label is added as a gas dissolved in sea water that is mixed uniformly with the sample at the start of the incubation.

Using published measurements of N_2-fixation rates obtained with the bubble-addition method, we calculated basin-wide and global budgets of marine N_2 fixation. The basin-wide averages are 14 Tg N yr^{-1} for the Atlantic, 63 Tg N yr^{-1} for the Pacific and 26 Tg N yr^{-1} for the Indian Ocean, giving a global total of 103 Tg N yr^{-1} (see Supplementary Information). Our own average N_2-fixation rates measured with the bubble-addition method and extrapolated over the Atlantic Ocean (25° N to 45° S), give an input of 14 Tg N yr^{-1}, which is identical to the value for the Atlantic Ocean calculated from the published data. By contrast, our average N_2-fixation rates measured with the dissolution method raise the Atlantic Ocean N_2-fixation rate to 24 Tg N yr^{-1}. If this relative difference applies equally to other ocean basins—where the diazotrophic communities may differ from those of the Atlantic Ocean—the global marine N_2-fixation rate based on direct measurements may have to be increased to 177 ± 8 Tg N yr^{-1}.

Our study demonstrates that field measurements of N_2 fixation made with the widely applied $^{15}N_2$ bubble-addition method have significantly and variably underestimated N_2-fixation rates in comparison with the dissolution method, with possible implications for the global marine nitrogen budget. Our data reveal regional variations in the magnitude of the underestimate, which may be related to the composition of the diazotrophic community. Specifically, the contribution of diazotrophs other than *Trichodesmium* may have been severely underestimated in previous field studies, leading to a biased view of the key players in this globally important process.

Received 3 November 2011; accepted 14 June 2012.
Published online 8 August 2012.

1. Karl, D. *et al.* Dinitrogen fixation in the world's oceans. *Biogeochemistry* **57,** 47–98 (2002).
2. Falkowski, P. G., Barber, R. T. & Smetacek, V. Biogeochemical controls and feedbacks on ocean primary production. *Science* **281,** 200–206 (1998).
3. Deutsch, C., Sarmiento, J. L., Sigman, D. M., Gruber, N. & Dunne, J. P. Spatial coupling of nitrogen inputs and losses in the ocean. *Nature* **445,** 163–167 (2007).
4. Altabet, M. A. Constraints on oceanic N balance/imbalance from sedimentary ^{15}N records. *Biogeosciences* **4,** 75–86 (2007).
5. Mahaffey, C., Michaels, A. F. & Capone, D. G. The conundrum of marine N_2 fixation. *Am. J. Sci.* **305,** 546–595 (2005).
6. Codispoti, L. A. An oceanic fixed nitrogen sink exceeding 400 Tg N a^{-1} vs the concept of homeostasis in the fixed-nitrogen inventory. *Biogeosciences* **4,** 233–253 (2007).
7. Montoya, J. P., Voss, M., Kahler, P. & Capone, D. G. A simple, high-precision, high-sensitivity tracer assay for N_2 fixation. *Appl. Environ. Microbiol.* **62,** 986–993 (1996).
8. Mohr, W., Grosskopf, T., Wallace, D. W. R. & LaRoche, J. Methodological underestimation of oceanic nitrogen fixation rates. *PLoS ONE* **5,** e12583 (2010).
9. Behrenfeld, M. J. & Falkowski, P. G. Photosynthetic rates derived from satellite-based chlorophyll concentration. *Limnol. Oceanogr.* **42,** 1–20 (1997).
10. Falkowski, P. G. Evolution of the nitrogen cycle and its influence on the biological sequestration of CO_2 in the ocean. *Nature* **387,** 272–275 (1997).
11. Mills, M. M., Ridame, C., Davey, M., La Roche, J. & Geider, R. J. Iron and phosphorus co-limit nitrogen fixation in the eastern tropical North Atlantic. *Nature* **429,** 292–294 (2004).
12. Moore, J. K., Doney, S. C., Lindsay, K., Mahowald, N. & Michaels, A. F. Nitrogen fixation amplifies the ocean biogeochemical response to decadal timescale variations in mineral dust deposition. *Tellus B* **58,** 560–572 (2006).
13. Naqvi, S. W. A. *et al.* Increased marine production of N_2O due to intensifying anoxia on the Indian continental shelf. *Nature* **408,** 346–349 (2000).
14. Duce, R. A. *et al.* Impacts of atmospheric anthropogenic nitrogen on the open ocean. *Science* **320,** 893–897 (2008).
15. Gruber, N. & Galloway, J. N. An Earth-system perspective of the global nitrogen cycle. *Nature* **451,** 293–296 (2008).
16. Gruber, N. & Sarmiento, J. L. Global patterns of marine nitrogen fixation and denitrification. *Glob. Biogeochem. Cycles* **11,** 235–266 (1997).
17. Zehr, J. P. *et al.* Unicellular cyanobacteria fix N_2 in the subtropical North Pacific Ocean. *Nature* **412,** 635–638 (2001).
18. Zehr, J. P. *et al.* Globally distributed uncultivated oceanic N_2-fixing cyanobacteria lack oxygenic photosystem II. *Science* **322,** 1110–1112 (2008).

19. Moisander, P. H. *et al.* Unicellular cyanobacterial distributions broaden the oceanic N_2 fixation domain. *Science* **327,** 1512–1514 (2010).
20. Langlois, R. J., LaRoche, J. & Raab, P. A. Diazotrophic diversity and distribution in the tropical and subtropical Atlantic Ocean. *Appl. Environ. Microbiol.* **71,** 7910–7919 (2005).
21. Langlois, R. J., Hummer, D. & LaRoche, J. Abundances and distributions of the dominant *nifH* phylotypes in the Northern Atlantic Ocean. *Appl. Environ. Microbiol.* **74,** 1922–1931 (2008).
22. Hamersley, M. R. *et al.* Nitrogen fixation within the water column associated with two hypoxic basins in the Southern California Bight. *Aquat. Microb. Ecol.* **63,** 193–205 (2011).
23. Fernandez, C., Farias, L. & Ulloa, O. Nitrogen fixation in denitrified marine waters. *PLoS ONE* **6,** e20539 (2011).
24. Farnelid, H. *et al.* Nitrogenase gene amplicons from global marine surface waters are dominated by genes of non-cyanobacteria. *PLoS ONE* **6,** e19223 (2011).
25. Halm, H. *et al.* Heterotrophic organisms dominate nitrogen fixation in the South Pacific Gyre. *ISME J.* **6,** 1238–1249 (2012).
26. Luo, Y. W. *et al.* Database of diazotrophs in global ocean: abundances, biomass and nitrogen fixation rates. *Earth Syst. Sci. Data Discuss.* **5,** 47–106 (2012).
27. Moore, C. M. *et al.* Large-scale distribution of Atlantic nitrogen fixation controlled by iron availability. *Nature Geosci.* **2,** 867–871 (2009).
28. Subramaniam, A. *et al.* Amazon River enhances diazotrophy and carbon sequestration in the tropical North Atlantic Ocean. *Proc. Natl Acad. Sci. USA* **105,** 10460–10465 (2008).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank G. Klockgether and T. Max for mass-spectrometry measurements. We thank S. Fehsenfeld for helping with sampling and H. Nurlaeli for experimental work on *Nodularia*. We also thank the captain and crew of RV *Meteor* and RV *Polarstern*, as well as the chief scientists, P. Brandt and A. Macke. We thank D. Desai for statistical analyses. This work is a contribution of the Sonderforschungsbereich 754 'Climate — Biogeochemistry Interactions in the Tropical Ocean', which is supported by the Deutsche Forschungsgemeinschaft. We thank the Max Planck Gesellschaft for financial support. We thank the Bundesministerium für Bildung und Forschung (BMBF) for financial support through the SOPRAN II (Surface Ocean Processes in the Anthropocene) project, grant number 03F0611A.

Author Contributions W.M. designed the dissolution method. T.G., T.B., H.S. and D.G. collected samples and performed *nifH* gene quantification. M.M.M.K. and G.L. did the measurements on the mass spectrometer. T.G. wrote the manuscript with W.M. and J.L.R.. M.M.M.K., G.L., R.A.S., D.W.R.W., J.L.R., W.M. and T.G. designed the experiments and analysed the data.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to J.L.R. (julie.laroche@dal.ca) and T.G. (tgrosskopf@geomar.de).