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Efficient CO_2 fixation by surface *Prochlorococcus* in the Atlantic Ocean

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Abstract

Nearly half of the Earth's surface is covered by the ocean populated by the most abundant photosynthetic organisms on the planet – *Prochlorococcus* cyanobacteria. However, in the oligotrophic open ocean the majority of their cells in the top half of the photic layer have levels of photosynthetic pigmentation barely detectable by flow cytometry suggesting low efficiency of CO_2 fixation compared to other phytoplankton living in the same waters. To test the latter assumption CO_2 fixation rates of flow cytometrically sorted ^{14}C-labelled phytoplankton cells were directly compared in surface waters of the open Atlantic Ocean (30° S to 30° N). CO_2 fixation rates of *Prochlorococcus* are at least 1.5-2.0 times higher than CO_2 fixation rates of the smallest plastidic protists and *Synechococcus* cyanobacteria when normalised to photosynthetic pigmentation assessed using cellular red autofluorescence. Therefore our data indicate that in oligotrophic oceanic surface waters pigment minimisation allows *Prochlorococcus* cells to harvest plentiful sunlight more effectively than other phytoplankton.

Introduction

Oceanic phytoplankton constitute only a minute fraction of the photosynthetic biomass on Earth (Falkowski, 2012), but they contribute almost half of the yearly global net primary production (Behrenfeld *et al.*, 2001; Falkowski *et al.*, 1998; Field *et al.*, 1998). *Prochlorococcus* (Pro) cyanobacteria are the most abundant phytoplankton in the ocean inhabiting nutrient-depleted environments from the equator up to 40-50 degrees of latitude (Campbell and Vaulot, 1993; Zubkov *et al.*, 2000). However, in surface waters the cellular pigment concentration of Pro are very low (~0.1 fg divinyl chlorophyll per cell) and consequently their carbon to chlorophyll ratios are very high (93.3-122 mg C per mg chlorophyll-*a*) in comparison to *Synechococcus* cyanobacteria and small picoeukaryotes (38-58 mg C per mg chlorophyll-*a*) (Bouman *et al.*, 2006; Veldhuis and Kraay, 2004).

From their discovery onwards Pro cells were almost exclusively enumerated by flow cytometry using their red autofluorescence and small size for identification (Chisholm *et al.*, 1988; Partensky *et al.*, 1999). Cellular red autofluorescence and chlorophyll pigment content are directly linked (Cavender-Bares *et al.*, 1999; Dusenberry *et al.*, 2001; Moore *et al.*, 1995), although the relation may not be always linear and can be affected by the pigment “packaging” or variations in accessory pigments predominantly for larger, more heterogeneous cells (Sosik *et al.*, 1989). Low red autofluorescence of Pro cells hampered their flow cytometric enumeration (Dusenberry and Frankel, 1994; Olson *et al.*, 1990) and technical improvements such as tightening of the beam spot, broadening of laser

excitation, the introduction of quartz flow cells with integrated lenses as well as the reduction of the sheath pressure were used to detect low pigmented Pro cells (Cavender-Bares *et al.*, 1998; Olson *et al.*, 1990). However, in the upper 40-80 m of stratified oligotrophic oceanic waters it has remained a challenge to enumerate Pro cells unambiguously using their red autofluorescence (Chisholm *et al.*, 1988; Malmstrom *et al.*, 2010; Partensky *et al.*, 1999; Ting *et al.*, 2002; Zubkov *et al.*, 1998). In such surface waters the flow cytometrically visible Pro population is typically shaped in the form of a "rising sun" emerging out of the background noise on a scatter plot of red autofluorescence (>650 nm) vs. 90° side light scatter (Charles *et al.* (2005) and Fig. S1). In some studies, an extrapolation based on the visible part of the Pro population was applied to correct for the missing part (e.g. Partensky *et al.*, 1996). Nucleic acid staining of bacterioplankton was proposed as an alternative solution to the Pro cell detection problem (Zubkov *et al.*, 2000). Later molecular identification confirmed that a distinct population observed following nucleic acid staining consists mainly of Pro cells (Gomez-Pereira *et al.*, 2013; Mary *et al.*, 2008; Zubkov *et al.*, 2007). Direct comparison of the two approaches for Pro enumeration, i.e. using red autofluorescence or cellular DNA-content/light scatter for identification, showed that in surface waters up to a half of the Pro cells are unaccounted when red autofluorescence is used as the sole identifier (Zubkov *et al.*, 2000).

High irradiance and resulting bleaching of photosynthetic pigments alongside potential nutrient-limitation in surface waters of oceanic subtropical gyres are common explanations for almost colourless Pro cells in surface ocean waters.

Because in deeper (>80 m) nutrient-replete but less illuminated parts of the water column Pro pigmentation is much more intense (Partensky *et al.*, 1999) CO_2 uptake studies have focused on Pro living in deeper waters (Chisholm *et al.*, 1988; Li, 1994).

We put to the test two alternative explanations that account for the extremely low photopigmentation of Pro cells in the nutrient-depleted surface waters: (a) pigment levels are decreased as a result of the combined effects of high irradiance and nutrient limitation, which diminishes capacity of the cells to cope with this stress, or (b) constitutively low cellular levels of photosynthetically active pigmentation are adequately physiologically balanced for these environmental conditions. In the former case Pro red autofluorescence normalised CO_2 fixation should be low compared to other phytoplankton, whereas red autofluorescence normalised CO_2 fixation of Pro cells will be comparable with, or higher than, red autofluorescence normalised CO_2 fixation by other phytoplankton cells if the latter were true.

Here, we present direct experimental evidence that red autofluorescence normalised CO_2 fixation of surface Pro is high compared to the smallest eukaryotic phytoplankton and *Synechococcus* cyanobacteria. These results demonstrate that the dim red autofluorescence of surface Pro does not prevent them attaining high CO_2 fixation rates across the Atlantic Ocean.

Materials and Methods

Sampling. Pre-dawn seawater samples were collected from 20 m depth in 20 L Niskin bottles attached to a standard conductivity-temperature-depth profiler

on the 20th cruise of the Atlantic Meridional Transect programme aboard the UK Royal Research Ship *James Cook* in October-November 2010 (Fig. S2). Seawater content of the entire Niskin bottle was decanted into an acid-rinsed polycarbonate carboy. To prevent exposure of photosynthetic cells to artificial light on board, the carboy was covered completely with two layers of dark plastic. Samples were processed immediately after collection. The sampling depth was chosen because it reflects the surface mixed layer while the influence of ship movement and contaminants at that depth are minimal. At selected stations (indicated in Fig. S2) additional samples were taken from the bottom of the thermocline in order to compare CO_2 fixation rates of deeper vs. surface phytoplankton communities.

Abundance measurements and definition of regional boundaries. Concentrations of the *Synechococcus* (Syn) and *Prochlorococcus* cyanobacteria were determined in unstained, fixed (1% paraformaldehyde (PFA), final concentration, Sigma-Aldrich, Germany) samples according to Olson et al. (1993) using a FACSort flow cytometer (Becton-Dickinson, UK). *Prochlorococcus* cells were counted both in unstained (Pro_{unst}) and stained, fixed samples (Pro_{st}) on the basis of their red autofluorescence and 90° side light scatter, and their nucleic acid content (green fluorescence) and 90° side light scatter, respectively. Subsamples for Pro_{st} counting were taken from a 20 L carboy and fixed with 1% PFA for 1h in the dark at room temperature and stained with SYBR Green I dye (Sigma-Aldrich, Germany) (Marie *et al.*, 1997). Cellular abundances of small (<2 µm) and large (2-5 µm) plastidic eukaryotes (Plast-S and Plast-L respectively) were determined in parallel from the same

sample. Prior to flow cytometric analyses a mixture of 0.5 µm and 1.0 µm multi-fluorescent beads (Polysciences, Germany) at a calibrated concentration (Zubkov and Burkill, 2006) was added to both stained and unstained samples. The beads were used as an internal standard for calculating absolute cell concentrations and for normalising cellular red autofluorescence.

Four major oceanic regions were identified using primarily Syn abundances: Northern subtropical gyre (NG), equatorial waters (EQ), Southern subtropical gyre (SG) and Southern temperate waters (ST) (Hartmann *et al.*, 2012).

Catalysed reporter deposition fluorescence *in situ* hybridisations (CARD-FISH) on flow cytometrically sorted cells. In order to confirm that the distinct, high-nucleic acid bacterial population observed by flow cytometry (Fig. S1) consists mainly of Pro cells, CARD-FISH hybridisations using the *Prochlorococcus*-specific probe PRO405 (West *et al.*, 2001) were carried out on sorted cells at selected stations covering each province (NG, EQ, SG). The *in silico* specificity of the probe was re-evaluated by running TestProbe (part of the Silva online software packages, www.arb-silva.de, (Quast *et al.*, 2013)) against the Silva SSU r117 reference database. In addition, the Eubacteria-targeted probe mix Eub338I-III (Amann *et al.*, 1990; Daims *et al.*, 1999) was used to determine overall hybridisation efficiency. The details of contamination free flow sorting of target cells and CARD-FISH are described in Gomez-Pereira *et al.* (2013). We analysed 7 stations in the NG, 3 stations in the EQ and 4 stations in the SG.

Total and cell-specific CO_2 fixation. Prior to each experiment, 60 ml Pyrex glass bottles (Fisher Scientific, UK) were acid-soaked overnight (10% HCl) and rinsed twice with 30ml sample seawater. After washing, 60 ml of seawater sample were added to each bottle and spiked with trace metal-clean ^{14}C radiolabelled sodium bicarbonate (34.66 mM $NaH^{14}CO_3$, DHI, Denmark). Samples were then incubated at ambient temperatures (regulated by a refrigerated water bath (Grant Instruments, UK)) in a 6 L water tank illuminated by a warm white light-emitting diode array (Photon Systems Instruments, Czech Republic) adjusted to a constant output of 500 µmol photons m^{-2} s^{-1}. The chosen light intensity equals half the irradiance reaching the water surface at noon-time in the equatorial region (Jitts *et al.*, 1976), because on average at 20 m depth the light intensity is reduced by 33-55%. Moreover, no photoinhibition occurs at this light intensity (Morel *et al.*, 1996). In contrast to incubations at ambient light, the constant light output made it possible to compare CO_2 fixation rates at different stations.

Two different concentrations of $NaH^{14}CO_3$ were used in order to determine total CO_2 fixation during a time series and to measure CO_2 fixation of flow cytometrically sorted phytoplankton populations, respectively. Time series were carried out to ensure linear uptake of label and to guarantee that the small volumes of sorted cells are representative of the whole community (Fig. S3). Subsamples of 1.6 ml were taken at 0 h and 10 h for flow cytometric analyses to ascertain that community composition remained unchanged for the duration of the experiment (Fig. S4).

For time series CO_2 fixation measurements, 3.7 kBq ml^{-1} $NaH^{14}CO_3$ was added to six 60 ml Pyrex glass bottles. Five bottles were incubated for 2, 4, 6,

8, 10 h in the light; and the remaining bottle was incubated for 10 h in the dark. At each discrete time point, the whole bottle was sacrificed by adding 1% PFA (final concentration) and incubated for 1 h at RT. Subsequently, the complete sample was filtered onto a 0.2 µm polycarbonate filter (Nuclepore, Whatman, UK), washed three times with ultra-clean water (MQ system, Millipore, Whatman, UK) and placed in a scintillation vial. Before addition of 5 ml scintillation cocktail (Goldstar, Meridian, UK), 1 ml of 10% HCl was added, the vial gently swirled and incubated for 10-30 min to fume out non-incorporated $NaH^{14}CO_3$. Dark CO_2 fixation rates were <3% of paired CO_2 fixation rates in the light in all experiments (Fig. S3).

To determine group-specific CO_2 fixation rates higher $NaH^{14}CO_3$ concentrations had to be used due to the small size of the organisms. To 60ml seawater sample in a Pyrex glass bottle 246 kBq ml^{-1} $NaH^{14}CO_3$ were added, the sample incubated for 10 h and then fixed with 1% PFA (final concentration). Three 1.6 ml subsamples were taken directly to determine total CO_2 fixation and to sort Pro_{st}. In order to sort adequate cell numbers of cyanobacteria (Pro_{unst} and Syn) 20 ml of the sample were concentrated on a 0.6 µm polycarbonate filter (Nuclepore, Whatman, UK) mounted in a filtration unit (Swinnex, Millipore, UK) using a syringe pump (KD Scientific, USA) at a flow rate of 2.5 ml min^{-1}. This pore size was selected as it was shown in an earlier publication (Zubkov *et al.*, 1998) that the Pro population with visible red autofluorescence had a cell diameter of 0.63±0.03 µm. Moreover, similar 90° side light scatter values of Pro_{unst} before and after concentration suggest that there is no selective enrichment of larger cells due to the concentration procedure (Student's t-test, p=0.871, Fig. S5). The remaining sample was

concentrated on a 0.8 µm polycarbonate filter (Nuclepore, Whatman, UK) the same way to enrich eukaryotic phytoplankton. Apart from the 0.6 µm concentrated fraction all samples were stained with SYBR Green I (Marie *et al.*, 1997), stored at 4° C and sorted flow cytometrically within 10 h.

In order to determine the influence of nutrients on CO_2 fixation, at three stations (NG and SG) a parallel incubation was carried out where 2.6 ml of nutrient-enriched seawater from 300 m depth were added to 60 ml of seawater sample from 20 m to simulate a mixing event (Fig. S2). The experiment was run in parallel to our standard 20 m incubations for 10 h under the same light regime. The sample was processed as described above for the standard incubations. The nutrient addition corresponds to a ~20 fold increase in nitrite/nitrate concentration (0.03 and 16.83 µmol L^{-1} average ambient concentration at 20 m and 300 m, respectively, Harris and Woodward (2014)) along the whole transect. At four stations (Fig. S2, EQ and SG), samples from the bottom of the thermocline were incubated in parallel at the same light intensity as surface samples to compare CO_2 fixation rates of phytoplankton groups living at the two depth.

Flow cytometric sorting. Different phytoplankton populations were sorted according to light-scattering properties (90° or side light scatter, SSC), relative concentration of SYBR Green I stain per particle (green fluorescence; FL1, 530 ± 30 nm), phycoerythrin-content (orange fluorescence; FL2, 580 ± 30 nm) and chlorophyll content (red fluorescence; FL3, >650 nm) using a FACSort instrument (Becton Dickinson, UK). Due to their low pigmentation in surface waters we used two approaches to sort Pro cells. A distinct bacterial

population, verified to be mainly Pro by CARD-FISH (>86%, Table 1), was sorted from unconcentrated, SYBR Green I stained samples (Pro_{st}) according to side scatter and green fluorescence properties. In addition, Pro was sorted according to red autofluorescence from 0.6 µm concentrated unstained samples (Pro_{unst}), as earlier studies indicated a cell diameter of >0.6 µm (Chisholm *et al.*, 1988; Vaulot *et al.*, 1990; Zubkov *et al.*, 2000). From the same sample Syn cells were sorted according to their phycoerythrin content. Plast-S and Plast-L populations were sorted from 0.8 µm concentrated, stained samples using side scatter, SYBR Green I stain and red autofluorescence as defining parameters. For each population 4-6 replicates of different cell numbers were sorted. Bacterial and eukaryotic cells were collected on 0.2 µm and 0.8 µm polycarbonate filters, respectively, and treated following the same procedure as for total CO_2 measurements (see above) before counting. Radio-assaying of samples was carried out using an ultra-low-level liquid scintillation counter (1220 Quantulus, Wallac).

Cell biomass estimation of *Prochlorococcus* and other phytoplankton. Cell diameters of Pro_{st} and Pro_{unst} surface populations were determined on AMT-4 at 11 stations spanning NG, EQ and SG regions using a size fractionation method (Zubkov *et al.*, 2000). Briefly, cell concentrations were measured in unfiltered sample and filtrates after filtering samples through polycarbonate filters (Nuclepore, Whatman, UK) of different pore size. The filter pore size versus the percentage of cells in the corresponding filtrates relative to cell concentration in the unfiltered sample were plotted to estimate the pore size that would retain 50% of cells. That pore size was interpreted as

a mean cell diameter. Average cell diameters of Pro_{st} (0.52±0.03 µm, n=30) and Pro_{unst} (0.6±0.05 µm, n=35) were significantly different (t-test, $p<0.001$). Mean cell biovolumes were calculated assuming a spherical cell shape. For Pro and Syn cells conversion factors of 184 and 211 fg C μm^{-3} (Heldal *et al.*, 2003) and cell diameters of 0.52±0.03 and 0.95±0.31 µm (Zubkov *et al.*, 2000) were applied. Details of all conversion factors used in this study can be found in Table 2. A conversion factor of 200 fg C μm^{-3} (Waterbury *et al.*, 1986) was used to calculate biomass-specific CO_2 fixation rates assuming spherical cell shape and average cell diameters of 2.0±0.1 and 3.1±0.3 µm for Plast-S and Plast-L cells, respectively (Hartmann *et al.*, 2012). These cell diameters were established for all studied regions on two consecutive AMT cruises including AMT-20 where the here presented CO_2 fixation rates were measured.

Data analyses. Cell specific CO_2 fixation rates were determined from average per cell values of each of the sorted replicates and converted to fg C $cell^{-1}$ h^{-1} according to Parsons et al. (1984). Statistical analyses were carried out using SigmaPlot. In case of normal distribution and equal variance t-tests were carried out for comparison. If the data was non-normally distributed or the equal variance test failed, Mann-Whitney Rank sum tests were used.

Results

Determination of *Prochlorococcus* cell abundance and CO_2 fixation rates. Due to the low red autofluorescence of Pro cells in surface waters and the resulting unreliability of detection we focused on a distinct population

within the bacterioplankton based on flow cytometrically determined cellular nucleic acid content and 90° light scatter, called Pro_{st} (Fig. S1) (Zubkov *et al.*, 2000). The taxonomic identity of cells within this population was verified by flow sorting followed by Card-FISH using a Pro-targeted probe (Pro405, West *et al*. (2001)) at 14 stations along the Atlantic Meridional Transect (AMT) (Table 1). The majority of flow sorted cells (86 to 94%) hybridised with the Pro-specific probe corroborating the results of previous molecular studies of the same distinct population (Gomez-Pereira *et al.*, 2013; Mary *et al.*, 2008; Zubkov *et al.*, 2007) (Table 1). As a control, Pro cells were in parallel enumerated on the basis of red autofluorescence in unstained samples (Pro_{unst}, Fig. S1) and compared to Pro_{st} cell numbers revealing a significant underestimation of Pro abundance in Pro_{unst} measurements (Fig. 1a).

To independently validate the molecular identification of Pro_{st}, fixation of $^{14}CO_2$ was determined in both Pro_{st} and Pro_{unst} sorted cells (Fig. S1). Both populations were photosynthetically active, the $^{14}CO_2$ fixation rate per cell of Pro_{unst} was 50% higher than that of Pro_{st} (Wilcoxon signed rank test, $p<0.001$) (Fig. 1b). A strong linear correlation between the $^{14}CO_2$ fixation of the Pro_{st} and Pro_{unst} cells ($R^2=0.93$, $p<0.001$) indicated that the Pro_{unst} cells comprised a subpopulation of the Pro_{st} population (Fig. 1).

There could be two reasons why the Pro_{unst} cells systematically fixed 50% more $^{14}CO_2$ than the Pro_{st} cells:

1) The presence of non-photosynthetic bacteria among the sorted Pro_{st} cells could lower the average cellular ^{14}C content (because the total measured $^{14}CO_2$ fixation was divided by the total number of sorted cells). The cells amongst the sorted Pro_{st} populations not hybridising with the Pro-targeted

probe could either be Pro cells with ribosomal contents below detection level of FISH (e.g. Pro cells were compromised or dead) or they could be by-sorted non-Pro cells displaying similar DNA fluorescence and side light scatter properties. A small proportion of sorted cells (5-6%) could not be detected by FISH with the universal bacterial probe (Table 1), which lends support to the former explanation. However, even in the extreme (assumed) case if all probe-negative cells were by-sorted non-Pro cells one could explain a discrepancy of only 6-14% between Pro_{st} and Pro_{unst} rather than the measured 50% difference.

2) On average larger Pro_{unst} cells which contain proportionally higher chlorophyll amounts resulting in detectable red autofluorescence, could fix more $^{14}CO_2$ than smaller Pro_{st} cells. Indeed, cell diameter estimates carried out on an earlier AMT cruise (AMT-4) using size fractionation revealed that Pro_{st} cells were systematically smaller than Pro_{unst} cells across the Atlantic Ocean (Table 2) (Zubkov *et al.*, 2000). These observations suggest that only larger or dividing Pro cells with higher red autofluorescence were visible above the threshold of the red fluorescence photomultiplier detector. Indeed, the abundance of Pro_{unst} cells was significantly lower than that of Pro_{st} cells (on average 58±18%, Fig. 1a). Consequently, the Pro_{unst} cells are not representative of the entire Pro population in the surface waters. Sorting of Pro_{unst} would therefore lead to overestimation of the actual cellular CO_2 fixation rates by Pro, and we used hereafter Pro_{st} cells for more realistic measurement of CO_2 fixation by Pro cells.

Pro cell-specific CO_2 fixation rates in the NG and SG were similar, but their rates were more than doubled in the EQ (t-test, p=0.002, Fig. S6 and Table

S1). The CO_2 fixation rates of Plast-L cells followed the same pattern (t-test, $p<0.001$), showing increased rates in the EQ (Fig. S6 and Table S1). In contrast, Plast-S as well as Syn cells showed similar rates in the NG, SG, and EQ, but significantly lower CO_2 fixation rates in the Southern temperate region (ST) (t-test, $p=0.042$ and $p=0.032$, respectively, Fig. S6 and Table S1).

Comparison of cellular, red autofluorescence normalised and biomass-specific CO_2 fixation of *Prochlorococcus*, *Synechococcus* and small eukaryotic phytoplankton. Although cellular CO_2 fixation rates positively correlated with cell sizes ($R^2=0.83$, Fig. S6), no such relationship was found between red autofluorescence normalised or biomass-specific CO_2 uptake (Table 2 for details on biomass in this study and Table S2 for a summary table of published biomass estimates). Because it was technically impossible to determine red autofluorescence of Pro_{st} with required precision, red autofluorescence of Pro_{unst} (upper estimates, Fig. S1b,d) were used for normalisation. Consequently, the derived normalised values for Pro should be treated as conservative lower estimations. Red autofluorescence normalised CO_2 fixation shows that Pro despite an order of magnitude lower red autofluorescence than Syn (Table 2) can fix up to 4 times more CO_2 than other small phytoplankton (t-test, $p\leq0.005$, Fig. 2a,b) while CO_2 fixation rates of Syn and plastidic eukaryotes are comparable.

Biomass-specific CO_2 fixation rates of plastidic eukaryotes were significantly lower than those of cyanobacteria (Mann-Whitney, $p<0.001$) while Syn showed on average 60% higher biomass-specific CO_2 fixation than Pro (t-test, $p=0.04$) (Fig. 2c,d). Due to the combined effect of comparatively high CO_2

uptake rates and high abundance Pro led microbial CO_2 fixation across the low latitude Atlantic Ocean (Mann-Whitney, $p \leq 0.038$, Fig. 2 e,f).

To assess if cellular CO_2 fixation and red autofluorescence of surface Pro could be influenced by a lack of nutrients, additional experiments with added nutrients were performed. Nutrient addition to surface samples in the form of deep water (300m depth) had no significant effect on CO_2 fixation rates of either Pro or Syn cells (t-test, $p>0.5$, Fig. 3) and did not influence red autofluorescence of Pro (t-test, $p=0.12$). Moreover, comparisons of CO_2 fixation rates of Pro populations from surface waters and deeper water (bottom of the thermocline) revealed no significant differences in CO_2 fixation rates when exposed to the same light conditions, despite stronger red fluorescence of deeper Pro cells (Fig. 4).

Discussion

Direct determination of group-specific CO_2 fixation rates using ^{14}C-tracer is technically challenging and has been attempted only in three other studies so far (Chisholm *et al.*, 1988; Jardillier *et al.*, 2010; Li, 1994) which were spatially restricted to small areas of the Atlantic Ocean. This new dataset provides for the first time insight into CO_2 fixation rates of four distinct phytoplankton groups across the Atlantic Ocean. Cellular CO_2 fixation rates measured in the equatorial region are comparable to those measured in the North East Atlantic (Jardillier *et al.*, 2010; Li, 1994) (Table S3). Our slightly lower values can be most likely attributed to the differences in light regime between the studies (artificial vs. ambient light). Inferred from estimates of diel synchronised cell

division in the photic layer (Vaulot *et al.*, 1995) and measurements of CO_2 fixation by Pro, inhabiting the deeper waters (Chisholm *et al.*, 1988) Pro could contribute more than a half to the total CO_2 fixation in the low latitude Ocean. These estimates concur with our results from surface waters (Fig. 2 e, f).

A combination of high sunlight irradiance (up to 3000 µmol photons m^{-2} s^{-1} (Jitts *et al.*, 1976)), slow rates of vertical mixing, low inorganic nutrient availability and potential preferential grazing pressure by mixotrophic protists (Hartmann *et al.*, 2013) creates a harsh habitat for *Prochlorococcus* cyanobacteria in surface waters of the low latitude Atlantic Ocean. How *Prochlorococcus* cells remain numerous, effective CO_2 fixers in these waters (Fig. 2) with virtually undetectable red cellular autofluorescence (indicative of extremely low photosynthetic pigmentation)?

Biosynthesis of photosynthetic pigments like chlorophyll requires certain inorganic nutrients, e.g. nitrogen and iron, which are depleted in the subtropical gyres, and constrained nutrient bioavailability can lead to reduced cellular pigmentation (Riemann *et al.*, 1989; Staehr *et al.*, 2002). However, both plastidic protists and *Synechococcus* cyanobacteria can easily be detected in the same waters by their photosynthetic pigmentation, suggesting that the required nutrients are still bio-available to those cells. Furthermore, CO_2 fixation rates of Pro remained unchanged in our deep water addition experiments to simulate mixing events, suggesting that Pro are not nutrient-limited, at least with regard to CO_2 fixation (Fig. 3). These findings are in accordance with a study in the equatorial Pacific Ocean (Vaulot *et al.*, 1995), where close to maximal (i.e. nutrient unlimited) growth rates of Pro were estimated. In addition, Pro cells populating the deeper parts of the mixed

layer, where nutrients are still scarce but irradiance is less intense, exhibit stronger red autofluorescence (Zubkov *et al.*, 1998).

Perhaps Pro cells produce only low amounts of photosynthetic pigmentation in order to achieve efficient CO_2 fixation with minimal effort using light energy for photosynthesis as well as for photoheterotrophy, i.e. redirecting a part of the collected light energy for actively importing organic molecules (Casey *et al.*, 2009; Zubkov, 2009). This would allow them to compete with Syn cells, which spend considerable energy on the production of photoprotective pigments (Aráoz and Häder, 1999; Raven, 1991). Molecular studies of cultured Syn and Pro showed that the Pro response to photo-damage is modelled to minimise energy demand, e.g. during high irradiance periods of the day the main metabolic processes are down regulated (Mella-Flores *et al.*, 2012). Lower biomass-specific CO_2 fixation rates of Pro in comparison to Syn (Fig. 2c,d) are, perhaps, a price worth paying to avoid constant repair of photo-damaged reaction centres in highly irradiated surface waters. That might be a reason (additional to inorganic nutrient limitation (Tarran *et al.*, 1999; Vaulot *et al.*, 1996)) for low concentrations of Syn cells in oligotrophic waters despite their higher biomass-specific CO_2 fixation rates. On the other hand, the difference in biomass-specific CO_2 fixation rates between Pro and Syn is comparatively small given the higher pigment content, up to 10 times at 1000 µmol m^{-2} s^{-1} light in cultures (Moore *et al.*, 1995) and according to red autofluorescence (Fig 2. a, b), of the latter.

High red autofluorescence-normalised CO_2 fixation by Pro is likely related to high geometrical absorption cross section owing to their small cell size (Bailey *et al.*, 2005; Morel *et al.*, 1993) and package effect, which states that the light-

harvesting effect of increased pigmentation is reduced due to a parallel decrease in the absorption cross section (Berner *et al.*, 1989; Dubinsky *et al.*, 1986). Light harvesting efficiency of Pro is further increased by the unique pigment – chlorophyll a_2 (Chisholm *et al.*, 1988; Goericke and Repeta, 1992) with absorption maximum that coincides with the wavelength of higher energy blue light. The cumulative outcome of these numerous adaptations has enabled Pro cells with minimal photosynthetic pigmentation to become highly efficient CO_2 fixers (Fig. 2).

Conclusion

The results presented here demonstrate that *Prochlorococcus* are highly efficient CO_2 fixers in surface waters of the Atlantic Ocean and their red autofluorescence normalised CO_2 fixation rates are higher than those of *Synechococcus* and small plastidic protists. These findings indicate that *Prochlorococcus* should be specially taken into account when photosynthetic pigmentation data is used for deducing biological CO_2 fixation in the oligotrophic open ocean.

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The authors declare that there are no conflicts of interest.

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Titels and legends to tables

Table 1. Identification of a flow cytometrically sorted, distinct population with high-nucleic acid content as *Prochlorococcus* cyanobacteria (Pro) in different regions of the Atlantic Ocean using Pro-targeted CARD-FISH (Pro405). Numbers are given as percentage of DAPI stained cells. For comparison percentage of positive signals for the eubacterial probe (EUB338I-III) are presented. Abbreviations: NG=Northern Gyre, EQ=equatorial region, SG=Southern Gyre, n=number of sampled stations, ± indicates standard error (SEM).

Table 2. Summary table of conversion factors used to calculate red autofluorescence normalised and biomass-specific CO_2 fixation for the different phytoplankton populations. Red autofluorescence values are based on mean red autofluorescence emitted by the individual populations and normalised to red autofluorescence of 1.0µm multifluorescent reference beads as measured by flow cytometry. Cell biovolume was calculated on the basis of a spherical shape of the cell. Prost=Prochlorococcus stained, Prounst=Prochlorococcus unstained, Syn=Synechococcus, Euk=eukaryotic phytoplankton, Plast-S and Plast-L=small (~2µm) and large (~3.1µm) plastidic eukaryotes, respectively, n.a.=not available

Fluorescence in situ hybridisation of flow cytometrically sorted high-nucleic acid bacterial cells

Region	Pro405 identification [%]	Eub338 identification [%]
NG (n=7)	86±3	94±1
EQ (n=3)	94±1	95±2
SG (n=4)	92±2	94±2

Summary table of conversion factors

Group	Red autofluo-rescence [relative units]	Cell diameter [µm]	Biovolume [μm^3]	Carbon conversion factor [fg C $cell^{-1}$]	Biomass [fg C μm^{-3}]
Pro_{st}	n.a.	0.52±0.03[a]	0.07±0.004	184[c]	16.2±0.9
Pro_{unst}	0.004±0.002	0.63±0.05 [a]	0.13±0.01	184 [c]	28.8±2.3
Syn	0.07±0.04	0.95±0.31 [a]	0.45±0.15	211 [c]	98.7±32.2
Euk	0.43±0.08				
Plast-S	n.a.	2.0±0.1[b]	4.2±0.2	200[d]	837±42
Plast-L	n.a.	3.1±0.3 [b]	15.6±1.5	200 [d]	3118±302

[a] (Zubkov *et al.*, 2000)

[b] (Hartmann *et al.*, 2012)

[c] (Heldal *et al.*, 2003)

[d] (Waterbury *et al.*, 1986)

Titels and legends to figures

Fig. 1. Assessment of *Prochlorococcus* abundance (a) and CO_2 fixation (b) using either pigmentation (Pro_{unst}) or DNA-content (Pro_{st}) to flow cytometrically separate them from other groups. Different colours indicate sampled regions in the Atlantic Ocean (NG=Northern Gyre, EQ=equatorial region, SG=Southern Gyre, ST=Southern temperate waters. Actual numbers of Pro are underestimated using pigmentation alone (a). A significant positive correlation between the two protocols suggests that Pro_{unst} is part of Pro_{st}. (b)

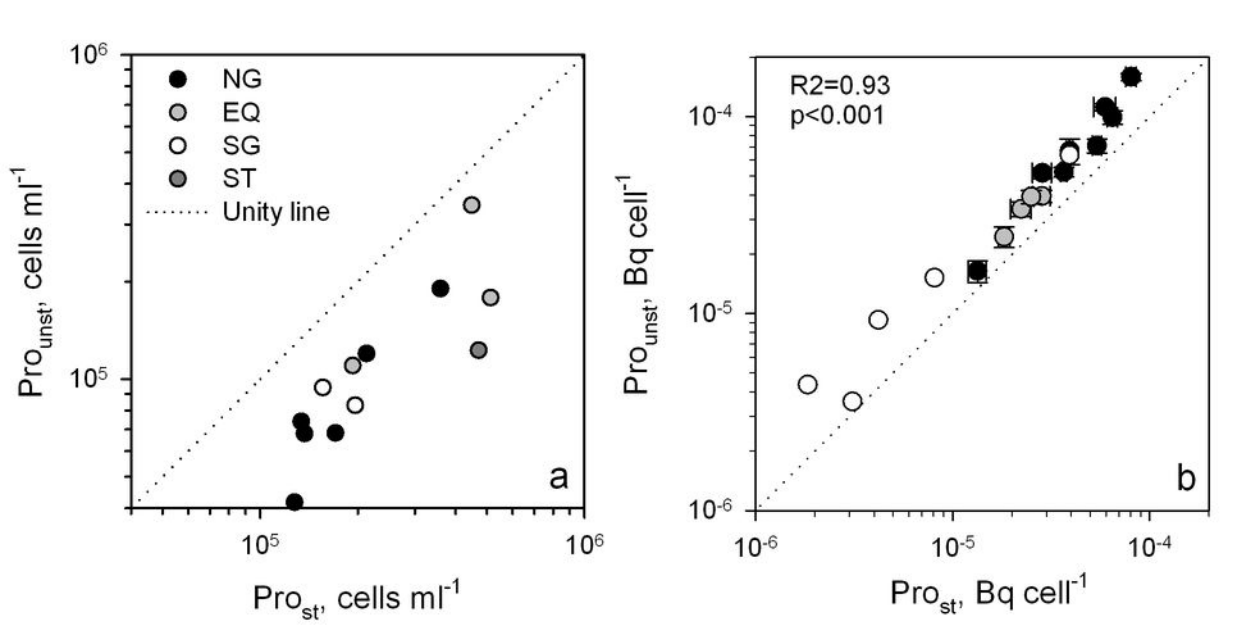
Fig. 2: Detailed (a, c, e) and average (b, d, f) red autofluorescence normalised, biomass-specific and population specific CO_2 fixation of *Prochlorococcus* (Pro) in comparison to *Synechococcus* (Syn) cyanobacteria, smaller and larger, plastidic eukaryotes (~2µm, Plast-S and ~3.1µm, Plast-L) in the Atlantic Ocean (ST=Southern temperate waters, SG=Southern Gyre, EQ=equatorial region and NG=Northern Gyre). Units on the y-axes are the same for a/b, c/d and e/f. Student's t-test confirmed significant differences in biomass-specific CO_2 fixation between all phytoplankton groups (p=0.001-0.047, see Result section for details).

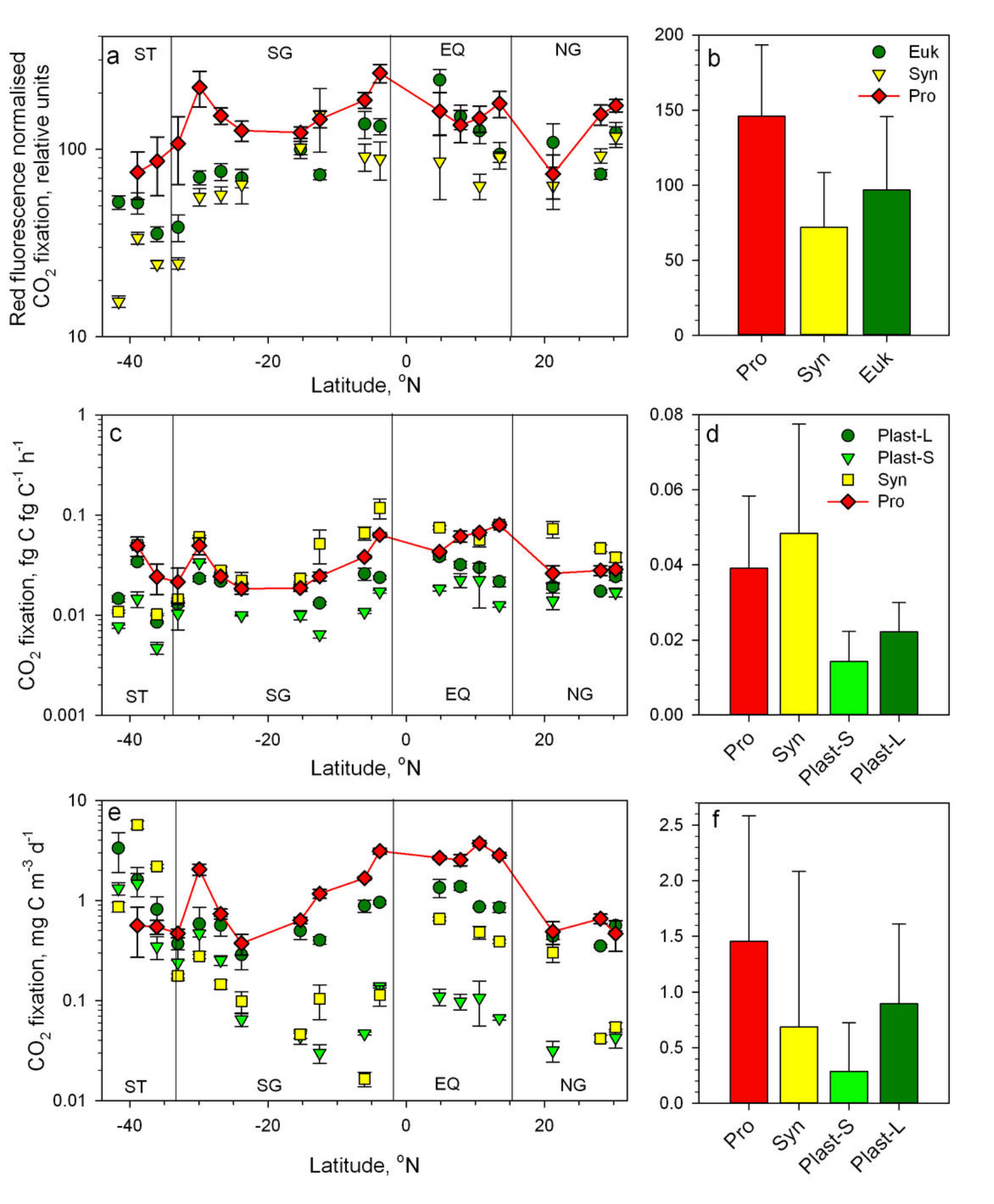
Fig. 3. Average per cell CO_2 fixation of surface Pro (empty) and Syn (pattern) with (white) and without addition of nutrients (grey, i.e. addition of sea water from 300m depth). NG=Northern Gyre, SG=Southern Gyre.

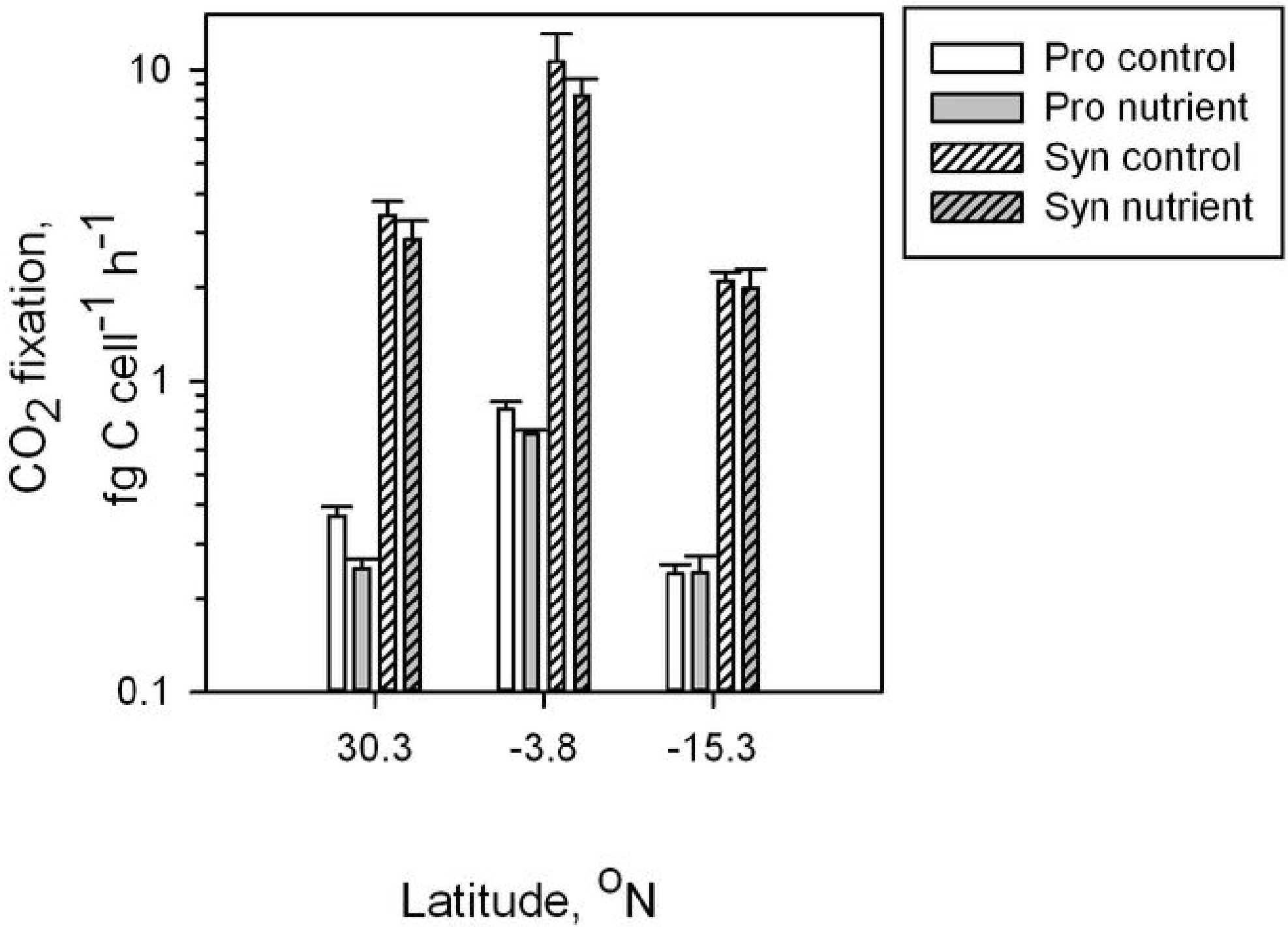
Fig. 4. Average biomass-normalised CO_2 fixation of Pro cells in surface waters (i.e. 20 m depth, light blue) and deeper water layers (dark blue) in the

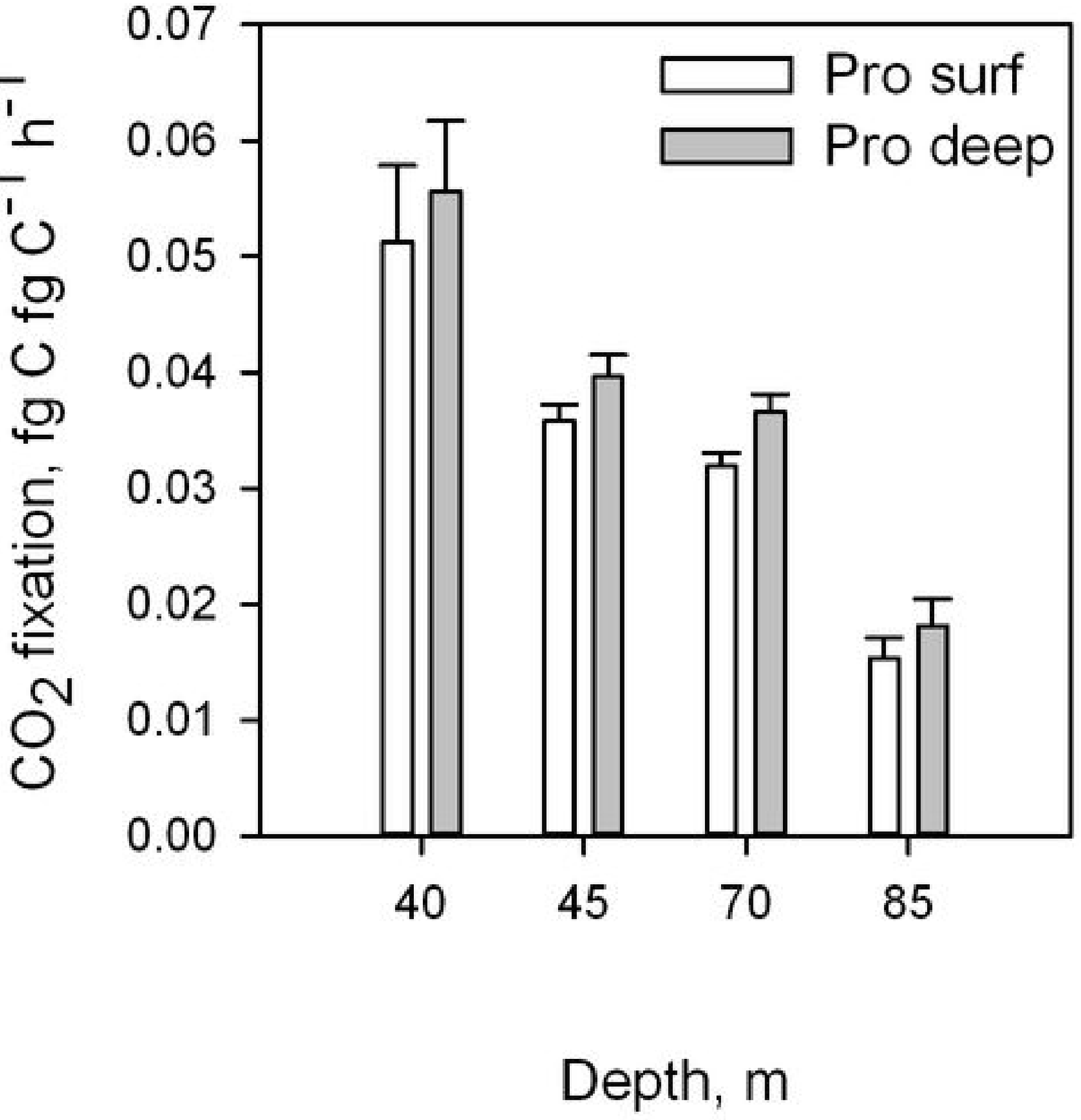
Equatorial region (EQ) and the Southern gyre (SG). Numbers on x-axis indicate depth of the deep water sample.

735


NG
EQ
SG
ST
Unity line
a
Pro_{unst}, cells ml^{-1}
Pro_{st}, cells ml^{-1}
10^5
10^6
R2=0.93
p<0.001
b
Pro_{unst}, Bq cell^{-1}
Pro_{st}, Bq cell^{-1}
10^{-4}
10^{-5}
10^{-6}


a
ST
SG
EQ
NG
Red fluorescence normalised
CO2 fixation, relative units
100
10
-40
-20
0
20
Latitude, °N
b
Euk
Syn
Pro
200
150
100
50
0
Pro
Syn
Euk
c
CO2 fixation, fg C fg C-1 h-1
1
0.1
0.01
0.001
ST
SG
EQ
NG
-40
-20
0
20
Latitude, °N
d
Plast-L
Plast-S
Syn
Pro
0.08
0.06
0.04
0.02
0.00
Pro
Syn
Plast-S
Plast-L
e
CO2 fixation, mg C m-3 d-1
10
1
0.1
0.01
ST
SG
EQ
NG
-40
-20
0
20
Latitude, °N
f
2.5
2.0
1.5
1.0
0.5
0.0
Pro
Syn
Plast-S
Plast-L


Pro control
Pro nutrient
Syn control
Syn nutrient
10
1
0.1
CO_2 fixation, fg C cell^{-1} h^{-1}
30.3
-3.8
-15.3
Latitude, ^{O}N


0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
CO_2 fixation, fg C fg C^{-1} h^{-1}
Pro surf
Pro deep
40
45
70
85
Depth, m