doi:10.1038/nature11338

## Material and Methods

### *Sampling*

In order to compare the commonly used $^{15}N_2$ tracer addition method to measure $N_2$ fixation [1] with the addition of $^{15}N_2$-enriched water as suggested by Mohr et al. (2010)[2], seawater was sampled on two cruises in the Atlantic Ocean, the first on board R/V Meteor (M80/1) on a longitudinal transect (23°W) between 15°N and 5°S, the second on board R/V Polarstern (ANT-XXVI/1) on a transect between 54°N and 54°S (Bremerhaven, Germany to Punta Arenas, Chile). In total 39 triplicate incubations were conducted with both methods in parallel. On the M80/1 cruise, seawater was sampled at 11 stations from the surface (bucket), 20 m depth and the chlorophyll maximum (CTD rosette sampler) at 7:00 in the morning, whereas on the ANT-XXVI/1 cruise, seawater was sampled at 6 stations at 16:00 from the ship's clean seawater supply which is installed at 11 m depth (keel of the ship).

### *Incubations*

Seawater samples were filled headspace-free (bubble addition method) or with a 100-150 ml headspace (dissolved method) into 4.5 L polycarbonate bottles and closed with Teflon® -coated butyl rubber septum caps. To determine $N_2$ fixation rates with the bubble-addition method, a 4.5 mL $^{15}N_2$ gas bubble (Sigma-Aldrich, ≥98 atom%) was injected through the septa into each of triplicate bottles (yielding a theoretical enrichment of ~12 atom% assuming a rapid isotopic equilibration between the added $^{15}N_2$ gas and the ambient dissolved $N_2$ of the water sample). After injection, bottles were gently inverted one hundred times. For comparison of $N_2$ fixation rates, we added $^{15}N_2$-enriched seawater to a second set of triplicate bottles (dissolution method). In detail, the preparation of the $^{15}N_2$-enriched seawater was started by degassing filtered seawater (0.2 µm filtered, Durapore) using a membrane flow-through system (Mini-Module, Membrana) in which the seawater flowed on the inside of the membrane and a vacuum (-960 mbar, water jet pump) was applied to the outer side of the membrane. The seawater flow rate was about 400 – 500 mL $min^{-1}$ and seawater was recirculated for the first 10-15 min of the degassing step. Degassed seawater was then filled directly from the flow-through system into evacuated gas-tight 3L Tedlar® bags without a headspace. Addition of $^{15}N_2$ gas was dependent on the amount of seawater in the Tedlar® bag and was added at a ratio of 10 ml $^{15}N_2$ per 1L seawater. The volume of degassed seawater in

the Tedlar® bag was estimated using a balance. During the Meteor cruise, dissolution of the $^{15}N_2$ gas was achieved by 'slapping' the bubble with a ruler. The 'slapping' lead to a dispersion of the large bubble into numerous small bubbles and thus an increase in the surface area to volume ratio which facilitated gas diffusion. After complete dissolution of the added $^{15}N_2$ gas ($^{15}N_2$-enriched seawater), an aliquot of the $^{15}N_2$ enriched water was collected for each preparation of enriched seawater and stored in an Exetainer until return to the laboratory where the isotopic composition was measured by membrane-inlet mass spectrometry[3]. Overall, the average concentration of $^{30}N_2$ in the prepared batches of enriched water was 246 µM (standard deviation =24.7 µM). This yielded a $^{15}N$-enrichment of about 2% when 100 mL enriched seawater are added to 4.5 L of incubation volume (depending on temperature and salinity). Next, 100-150 ml of $^{15}N_2$-enriched seawater were added to each of triplicate bottles before capping the bottles headspace-free with Teflon®-coated butyl rubber septum caps. Bottles were inverted 100 times. Primary production rates were determined in all incubation bottles by the addition of $NaH^{13}CO_3$ (~3.5 atom% final) after the addition of $^{15}N_2$ gas or $^{15}N_2$-enriched water. All bottles were placed into on-deck incubators with a surface seawater flow-through and a 25% in situ light level established with a light foil layer (Blue Lagoon, Lee Filters). Incubations were stopped after 24 hours by filtering 2-3 liters of each incubation onto pre-combusted (450°C, 5 hours) GF/F filters (Whatman) under gentle vacuum (-200 mbar). Filters were oven-dried (50°C, 24 hours) (Meteor) and stored over desiccant until analysis or frozen at -20°C directly after filtration (Polarstern). On the Meteor, the remainder of the samples was pooled for nucleic acid sampling within each set of triplicate bottles and a total of 2 L were filtered onto Durapore filters (47 mm, 0.2 µm pore size; Millipore). Samples were shock-frozen in liquid nitrogen and stored at -80°C until analysis.

To determine the natural abundance (*NA-control*) of $^{15}N$ and $^{13}C$ isotopes in the incubations, two different controls were incubated alongside with the six experimental bottles. The control for the bubble-addition method was untreated seawater, while the control for the dissolved method received an aliquot of seawater which had previously been degassed but instead of the addition and dissolution of $^{15}N_2$ for the preparation, ambient air was used. Both controls were processed as described above for the experimental bottles.

***Elemental stoichiometry and isotopic composition of particulate organic material***

GF/F filters were acidified over fuming HCl overnight in a dessicator. Filters were then oven-dried for 2 hours at 50°C and pelletized in tin cups. Samples were analyzed for particulate

organic carbon and nitrogen (POC and PON) and isotopic composition using a CHN analyzer coupled to an isotope ratio monitoring mass spectrometer. Caffeine standards, calibrated against IAEA standard N1 and N2, were measured every 6 samples.

***Calculation of nitrogen and carbon fixation rates***

$N_2$ fixation rates were calculated based on the final isotopic composition of the particulate organic nitrogen after the incubation using the following equation (1):

$$N_2 fixation\ rate = \frac{\left(A^{PN}_{sample} - A^{PN}_{NA-control}\right)}{\left(A_{N_2} - A^{PN}_{NA-control}\right)} x \frac{[PN]}{\Delta t} \quad (1)$$

With A = atom% $^{15}N$ in the particulate organic nitrogen (PN) in incubations to which $^{15}N_2$ was added ( $A^{PN}_{sample}$), in control incubations ( $A^{PN}_{NA-control}$) which were simultaneously incubated with the other bottles or in the dissolved $N_2$ pool ( $A_{N_2}$ ). For the bubble-addition method, the atom% in the $N_2$ pool was calculated from the predicted$^{15}N_2$ concentrations according to Mohr *et al*. (2010) [2]. For the dissolution method the $^{15}N_2$ concentration was calculated from the MIMS measurement value in the batch of enriched water for individual experiments and the measured volume of enriched water added to the incubation bottle. Carbon fixation rates were calculated as described for $N_2$ fixation rates. All rates are displayed as means of triplicate incubations with a standard error.

***DNA extraction and determination of nifH phylotype gene copy number***

DNA was extracted according to Langlois et al. 2008 using the AllPrep DNA/RNA extraction kit (QIAGEN) following the manufacturer's instructions[4]. Amplification of *nifH* genes was performed on an ABI-PRISM 7000 thermocycler, using phylotype specific probes and primers described in Langlois et al. 2008 [4] and Foster et. al. 2007 [5] (Table S1). *nifH* gene copy numbers were calculated based on the attained Ct values and a linear regression from plasmid standards ranging from $10^7$ to $10^1$ copies and simultaneously amplified on the same plate.

Table S1. Primers and Probes used in Taqman assays during this study. All sequences reported in 5'-3' direction.

| | Reverse (5'-3') | Pos | Forward (5'-3') | Pos | Probe (5'-3') | Pos |
|---|---|---|---|---|---|---|
| UCYN-A | TCAGGACCACCG GACTCAAC | 127-146 | TAGCTGCAGAAAGA GGAACTGTAGAAG | 50-76 | TAATTCCTGGCT ATAACAAC | 98-117 |
| Filamentous (Fil) | GCAAATCCACCG CAAACAAC | 256-275 | TGGCCGTGGTATTAT TACTGCTATC | 165-189 | AAGGAGCTTAT ACAGATCTA | 206-225 |
| Croco (UCYN-B) | TCAGGACCACCA GATTCTACACAC T | 122-146 | TGCTGAAATGGGTTC TGTTGAA | 54-75 | CGAAGACGTAA TGCTC | 87-102 |
| UCYN-C | GGTATCCTTCAA GTAGTACTTCGT CTAGCT | 83-112 | TCTACCCGTTTGATG CTACACACTAA | 1-26 | AAACTACCATTC TTCACTTAGCAG | 32-55 |
| GamAO | AACAATGTAGAT TTCCTGAGCCTT ATTC | 294-321 | TTATGATGTTCTAGG TGATGTG | 240-266 | TTGCAATGCCTA TTCG | 275-290 |
| Het-1 (Rich-Rizo) | AATACCACGACC CGCACAAC | 158-177 | CGGTTTCCGTGGTGT ACGTT | 105-124 | TCCGGTGGTCCT GAGCCTGGTGT | 133-155 |
| Het-2 (Rich-Hemi) | AATGCCGCGACC AGCACAAC | 158-177 | TGGTTACCGTGATGT ACGTT | 106-124 | TCTGGTGGTCCT GAGCCTGGTGT | 133-155 |

***Statistical analysis***

To test if the grouping of the Meteor dataset into Equatorial and Tropical North Atlantic stations was statistically significant, we used PRIMER (v. 6[6]) to perform a principal component analysis (PCA) and an analysis of similarity (ANOSIM) with the input parameters sample depth, phosphate concentration, *Trichodesmium* dominance (filamentous *nifH* abundance relative to other *nifH* genes), temperature, oxygen, ammonium concentration, salinity and the measured $N_2$ fixation rates for the bubble addition and dissolution methods (Fig. S2). The PCA showed that temperature, oxygen and phosphate were driving a spread of the data according to water depth, while the dominance of *Trichodesmium*, the measured $N_2$ fixation rates, salinity and ammonium were responsible for the separation in latitude (Fig. S2A). The ANOSIM indicated that the division into the equatorial and tropical areas was highly significant ($p<0.01$).

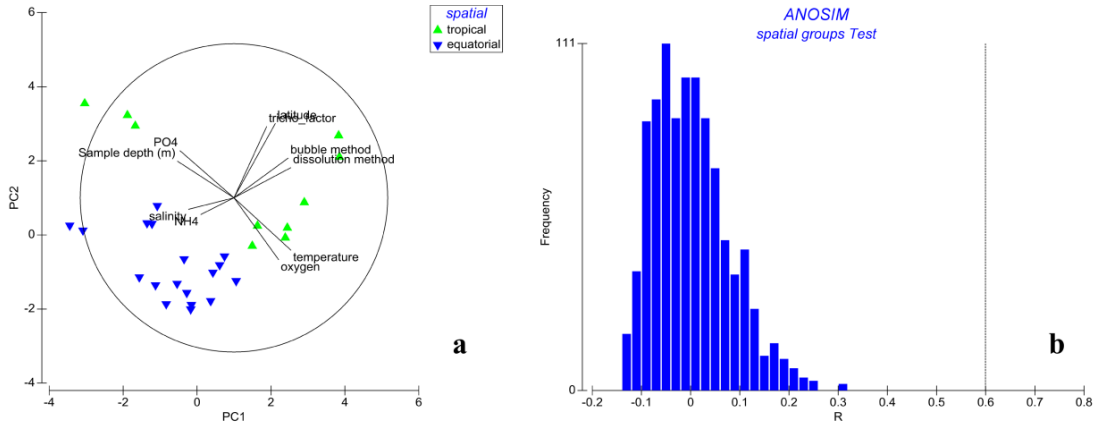


Figure S2. A: PCA analysis showing the samples of the METEOR 23°W transect and the clustering into the tropical (green triangles) and equatorial (blue triangles) groups. The blue lines show the strength and the direction of the influence of the variable over the direction of the data distribution. B: The observed R value of 0.6 (black line) is to the right beyond the distribution function of random sampling of the variables (blue frequency bars), hence showing the statistical significance ($p<0.01$) of the grouping in the PCA of Fig. S2A.

***Areal rates***

Areal rates were calculated for the Meteor cruise (15°N – 5°S) according to equation (2):

$$N_{fix}\ (\mu mol\ N\ m^{-2}\ d^{-1}) = \frac{(r_0 + r_{20})}{2} x20m + \frac{(r_{20} + r_{DCM})}{2} x(z_{DCM} - 20m) \qquad (2)$$

With the areal $N_2$ fixation rate $N_{fix}$, $r_0$, $r_{20}$ and $r_{DCM}$ the $N_2$ fixation rates at surface, 20 meters and the chlorophyll maximum respectively and $z_{DCM}$ the depth of the chlorophyll maximum. Standard errors for areal $N_2$ fixation rates were propagated according to the propagation of errors for linear combinations [7].

The areal $N_2$ fixation rates (µmol N $m^{-2}$ $d^{-1}$) calculated according to equation 2 showed a strong linear correlation (Fig. S3, $r^2$ = 0.92) with the $N_2$ fixation rates (nmol N $l^{-1}$ $d^{-1}$) measured for the surface waters during the Meteor cruise. This linear correlation (equation 3) was subsequently used to convert the $N_2$ fixation rates at 11m water depth from the Polarstern cruise to areal $N_2$ fixation rates. Note that the areal rates here only cover the surface mixed layer and may not capture the full profile of $N_2$ fixation, particularly sub-surface features.

$$N_{2\,fix}\ (\mu mol\ N\ m^{-2}\ d^{-1}) = 35.872\ x\ N_{2\,fix(surface)} (nmol\ N\ L^{-1}\ d^{-1}) \qquad (3)$$

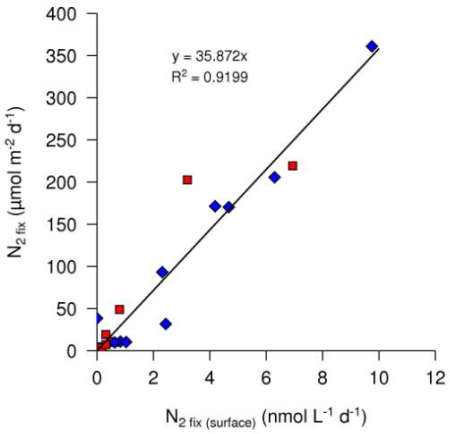


Figure S3. Correlation between $N_2$ fixation rates measured for the surface waters and calculated (equation 2) areal rates for the Meteor transect for both the bubble addition (red squares) and the dissolution method (blue diamonds). The black line represents the linear relationship for the entire data set with both methods, forced through zero ($r^2 = 0.92$). The slopes of the regressions for the individual regressions of the two methods separately are 36.826 ($r^2 = 0.946$) for the dissolution and 35.851 ($r^2 = 0.8624$) for the bubble-addition method.

***Global $N_2$ fixation estimates from literature values***

For the global estimates of N gain via $N_2$ fixation, published values of bulk water $N_2$ fixation rates measured with the bubble-addition method (Table S2) were weighted according to the number of stations and averaged over six ocean basins: North and South Pacific, North and South Atlantic and North and South Indian Ocean (Table S3). The basin wide average was multiplied by the dimension of the basin, whose extent was defined by the most northerly and most southerly observation of $N_2$ fixation mentioned. Since the Indian Ocean is poorly constrained in terms of measurements by the bubble-addition method, we used average rates from the North and South Pacific for the North and South Indian Ocean, respectively.

Table S2: Collection of publications using the bubble-addition method in the Atlantic and Pacific Oceans. S.e., standard error. [+] Treated as outlier, * as quoted in Mahaffey2005, ** Considering stations 11, 12, 21, 22, 31, 32, 41, 51, 52 and 71 as upwelling stations, [‡] values from supporting online material, *** used for North and South Atlantic with half the stations weighted

| Publication | Ocean domain | North limit (°N) | South limit (°N) | West limit (°E) | East limit (°E) | Stations | Areal rate (µmol N $m^{-2}$ $d^{-1}$) | S.e. |
|---|---|---|---|---|---|---|---|---|
| Rees *et al.* 2009 | Atlantic, English channel[8] | 50.2 | 49 | -5 | -4 | 2 | 350 | |
| Fernandez *et al.* 2010 | Atlantic, North[9] | 40 | 15 | -29 | -28 | 8 | 18 | 3 |
| Voss *et al.* 2004 | Atlantic, Western Tropical North[10] | 10 | 10 | -55 | -40 | 4 | 24 | 9 |
| Voss *et al.* 2004 | Atlantic, Eastern Tropical North[10] | 10 | 5 | -25 | -15 | 6 | 140 | 32 |
| Voss *et al.* 2004 | Atlantic, Equatorial[10] | 0 | 0 | -26 | -24 | 2 | 4 | |
| Fernandez *et al.* 2010 | Atlantic, Equatorial[9] | 15 | -5 | -29 | -28 | 14 | 60 | 5 |
| Mourino-Carballido *et al.* 2011 | Atlantic, North[11] | 30 | 16 | -29 | -15 | 6 | 11 | 4 |
| Mourino-Carballido *et al.* 2011 *** | Atlantic, Equatorial[11] | 16 | -12 | -29 | -28 | 7 | 56 | 19 |
| Sohm *et al.* 2011 | Atlantic, South East[12] | -14 | -11 | -5 | 0 | 2 | 24 | |
| Sohm *et al.* 2011 | Atlantic, South East[12] | -14.75 | -14.75 | 12 | 12.2 | 1 | 85 | |
| Fernandez *et al.* 2010 | Atlantic, South[9] | -5 | -30 | -29 | -28 | 12 | 7 | 1 |
| Mourino-Carballido *et al.* 2011 | Atlantic, South[11] | -12 | -31 | -36 | -28 | 7 | 10 | 4 |
| Shiozaki *et al.* 2009 | Pacific, North[13] | 44 | 30 | -155 | -155 | 3 | 0 | |
| Montoya *et al.* 2004 | Pacific, Eastern North Gyre[14] | 35 | 25 | -160 | -125 | 10 | 520 | 160 |
| Needoba *et al.* 2007 | Pacific, North East[15] | 34 | 34 | -129 | -129 | 24 | 15 | |
| Hamersley *et al.* 2011 | Pacific, North East[16] | 34 | 34 | -119 | -119 | 15 | 150 | |
| White *et al.* 2007 | Pacific, North East[17] | 30 | 22 | -122 | -122 | 4 | 106 | 55 |
| Shiozaki *et al.* 2009 | Pacific, North[13] | 30 | 0 | -155 | -155 | 8 | 55 | 16 |
| Sohm *et al.* 2011 [‡] | Pacific, North[18] | 24 | 19 | -160 | -154 | 23 | 143 | 21 |
| Sohm *et al.* 2011 [‡] | Pacific, North[18] | 23 | 19 | -162 | -156 | 11 | 216 | 21 |
| Sohm *et al.* 2011 [‡] | Pacific, North[18] | 28 | 23 | -180 | -161 | 8 | 137 | 16 |
| Church *et al.* 2009 | Pacific, ALOHA[19] | 22.75 | 22.75 | -158 | -158 | 34 | 111 | 11 |
| Zehr *et al.* 2001 | Pacific, ALOHA[20] | 22.75 | 22.75 | -158 | -158 | 1 | 95 | |
| Montoya *et al.* 2004 | Pacific, ALOHA[14] | 22.75 | 22.75 | -158 | -158 | 7 | 66 | 19 |
| Grabowski *et al.* 2008 | Pacific, ALOHA[21] | 22.75 | 22.75 | -158 | -158 | 9 | 72 | 4 |
| Dore *et al.* 2002 * | Pacific, ALOHA[22] | 22.75 | 22.75 | -158 | -158 | 4 | 69 | 13 |
| Voss *et al.* 2006 ** | Pacific, North West[23] | 13.5 | 10 | 108 | 110.5 | 10 | 26 | 17 |
| Voss *et al.* 2006 | Pacific, North West[23] | 13.5 | 10 | 108 | 110.5 | 18 | 71 | 16 |
| Bombar *et al.* 2010 | Pacific, North West[24] | 13 | 10 | 108 | 111 | 4 | 23 | 3 |
| Bombar *et al.* 2010 | Pacific, North West[24] | 13 | 10 | 108 | 111 | 7 | 138 | 45 |
| Bombar *et al.* 2010 | Pacific, North West[24] | 13 | 10 | 108 | 111 | 4 | 88 | 49 |
| Bombar *et al.* 2010 | Pacific, North West[24] | 13 | 10 | 108 | 111 | 4 | 59 | 23 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Fernandez *et al.* 2011 | Pacific, South East[25] | 2 | -18 | -86 | -74 | 8 | 8 | 2 |
| Raimbault *et al.* 2008 | Pacific, South West[26] | -7 | -15 | -141 | -134 | 4 | 110 | 15 |
| Fernandez *et al.* 2011 | Pacific, South East[25] | -13 | -20 | -78 | -70 | 8 | 190 | 29 |
| Raimbault *et al.* 2008 | Pacific, South West[26] | -15 | -20 | -133 | -123 | 4 | 70 | 35 |
| Garcia *et al.* 2007 | Pacific, South West[27] | -20 | -22 | 166 | 167 | 6 | 290 | 86 |
| Raimbault *et al.* 2008 | Pacific, South West[26] | -20 | -30 | -123 | -101 | 4 | 60 | 15 |
| Raimbault *et al.* 2008 | Pacific, South West[26] | -30 | -33 | -100 | -81 | 6 | 30 | 2 |
| Raimbault *et al.* 2008 | Pacific, South West[26] | -33 | -35 | -80 | -72 | 2 | 90 | 47 |
| Montoya *et al.* 2004+ | Pacific, Arafura Sea[14] | -10 | -20 | 120 | 150 | 2 | 3955 | |
| Montoya *et al.* 2004 | Pacific, Kaneohen Bay[14] | -9.5 | -9.5 | 135 | 135 | 6 | 24 | 12 |
| Montoya *et al.* 2004 | Pacific, Arafura Sea[14] | -10 | -20 | 120 | 150 | 7 | 126 | 47 |

Table S3: Global rates of annual $N_2$ fixation. The rates are basin wide averages, obtained by station weighted averaging over the four domains (North Atlantic, South Atlantic, North Pacific and South Pacific Ocean) from table S2. * For the Indian Ocean areal rates of the Pacific Ocean were used.

| Ocean Basin | North limit (°N) | South limit (°N) | Stations | Areal rate (μmol N $m^{-2}$ $d^{-1}$) | Area ($10^6$ $km^2$) | Areal rate (mol N $m^{-2}$ $yr^{-1}$) | Basin (mol N $y^{-1}$) | Basin rate (tg N $y^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| Atlantic North and Equatorial (weighted average) | 50 | -5 | 46 | 64 | 39.2 | 0.023 | 9.08E+11 | 12.7 |
| Atlantic South (weighted average) | -5 | -30 | 26 | 19 | 15.9 | 0.007 | 1.08E+11 | 1.5 |
| Pacific North (weighted average) | 30 | 0 | 208 | 115 | 56.3 | 0.042 | 2.36E+12 | 33.0 |
| Pacific South (weighted average) | 0 | -35 | 55 | 103 | 56.1 | 0.038 | 2.11E+12 | 29.5 |
| Indian North | 25 | 0 | 0 | 115* | 15.8 | 0.042* | 6.62E+11 | 9.3 |
| Indian South | 0 | -35 | 0 | 103* | 31.8 | 0.038* | 1.20E+12 | 16.7 |
| Global Sum | | | | | 215.1 | | | **102.7** |

Recently, the annual contribution of $N_2$ fixation to the Atlantic and Pacific Ocean has been estimated at 63 Tg N yr-1 based on $N_2$ fixation rate measurements made with the bubble-addition method and the acetylene reduction assay [28]. This estimate is comparable in magnitude to the 76 Tg N yr-1 obtained in this study for Atlantic and Pacific Ocean combined.

## Supplementary Online Material and Figures

***Comparison of mean $N_2$ fixation rates measured by the bubble-addition and the dissolution methods in the tropical and equatorial regions***

A pairwise comparison (t-test for dependent samples) of the mean $N_2$ fixation rates determined with the bubble-addition and the dissolution methods within the tropical and equatorial areas (Fig. S4) indicated that the underestimation by the bubble-addition method was statistically significant in both regions. Moreover, this underestimation was substantially higher in the equatorial region, which was dominated by diazotrophs other than *Trichodesmium.*

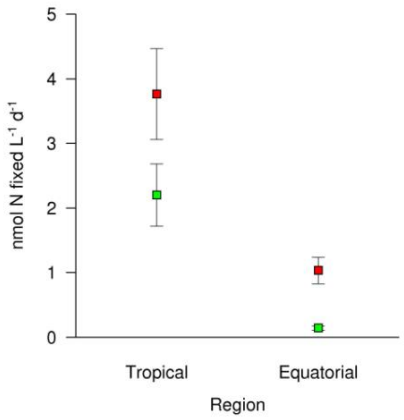


Figure S4: Plot of means with standard error for the dissolution method (red boxes) and bubble-addition method (green boxes). Rates were pooled over all depths for the Equatorial (4.5°N - 5°S) and the Tropical North Atlantic (15°N - 5°N) region. The mean $N_2$ fixation rates measured by the dissolution and the bubble-addition methods were significantly different for both the equatorial region (t-test for dependent samples, $p < 0.01$, $df = 61$), and the tropical region (t-test for dependent samples, $p < 0.01$, $df= 38$). The means of the bubble and dissolution methods over the entire dataset significantly differed by a factor of 2 (1.0 +/- 0.2

nmol N $L^{-1}$ $d^{-1}$(n=116) and 2.0 +/-0.3 nmol N $L^{-1}$ $d^{-1}$ (n=115) (means and s.e.m. of the bubble-addition and the dissolution method, respectively (t-test for dependent samples, $p < 0.01$, df = 114)).

Table S4: Mean and standard deviation for the 39 parallel comparison experiments. Date and start time mark the beginning of an 24 hour incubation. n.d. = not detectable.

| Date | Start time | Depth (m) | Lat ( °N) | Lon ( °E) | Bubble-addition method (nmol N L$^{-1}$ d$^{-1}$) | std. dev. (n=3) | dissolution method (nmol N L$^{-1}$ d$^{-1}$) | std. dev. (n=3) |
|---|---|---|---|---|---|---|---|---|
| 28.10.2009 | 16:00 | 11 | 25.3 | -16.6 | 3.88 | 0.71 | 3.63 | 0.47 |
| 21.11.2009 | 11:00 | 0 | 13.8 | -23 | 6.94 | 0.2 | 9.98 | 0.6 |
| 21.11.2009 | 11:00 | 20 | 13.8 | -23 | 7.15 | 0.66 | 12.44 | 0.57 |
| 21.11.2009 | 11:00 | 42 | 13.8 | -23 | n.d. | - | n.d. | - |
| 19.11.2009 | 06:00 | 0 | 9 | -23 | 3.2 | 0.39 | 4.77 | 2.03 |
| 19.11.2009 | 06:00 | 20 | 9 | -23 | 5.74 | 1.2 | 4.09 | 1.72 |
| 19.11.2009 | 06:00 | 59 | 9 | -23 | 0.07 | 0.08 | 0.09 | 0.88 |
| 17.11.2009 | 05:00 | 0 | 6.8 | -23 | 0.32 | 0.12 | n.d. | - |
| 17.11.2009 | 05:00 | 20 | 6.8 | -23 | 0.13 | 0.06 | n.d. | - |
| 17.11.2009 | 05:00 | 64 | 6.8 | -23 | n.d. | - | 1.75 | 0.08 |
| 31.10.2009 | 08:00 | 0 | 5 | -23 | 0.8 | 0.17 | 6.44 | 4.69 |
| 31.10.2009 | 08:00 | 20 | 5 | -23 | 1.32 | 0.88 | 3.24 | 1.85 |
| 31.10.2009 | 08:00 | 71.3 | 5 | -23 | n.d. | - | 1.01 | 0.35 |
| 03.11.2009 | 16:00 | 11 | 4.6 | -23 | 0.45 | 0.02 | 0.92 | 0.04 |
| 15.11.2009 | 09:00 | 0 | 3.5 | -23 | 0.48 | 0.21 | 1.06 | 0.79 |
| 15.11.2009 | 09:00 | 20 | 3.5 | -23 | 0.19 | 0.13 | n.d. | - |
| 15.11.2009 | 09:00 | 63 | 3.5 | -23 | n.d. | - | n.d. | - |
| 02.11.2009 | 06:00 | 0 | 2 | -23 | 0.31 | 0.12 | 4.29 | 1.81 |
| 02.11.2009 | 06:00 | 20 | 2 | -23 | 0.42 | 0.06 | 2.44 | 0.39 |
| 02.11.2009 | 06:00 | 76.6 | 2 | -23 | n.d. | - | 1.24 | 1.03 |
| 05.11.2009 | 08:00 | 0 | 0 | -23 | 0.31 | 0.11 | 4.23 | 4.4 |
| 05.11.2009 | 08:00 | 20 | 0 | -23 | 0.14 | 0.16 | n.d. | - |
| 05.11.2009 | 08:00 | 58.5 | 0 | -23 | n.d. | - | n.d. | - |
| 13.11.2009 | 06:00 | 0 | 0 | -23 | 0.04 | 0.03 | 0.75 | 1.48 |
| 13.11.2009 | 06:00 | 20 | 0 | -23 | 0.01 | 0.07 | 0.02 | 0.31 |
| 13.11.2009 | 06:00 | 55 | 0 | -23 | n.d. | - | 0.75 | 0.3 |
| 07.11.2009 | 08:00 | 0 | -1.3 | -23 | 0.6 | 0.89 | 2.37 | 1.17 |
| 07.11.2009 | 08:00 | 20 | -1.3 | -23 | 0.1 | 0.14 | 1.34 | 0.2 |
| 07.11.2009 | 08:00 | 70.2 | -1.3 | -23 | n.d. | - | 0.89 | 0.1 |
| 11.11.2009 | 08:00 | 0 | -2 | -23 | 0.04 | 0.06 | 0.64 | 0.64 |
| 11.11.2009 | 08:00 | 20 | -2 | -23 | 0.01 | 0.15 | 0.1 | 0.04 |
| 11.11.2009 | 08:00 | 64.4 | -2 | -23 | n.d. | - | n.d. | - |
| 09.11.2009 | 06:00 | 0 | -4.5 | -23 | 0.02 | 0.02 | 0.84 | 0.73 |
| 09.11.2009 | 06:00 | 20 | -4.5 | -23 | 0.13 | 0.14 | 0.07 | 0.23 |
| 09.11.2009 | 06:00 | 68.5 | -4.5 | -23 | n.d. | - | n.d. | - |
| 12.11.2009 | 16:00 | 11 | -24.7 | -29.5 | 1.26 | 0.07 | 1.06 | 0.13 |
| 14.11.2009 | 16:00 | 11 | -28.1 | -35.8 | 4.93 | 1.41 | 5.01 | 0.44 |
| 18.11.2009 | 16:00 | 11 | -38.1 | -48.2 | 0.1 | 0.01 | 0.44 | 0.13 |
| 21.11.2009 | 16:00 | 11 | -44.6 | -56.4 | 0.18 | 0.06 | 0.54 | 0.21 |

***Magnitude of the underestimation by the bubble-addition method relative to the dissolution method***

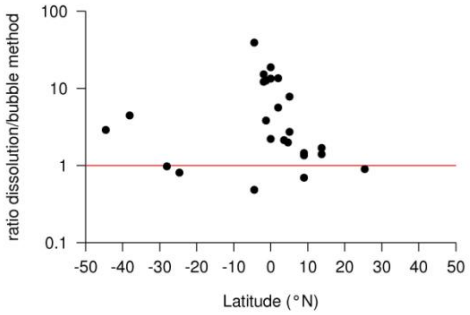


Figure S5: Ratio of $N_2$ fixation rates measured by dissolution method relative to the bubble-addition method (note the logarithmic scale) as a function of latitude. Ratios were calculated for the paired measurements where $N_2$ fixation rates were detectable with both methods (n=25). Note that the ratio is particularly large (i.e. the underestimation of $N_2$-fixation rates by the bubble method is large) in the equatorial regions where diazotrophs other than *Trichodesmium* dominate.

***Summary and implications of the results presented in Mohr et al. (2010)***

Mohr et al. (2010) showed that the equilibration of a $^{15}N_2$ gas bubble in seawater is slow, taking up to 12 hours to reach ~ 80% of the calculated value based on complete equilibration of the $^{15}N_2$ label with seawater (Fig. S6, upper panel). In contrast, the $^{15}N_2$ label added as dissolved $^{15}N_2$ remained constant with time (Fig. S6, lower panel). Mohr et al. (2010) shows that the strength of agitation (their Figure 2 and 4), bottle size and bubble size (their Figure 3 and 4), affect the kinetics of the $^{15}N_2$ gas equilibration with the surrounding seawater. The consequence of the slow and variable equilibration time of the $^{15}N_2$ gas in field incubations carried out by the bubble addition method[1] is that the rates will be underestimated in most cases.

In view of the slow equilibration of $^{15}N_2$ gas with its surrounding water and the resulting underestimation of the $N_2$ fixation rate, we suggest that, in the future, gas exchange/equilibration in the acetylene reduction assay (ARA) may also need to be considered to ensure accuracy of the method.

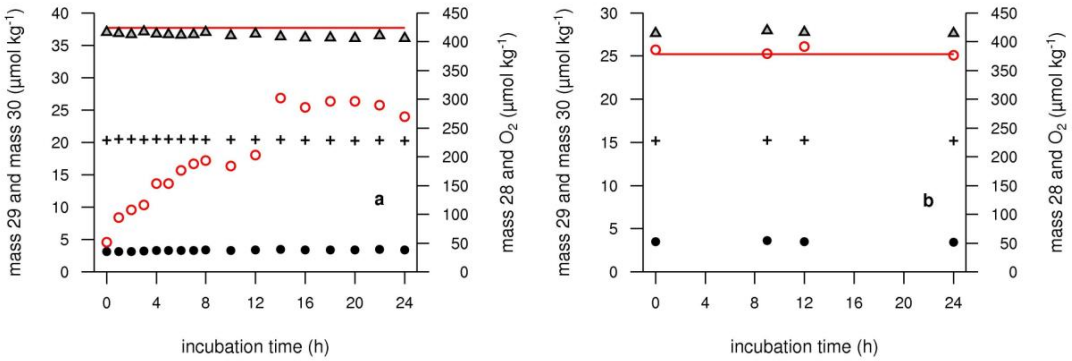


Figure S6: Membrane inlet mass spectrometry measurements of $^{28}N_2$, $^{29}N_2$, $^{30}N_2$, and dissolved oxygen ($^{32}O_2$) concentrations versus equilibration time for the bubble-addition method (a) and the dissolution method (b). The calculated (expected) values for $^{15}N_2$ (mass 30) are 37.7 and 25.2 µmol kg$^{-1}$ for the bubble-addition and the dissolution method, respectively (red solid lines) (data redrawn from Mohr et al. (2010).

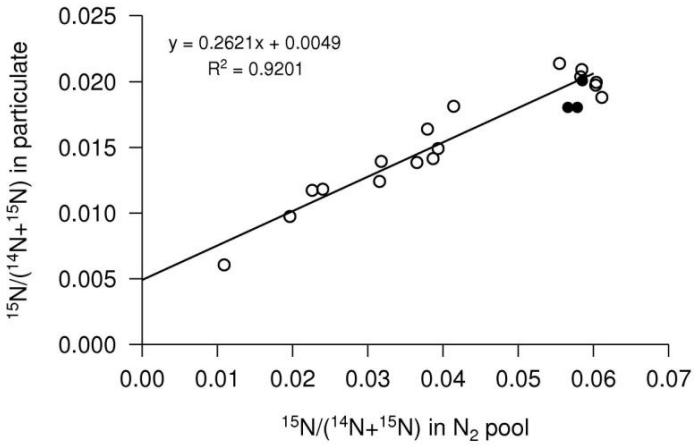


Figure S7: Linear correlation between the $^{15}N/(^{14}N+^{15}N)$ ratio for dissolved $N_2$ and the corresponding $^{15}N/(^{14}N+^{15}N)$ ratio for particulate nitrogen from the *Crocosphaera watsonii* culture after 12 h incubation. The linear relationship shows that $^{15}N$-assimilation by these diazotrophs is directly proportional to the $^{15}N$-labeling % for dissolved $N_2$ in the medium. The slope of the regression line is 0.26 indicating that the N-based doubling times for *Crocosphaera* were ~2 days in our incubations, which is comparable to published growth rates[29]. The different levels of $^{15}N$ enrichment in seawater were achieved by pre-equilibrating

a gas bubble for times varying from 1 to 24 hours prior to the inoculation with the *Crocosphaera* cultures. For comparison, the values of incubations to which dissolved $^{15}N_2$ was added are displayed (filled circles). Data redrawn from Mohr et al. (2010).

***Considerations about the change in gas composition in an incubation***

An incubation of seawater in a bottle always presents a situation that is ideally close to, but not identical to *in situ* conditions. Since a bottle offers a very limited space, it is possible that in a 24 hour incubation some organisms or particulate matter sink to the bottom of the bottle, where oxygen will get more depleted than in the upper part of the bottle. However, such bottle effects are inherent to any kind of incubation and would not differentially affect the methods used here. In both the bubble-addition and the dissolution method a minor part of the gas composition is replaced by the added $^{15}N_2$ label. This volume needs to be kept as low as possible, but on the other hand the desire is to maximize the addition of $^{15}N_2$ label, in order to increase the detection limit. By adding 100 mL of degassed water to a 4.5 L incubation (= 2.2%), the oxygen concentration for example drops theoretically from 225 µM to 220 µM. Such a change in oxygen concentration is within the natural variability of surface oxygen concentration due to photosynthesis and respiration. In a bubble-addition type of incubation with 1 mL $^{15}N_2$ gas per L of seawater the drop in oxygen concentration would be from 225 µM to ~216 µM, since the bubble would hold ~20% oxygen after complete equilibration. The manipulation in the gas composition is therefore similar in both methods and within the range of natural variability. In Mohr et al, (2010), the concentrations of the nitrogen isotopes as well as dissolved oxygen were measured in water samples that were incubated with a $^{15}N_2$ gas bubble as well as with dissolved $^{15}N_2$ additions (see Fig. S6 above). The measured data confirm the theoretical changes mentioned here.

The strong linear correlation between the isotopic composition of dissolved $N_2$ in the medium and the N-isotopic composition of *Crocosphaera* biomass (Fig. S7) shows that variations in $^{15}N_2$, and total dissolved $N_2$ concentrations themselves have no significant effect on the $N_2$-fixation rate.

***Examples of potential bias introduced by the species composition for the bubble-addition method***

There are several ways in which the diazotrophic community composition can affect the magnitude of underestimation in a bubble-addition method incubation. One example is via

the time of $N_2$ fixation activity relative to the beginning of the incubation. Diazotrophic communities dominated by diazotroph species fixing at night (e.g. *Crocosphaera*) will suffer less underestimation than one that is dominated by diazotrophs fixing during the day (e.g. UCYN-A) when the incubation is set up at sunrise, since the $^{15}N_2$ label had more time to equilibrate with the water phase [2] and vice versa.

The second way is via the relative position in the water, i.e. the "closeness" of the diazotroph to the source of label, the $^{15}N_2$ bubble. The following experiment with *Nodularia*, a buoyant diazotrophic cyanobacterium, suggests that this may also happen in natural communities. A culture of the heterocystous cyanobacterium *Nodularia spumigena* IOW-2000/1 was grown in modified artificial seawater [30] supplemented with phosphate and trace metals (ASW). The culture was transferred twice into fresh medium before the experiment. For the experiment, three 450 ml sub-cultures (1 week after last transfer) were transferred from culture flasks into 1.15 L sterile glass bottles. The cultures were supplemented with fresh media up to ~ 1.13 L. Cultures were left over-night to allow filaments to float to the top of the bottle. The next day, cultures were filled with fresh media from the bottom with a syringe in order to keep the floating filaments in place. The bottles were closed headspace-free with Teflon®-coated butyl rubber septum caps and 5 mL of $^{15}N_2$ gas were injected just underneath the septum. At the end of the ~ 6 h incubation period during the day time, the floating filaments were separated from the non-floating ones and both were separately filtered onto pre-combusted GF/F filters (Whatman). A natural abundance sample was also filtered at the end of the incubation. Filters were treated as previously described.

The nitrogen fixation rate measured in the floating fraction was significantly higher (t-test, $p < 0.05$, $n = 3$) than the measured rate of the bulk phase (Fig. S8). However, $N_2$ fixation rates measured with the $^{15}N_2$ isotope added as a dissolved gas (dissolution method) measured during the same experiment were approximately 2 and 6 times higher than those measured with the bubble-addition method for the floating and the non-floating fractions, respectively. Although our experimental design does not rule out that the floating and sinking fraction may have been in a different physiological state and fixing $N_2$ at different rates, the right panel of Figure S8 shows that a small layer of filaments close to the bubble of pure $^{15}N_2$ gas would have been exposed to very high concentration of $^{15}N_2$ gas during the incubation period. The calculated rates for the floating and non-floating fraction are in agreement with this hypothesis and suggest that such situation may also develop during calm field conditions. The culture results presented here are also congruent with the observation of less discrepancy

between the bubble-addition and dissolution method in areas which were dominated by *Trichodesmium* vs. areas not dominated by *Trichodesmium* which suffered from larger discrepancies.

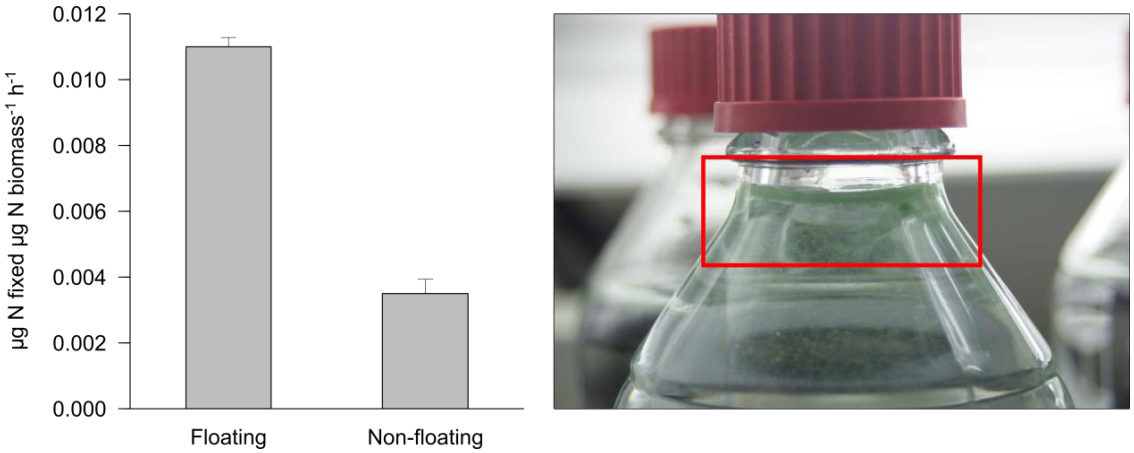


Figure S8. Left: Biomass-specific $N_2$ fixation rates of *N.spumigena* measured with the bubble-addition method. Filaments at the very surface of the incubation bottle (floating) were analyzed separately from the bulk liquid phase (non-floating). Error bars indicate standard errors of triplicate incubations. Right: Filaments of *Nodularia* in the incubation bottles. Red box marks the floating fraction analyzed in this experiment.

***Literature survey of incubation conditions used for $^{15}N_2$ fixation measurements***

Since many factors besides the diazotrophic species composition (see upper section) have the potential to influence the magnitude of difference between the two methods, like bottle size, incubation time and bubble size, it is of interest to know how our experimental conditions compared to those in other studies. Table S5 gives an overview of the published literature and the experimental setup used. The global mean of $^{15}N_2$ gas added per liter of sample is 2.6 mL $L^{-1}$, the median is 1.0 mL $L^{-1}$. In this study we used 1 mL $L^{-1}$ of $^{15}N_2$ gas, which is a representative value of the previously published work. The incubation time and bottle size in this study were 24 h and 4.5 liters, respectively, both representative of conditions used for whole water incubations in oligotrophic regions. In addition, a 24 h incubation also ensures that the $N_2$ fixation measurement covers a full daily cycle. However, the table below shows the range of incubation times used and that the underestimation or rates may be highly variable especially considering the timing effect of incubation vs. the timing of $N_2$ fixation activity.

Table S5: Published literature about studies using the $^{15}N_2$ tracer addition (bubble-addition) method and the parameters used in their incubation setup.

| Reference | Year published | Ocean | Study area or organism | Incubation time [h] | $^{15}N_2$ [ml $L^{-1}$] | bottle size [L] |
|---|---|---|---|---|---|---|
| Benavides *et al.* | 2011[31] | Atlantic | NE Atlantic | 24 | 1.6 | 1.24 |
| Bombar *et al.* | 2011[32] | South China Sea | Mekong River Plume | 6-12 | 0.9 | 2.3 |
| Bombar *et al.* | 2010[33] | South China Sea | upwelling | 6 | 1.1 | 2.3 |
| Bonnet *et al.* | 2009[34] | Pacific | Equatorial W Pacific | 24 | 0.7 | 4.5 |
| Bonnet *et al.* | 2011[35] | Mediterranean | | 24 | 0.9 | 4.5 |
| Burns *et al.* | 2006[36] | Atlantic / Pacific | *Trichodesmium* | 4 | 0.4 | 0.25 |
| Capone *et al.* | 2005[37] | Atlantic | *Trichodesmium* | 2 | 0.3 | 0.31 |
| Chen *et al.* | 2008[38] | South China Sea | Kuroshio and South China Sea Basin, *Trichodesmium* | 3 | 1.7 | 0.12 |
| Chen *et al.* | 2011[39] | Pacific | NW Pacific / Kuroshio / *Trichodesmium* | 3-5 | 1.7 | 0.12 |
| Chen *et al.* | 2011[39] | culture | *Trichodesmium* | 24 | 5 | 1.2 |
| Church *et al.* | 2009[19] | Pacific | N Pacific subtropical gyre, station ALOHA | 24 | 0.7 | 4.5 |
| Degerholm *et al.* | 2008[40] | Baltic Sea | | 4 | 18.4 | 0.25 |
| Dore *et al.* | 2002[22] | Pacific | subtropical N Pacific | 24 | 0.2 | 4.7 |
| Falcón *et al.* | 2004[41] | Atlantic / Pacific | Unicellular | 24 | 0.64 | 0.014 |
| Fernandez | 2010[42] | Atlantic | Atlantic | 24 | 1 | 2 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *et al.* | | | | | | |
| Fernandez *et al.* | 2011[25] | Pacific | tropical SE Pacific | 24 | 2 | 2 |
| Fong *et al.* | 2008[43] | Pacific | N Pacific subtropical gyre | 24 | 0.7 | 4.5 |
| Gandhi *et al.* | 2011[44] | Indian Ocean | Arabian Sea | 4 | 1.6 | 1.25 |
| Garcia *et al.* | 2011[45] | culture | *Trichodesmium* | 12 | 1 | 0.16 |
| Garcia *et al.* | 2007[27] | Pacific | SW Pacific | 12 | 1.7 | 1.2 |
| Goebel *et al.* | 2010[46] | Atlantic | tropical Atlantic | 24 | 0.1 | 3.8 |
| Grabowski *et al.* | 2008[21] | Pacific | ALOHA | 24 | 0.25 | 4.5* |
| Grosse *et al.* | 2010[47] | South China Sea | Mekong River Plume | 6-12 | 0.9 | 2.3 |
| Holl *et al.* | 2007[48] | Atlantic | W Gulf of Mexico / *Trichodesmium* | 6 | 0.96 | 0.25 |
| Ibello *et al.* | 2010[49] | Mediterranean | | 24 | 1 | 4.6 |
| Konno *et al.* | 2010[50] | Pacific | NW Pacific | 24-72 | 2-4 | 0.25-0.5 |
| Law *et al.* | 2011[51] | Pacific | SW Pacific | 12-36 | 1 | 2.4 |
| Maranon *et al.* | 2010[52] | Atlantic | central Atlantic | 24 | 1 | 2 |
| Mills *et al.* | 2004[53] | Atlantic | eastern tropical N Atlantic | 24 | 0.85 | 1.18 |
| Moisander *et al.* | 1996[54] | Baltic Sea | Field and culture | 2 | 40 | 0.0245 |
| Moore *et al.* | 2009[55] | Pacific | Fiji to Hawaii | 24 | 0.7 | 4.5 |
| Mourino-Carballido | 2011[56] | Atlantic | tropical and subtropical Atlantic | 24 | 1 | 2 |
| Moutin *et al.* | 2008[57] | Pacific | tropical S Pacific | 24 | 1.7 | 0.6 |
| Mulholland | 2005[58] | culture | *Trichodesmium* | 1 | 1 | 0.16 |

| | | | | | | |
|---|---|---|---|---|---|---|
| and Bernhardt | | | | | | |
| Mulholland *et al.* | 2004[59] | culture | *Trichodesmium* | 2 | 1 | 0.16 |
| Needoba *et al.* | 2007[15] | Pacific | oligotrophic N Pacific Ocean | 48 | 2 | 4 |
| Orcutt *et al.* | 2001[60] | Atlantic | BATS/*Trichodesmium* | 6 | 14.3 | 0.014 |
| Ploug *et al.* | 2010[61] | Baltic Sea | *Aphanizomenon* | 3-6 | 8 | 0.25 |
| Raimbault and Garcia | 2008[26] | Pacific | S Pacific Ocean | 24 | 3.5 | 0.58 |
| Rees *et al.* | 2009[8] | Atlantic | western English Channel | 24 | 2 | 0.64 |
| Rees *et al.* | 2006[62] | Mediterranean | E Mediterranean Sea | 24 | 2 | 10 |
| Ridame *et al.* | 2011[63] | Mediterranean | | 24 | 1.1 | 4.5 |
| Rijkenberg *et al.* | 2011[64] | Atlantic | subtropical NE Atlantic | ≥24 | 1 | 4 |
| Sandroni *et al.* | 2007[65] | Mediterranean | NW Mediterranean Sea | 12 | 1.7 | 0.6 |
| Shiozaki *et al.* | 2009[13] | Pacific | NW Pacific (along 155°E) | 24 | 0.5 | 4.5 |
| Sohm *et al.*, A | 2011[12] | Atlantic | South Atlantic Gyre | 24 | 0.7 | 4.5 |
| Sohm *et al.*, B | 2011[18] | Pacific | whole water and *Trichodesmium* | 24 | 0.7 | 4.5 |
| Ternon *et al.* | 2011[66] | Mediterranean | | 24 | 1.1 | 4.5 |
| Turk *et al.* | 2011[67] | Atlantic | tropical NE Atlantic | 6 | 2 | 1 |
| Twomey *et al.* | 2007[68] | Indian | SW coast of Australia | 6 | 0.8 | 4 |
| Voss *et al.* | 2004[10] | Atlantic | tropical N Atlantic, transect at 10°N | 6 | 1 | 1 |
| Voss *et al.* | 2006[23] | South China Sea | Off Vietnam | 6 | 1.1 | 2.3 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Wannicke *et al.* | 2010[69] | Atlantic | NE Atlantic | 1-20 | 1 | 2.5 |
| Wasmund *et al.* | 2005[70] | Baltic Sea | | 2 | 2 | 0.25 |
| Wasmund *et al.* | 2001[71] | Baltic Sea | | 8 | 4 | 0.25 |
| Watkins-Brandt *et al.* | 2011[72] | Pacific | North Pacific | 24 | 0.5 | 4.4 |
| White *et al.* | 2007[17] | Pacific | Gulf of California | 24 | 0.3 | 2 |
| Yogev *et al.* | 2011[73] | Mediterranean | E Mediterranean Sea | 24-30 | 2 | 4.5 |
| **Mean** | | | | **15** | **2.6** | **2.2** |
| **Median** | | | | **18** | **1.0** | **2.0** |
| **This study** | **2012** | **Atlantic** | | **24** | **1** | **4.5** |

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