Cell-specific CO_2 fixation rates of two distinct groups of plastidic protists in the Atlantic Ocean remain unchanged after nutrient addition

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Abstract

To assess the role of open ocean ecosystems in global CO_2 fixation we investigated how picophytoplankton, that dominate primary production, responded to episodic increases in nutrient availability. Previous experiments have shown nitrogen alone, or in combination with phosphorus or iron, to be the proximate limiting nutrient(s) for total phytoplankton grown over several days. Much less is known about how nutrient up-shift affects picophytoplankton CO_2 fixation over the duration of the light period. To address this issue we performed a series of small volume (8-60 ml) - short term (10-11 hours) nutrient addition experiments in different regions of the Atlantic Ocean using NH_4Cl, $FeCl_3$, K medium, dust and nutrient-rich water from 300 m depth. We found no significant nutrient stimulation of group-specific CO_2 fixation rates of two taxonomically- and size-distinct groups of plastidic protists. The above was true regardless of the region sampled or nutrient added suggesting this is a generic phenomenon. Our findings show that at least in the short term (i.e. daylight period) nutrient availability does not limit CO_2 fixation by the smallest plastidic protists, whilst their taxonomic composition does not determine their response to nutrient addition.

Introduction

Although responsible for more than 70% of global marine primary production (Chen et al., 2003) the oligotrophic open ocean is characterised by consistently low chlorophyll *a* concentrations, i.e., standing stocks of photosynthetic biomass, and primary production rates. One of the key factors regulating phytoplankton growth and CO_2 fixation in these regions is thought to be the availability of macro- (e.g., nitrogen and phosphorus) and/or micronutrients (e.g., iron) (Arrigo et al., 2005). Hence, it is no surprise that a considerable amount of effort has been put into understanding how open ocean photosynthetic communities respond to nutrient addition (Graziano et al., 1996; McAndrew et al., 2007; Davey et al., 2008; Moore et al., 2008; Mahaffey et al., 2012).

With an average surface phosphate concentration of 9 nM (Mather et al., 2008), the North Atlantic subtropical gyre has long been considered to be phosphorus limited. Indeed, as little as 1.8 nM of bioavailable phosphate has been measured in this region (Zubkov et al., 2007). The drawdown of phosphate is believed to be facilitated by the external supply of nitrogen through nitrogen fixation (Reynolds et al., 2007), which is in turn controlled by dissolved iron availability (Moore et al., 2009). Conversely, due to low iron availability nitrogen fixation rates in the South Atlantic subtropical gyre are much lower (Moore et al., 2009) and hence the average surface phosphate concentration is more than 20 times higher (210 nM, Mather et al., 2008). Nitrate and ammonium concentrations, on the other hand, are typically around 10 and 50 nM, respectively, in both subtropical gyres (Rees et al., 2006).

In the oligotrophic open ocean phytoplankton biomass is dominated by the picophytoplankton, encompassing cyanobacteria of the genera *Prochlorococcus* and *Synechococcus* as well as taxonomically diverse photosynthetic picoeukaryotes (see Zubkov et al., 2000). In the case of cyanobacteria, discrete genetic lineages with specific light and/or

nutrient requirements have been found to dominate different regions of the Atlantic Ocean (Johnson et al., 2006; Zwirglmaier et al 2007). The photosynthetic picoeukaryotes, hereafter referred to as plastidic protists, also show marked differences in their taxonomic composition over large spatial scales (Kirkham et al., 2013). Furthermore, two distinct populations defined according to their average cell size as small (<2 μm, Plast-S) and large (2-3 μm, Plast-L) plastidic protists, have been shown to be taxonomically distinct, with Plast-S being dominated by Pelago-, Chryso- or Prymensiophyceae, depending on the oceanic region, and Plast-L always being dominated by the latter algal class (> 40% of cells; Jardillier et al., 2010; Grob et al., 2011).

To determine whether open-ocean primary production is limited by the availability of phosphorus (P), nitrogen (nitrate + ammonium, N) and/or iron (Fe), several nutrient addition experiments have been carried out in the oligotrophic North Atlantic Ocean. When incubating for 24-48 hours, previous studies have shown that whereas chlorophyll *a* synthesis and primary production increased after adding N alone, cell abundance did so only when combining N with P or Fe (Graziano et al., 1996; Mills et al, 2008; Davey et al., 2008; Moore et al., 2008). Similar increases in photosynthetic biomass and production have also been observed after adding nutrient-rich deep seawater instead of discrete inorganic compounds and incubating for 48 hours (McAndrew et al., 2007) or for up to 7 days (Mahaffey et al., 2012). Little is still known, however, about the response of taxonomically distinct groups to nutrient enrichment over the duration of the daylight period, hereafter referred to as daytime, when they are actively fixing CO_2 and nutrients into organic matter using light as their energy source.

In order to assess the daytime response of picophytoplankton to episodic increases in nutrient availability, a series of small volume (8-60ml) - short term (10-11h) nutrient addition

80 experiments were carried out in the tropical and subtropical northeast Atlantic Ocean during
81 the SOLAS (Surface Ocean Lower Atmosphere Study) cruise in January-February 2008 and
82 in the North and South Atlantic Gyre during the Atlantic Meridional Transect cruise,
83 AMT20, in October-November 2010 onboard the *RRS Discovery* and *RRS James Cook*,
84 respectively. The exact location of all the stations sampled, as well as the incubation volumes
85 and nutrients added, are summarised in Table 1. In each case we carried out $NaH^{14}CO_3$
86 radiotracer incubations as previously described (see Jardillier et al., 2010 and Grob et al.,
87 2011) running control and amended experiments in parallel. After incubation, we measured
88 total inorganic carbon uptake (both cruises), as well as cell-specific uptake rates of plastidic
89 protists (AMT20; see figure legends for details), as described elsewhere (Jardillier et al., 2010
90 and Grob et al., 2011).

91 Statistically significant differences in total CO_2 fixation and group-specific uptake rates
92 between treatments (control versus amended) were assessed by applying the non-parametric
93 Mann-Whitney test when the hypothesis of normal distribution was rejected (Shapiro-Wilk
94 test) and t-test when accepted, the latter after checking for equal variances. All tests were
95 performed using the software R (www.r-project.org).

96 **Results and discussion**

97 In the present work we found a consistent lack of response by photosynthetic picoplankton
98 communities from different regions of the oligotrophic Atlantic Ocean to the addition of
99 individual ($FeCl_3$ or NH_4Cl) or mixed (K medium, dust or deep seawater) nutrients (Figs. 1
100 and 2). Neither total uptake nor group-specific CO_2 fixation rates for Plast-S and Plast-L
101 showed a significant difference between control and amended experiments ($p > 0.05$ in all
102 cases). This is, to the best of our knowledge, the first time that group-specific CO_2 fixation
103 rate responses to nutrient addition have ever been reported for natural eukaryotic populations.

Total CO_2 fixation, carried out by *Prochlorococcus*, *Synechococcus* and plastidic protists combined (Jardillier et al., 2010), was not stimulated following the addition of different types of nutrients regardless of the oceanic region sampled. Indeed, samples taken every two hours in the tropical and subtropical northeast Atlantic (SOLAS) showed a parallel, but not significantly different increase in CO_2 uptake in both control and amended experiments throughout the incubation period in all ten samples (Fig. 1; p=0.6). The same results were observed for the subtropical North and South Atlantic gyres samples (AMT20; Fig. 2a; p=0.34). These results are particularly interesting considering that SOLAS samples were incubated under ambient light conditions, i.e., increasing irradiance from dawn to noon and then decreasing until dusk, whereas AMT20 samples were incubated under constant artificial light at 500 μmol photons m^{-2} s^{-1}, i.e., under non-limiting light conditions.

Group-specific CO_2 fixation rates also remained unchanged after the addition of nutrient-rich waters from 300 m, with no significant difference between control and amended samples for Plast-S (Fig. 2b; p=0.86) or Plast-L (Fig. 2c; p=0.57). It is of significant interest that fluorescence in situ hybridisation (FISH) analyses performed on Plast-S and Plast-L sorted from our SOLAS samples (Jardillier et al., 2010) and during a previous AMT cruise (AMT19; Grob et al., 2011) showed significant differences in the taxonomic composition of these two groups. Given that the distinction between Plast-S and Plast-L is not only consistent across large oceanic areas but also along the same transect sampled during AMT20, we have no reason to believe that this was not the case in our samples. Hence, the taxonomic composition of the open-ocean photosynthetic community does not seem to account for the lack of nutrient stimulation of primary production observed here at the daytime scale.

Choosing the right incubation time and volume is critical to better estimate marine primary production. Here, we performed short term incubations (i.e. 10-11 hrs), corresponding to the daylight period at the time of the experiments, to assess CO_2 fixation rates of the key players while they are photosynthetically active and before cell division. Indeed, in the open ocean this latter process is known to occur at dusk in the case of *Synechococcus* and picoeukaryotes (Jacquet et al., 2002) and at night time for *Prochlorococcus* (Vaulot et al., 1995; Jacquet et al., 2002). Using small volume (8-60ml) incubations, cell-specific CO_2 fixation rates of all major pigmented groups, i.e., *Prochlorococcus*, *Synechococcus*, Plast-S and Plast-L, have previously been determined, confirming that they are actively fixing CO_2 throughout the experiment (Jardillier et al., 2010; Hartmann et al., 2014). In the case of protists, this method was sensitive enough to detect a marked 4-(SOLAS) to 5-(AMT20) fold difference in fixation rates between the Plast-S and Plast-L groups, without any cell loss being observed (Jardillier et al. 2010; Grob et al., 2011; this work), i.e., Plast-S and Plast-L cell numbers at the beginning and end of our incubations were not significantly different (Fig. 3; $p=0.17$). All of the above suggests that if there was any negative effect on these populations due to bottle enclosure, e.g., a drop in biomass due to stress, as previously reported for incubation experiments using 70- 1000 ml volumes (e.g., Fernández et al., 2003; Calvo-Díaz et al., 2011), it was negligible. We are therefore confident that neither grazing nor viral lysis, the two main causes of mortality for open-ocean picophytoplankton (Worden and Not, 2009), nor bottle enclosure, had a major impact on our CO_2 fixation measurements.

Short-term incubations have previously been shown to be successful in detecting significant changes in *Prochlorococcus* and low nucleic acid bacteria (LNA) cell-specific ^{35}S-methionine uptake rates in response to dust additions (6 hrs), with equivalent results being observed at the bacterioplankton community level (uptake ml^{-1}) after 6-8 and 24 hrs (Hill et

al., 2010). Similarly, significant differences in ATP and methionine uptake rates were observed for the two groups mentioned above after incubating for 10hrs under light versus dark conditions (Gómez-Pereira et al., 2013). There is also evidence of microbial communities (including photosynthetic organisms) being capable of acquiring nitrate and ammonium at timescales shorter than 4 hrs across the Atlantic Ocean (Rees et al., 2006), whilst transcriptional responses to nutrient addition can be observed after 6 hrs in natural *Prochlorococcus* communities (Shi et al., 2012) or after 4 hrs in a red tide dinoflagellate (Morey et al., 2011). More importantly, experiments performed over the light period (dawn to dusk) off the Oregon coast have previously shown an increase in CO_2 uptake after the separate addition of N, P and Fe in 4 out of 5 sampled stations (Glooschenko and Curl, 1971). In light of the evidence presented above, we are confident that any changes in the CO_2 fixation rates of the photosynthetic community would have been detectable at the volume and time scales chosen here, had they occurred.

In the tropical and subtropical northeast Atlantic Plast-S and Plast-L are responsible for about 40% of the total primary production (Jardillier et al., 2010). Although as individual cells these two groups take up CO_2 at different rates, their biomass-specific CO_2 fixation rates are equivalent (Grob et al., 2011). Not only that, but the biomass-specific uptake rates measured during SOLAS for *Prochlorococcus*, *Synechococcus* and plastidic protists were very similar (Jardillier et al., 2010), suggesting that there might be an upper limit on the biomass that can accumulate in the system rather than on individual growth rates (Grob et al., 2011). The fact that the photosynthetic activity of Plast-S (Fig. 2a) and Plast-L (Fig. 2b) plastidic protists did not vary significantly after nutrient addition further supports this idea. We therefore believe that our results favour the notion of top down regulation of the contribution of these two ecologically important groups to open-ocean primary production.

Our findings also suggest that the lack of response to nutrient addition during the daytime could be a widespread feature, being consistent under different incubation irradiances (i.e., ambient in SOLAS versus constant artificial light in AMT20). This is not the first time that open-ocean photosynthetic communities have been found to be unresponsive to the addition of nutrients thought to be limiting. For instance, the addition of iron did not stimulate growth or primary production in the sub-tropical South Pacific suggesting that picophytoplankton occupying this region are well adapted to the extremely low concentrations found there (Bonnet et al., 2008). In other regions the lack of response to the addition of different nutrients has been attributed to possible growth limitation by some other factor (Caron et al., 2000) or most likely to differences in the initial environmental (mainly, specifically P-availability) and biological conditions (such as community composition) between samples (Martínez-García et al., 2010). Thus, although low phytoplankton standing stock and primary production rates in a low-nutrient environment are considered to be signs of limitation, these two parameters do not necessarily increase after nutrient addition, further supporting the validity of our results.

Understanding how the open-ocean photosynthetic community responds to episodic increases in nutrient availability is crucial to better understand the role of these ecosystems in the global carbon cycle, especially under the changing environmental conditions related to climate. Here, we have clearly added to our understanding of the functioning of this important ecosystem by showing that the organisms studied seem to be well adapted to the prevailing open-ocean environmental conditions, as already proposed for the oligotrophic south-east Pacific Ocean (Bonnet et al., 2008). Potentially, top-down regulation of open-ocean picophytoplankton CO_2 fixation capacity is the most likely controlling factor, i.e., as

198 opposed to nutrient limitation or differences in taxonomic composition, that strongly warrants
199 future investigation.

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313 Scanlan, D.J. (2007) Basin-scale distribution patterns of picocyanobacterial lineages in the
314 Atlantic Ocean. Environ Microbiol 9**:** 1278-1290.

315 Table1. Summary of nutrient addition experiments performed and variables measured across the Atlantic Ocean during SOLAS and AMT20
316 cruises. Seawater for all experiments was collected at the surface (5-20m). Nutrient concentrations in samples where 300m water was added
317 were estimated based on data provided by Harris and Woodward (2014) for AMT20 or measured by Dr. E. Achterberg during SOLAS (data
318 made available by the British Oceanographic Data Centre). K medium (Keller et al., 1987) contains $NaNO_3$, NH_4Cl, Na_2 ß-glycerophosphate,
319 H_2SeO_3, Tris-base (pH7.2), Na_2EDTA, a mixture of trace metals ($FeCl_3$, $MnCl_2$, $ZnSO_4$, $CoCl_2$, $CuSO_4$, Na_2MoO_4, $NiSO_4$) and vitamins (B_1, H,
320 B_{12}). Dust was collected onboard the ship using polypropylene filters and prepared for addition as described in Hill et al. (2010). Temp, Pi and N
321 represent temperature, inorganic phosphorus, and nitrogen (nitrate + nitrite), respectively.

322

323 Table 1.

Cruise name (number and ship)	Time of sampling	Region sampled	Incubation volume	Sampling location	*in situ* temp (°C)	Nutrient added (concentration)	Variables measured
SOLAS (D326, RRS Discovery)	Jan-Feb 2008	Tropical and Subtropical Northeast	8 ml	15.54°N-25.39°W	23.2	NH_4Cl (5nM)	Total CO_2 fixation rates
				15.54°N-25.39°W	23.2	Fe(III)Cl (1nM)	
				13.02°N-25.82°W	24.5	Fe(III)Cl (1nM)	
				12.65°N-27.11°W	24.5	K medium (2nM of Pi, 180nM of N)	
				12.65°N-27.11°W	24.5	300m water (5- and 400-fold increase in Pi and N)	
				12.59°N-30.00°W	24.3	K medium (180nM of N, 2nM of Pi)	
				12.59°N-30.00°W	24.3	300m water (3- and 500-fold increase in Pi and N)	
				12.54°N-32.69°W	25.0	Fe(III)Cl (1nM)	
				12.51°N-35.78°W	24.9	Fe(III)Cl (1nM)	
				12.59°N-33.25°W	24.6	Dust (10nM of Pi, 100nM of N)	
AMT20 (JC059, RRS James Cook)	Oct-Nov 2010	North and South Subtropical Gyre	60 ml	38.28°N -25.64°W	20.1	300m water, 2.6 ml added resulting in a 4- and 20-fold increase in Pi and N concentrations, on average	Total and group-specific CO_2 fixation rates, the latter for small and large plastidic protists
				34.22°N -29.76°W	23.5		
				32.43°N -31.80°W	24.2		
				30.30°N -34.18°W	25.3		
				25.98°N -38.78°W	26.5		
				23.76°N-41.11°W	26.7		
				18.69°N -37.52°W	27.1		
				3.85°S -25.01°W	27.2		
				15.33°S -21.84°W	23.9		

324

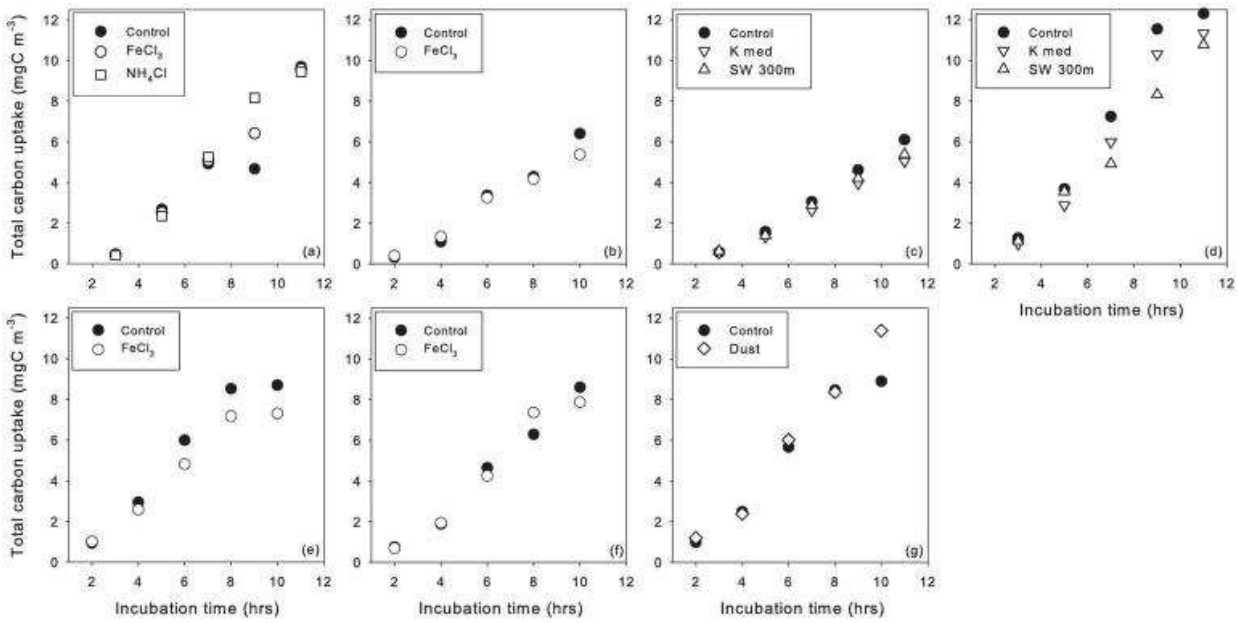
Figure legends

Fig. 1. Total CO_2 fixation (mgC m^{-3}) measured every 2hrs for 10-11hrs in the control (black circles) and nutrient addition (white circles, diamonds, squares and triangles) experiments during SOLAS (Surface Ocean Lower Atmosphere Study, January-February 2008), corresponding to the sum of the uptake by *Prochlorococcus*, *Synechococcus* and plastidic protists (Jardillier et al., 2010). Samples were inoculated with 74 KBq bicarbonate ($NaH^{14}CO_3$) and incubating in glass vials on deck within 10% of the in situ light and temperature conditions. Replicas sacrificed every 2hrs were fixed with paraformaldehyde 1% (w/v) final concentration (Sigma-Aldrich, UK) for 1h in the dark and the cells were collected onto 0.2 µm pore-size polycarbonate filters (Nuclepore, Whatman, UK), washed with deionised water and purged of residual inorganic carbon with 1% (v/v) HCl before adding scintillation cocktail (Goldstar, Meridian, UK) and measuring using a Tri-Carb 3100 scintillation counter (Perkin Elmer, Cambridge, UK). Nutrient additions consisted of ammonium (NH_4Cl), iron ($FeCl_3$), K medium (K med), dust and seawater collected at 300m depth (SW 300m) as described in Table 1. Each figure (a-g) corresponds to a different day and sampling station.

Fig. 2. Total (a; mgC m^{-3}) and group-specific CO_2 fixation rates (Bq x 10^3 $cell^{-1}$) for small (b; Plast-S) and large (c; Plast-L) plastidic protists measured during AMT20 in control (black boxes) and nutrient addition (white boxes) experiments, including standard errors. Samples were inoculated with 246 KBq ml^{-1} $NaH^{14}CO_3$ and incubated for 10h in acid washed glass bottles at an intensity of 500 µmol photons m^{-2} s^{-1}, using a warm white light emitting diode (LED) array (Photon Systems Instruments, Drasov, Czech Republic) to mimic the irradiance that would reach the sampling depth based on solar noon levels measured in the Equator (Jitts et al., 1976). Samples were fixed as described in Fig. 1 and concentrated using a syringe-

pump method (Zubkov & Tarran, 2008) before staining with SYBR-Green I dye (Marie et al., 1997) to differentiate Plast-S and Plast-L populations based on their flow cytometry red autofluorescence, nucleic acid content and scattering signals according to Zubkov et al. (2007b) and as shown in Fig. S1 of Grob et al. (2011), using a FACSort flow cytometer (Becton Dickinson, Oxford, UK). Rates and standard errors were determined by sorting as many cells as possible (between 100 and 5000 depending on their abundance) in 1, 2, 3 and 4 min and collected them on 0.8 μm pore-size polycarbonate filters (Nucleopore, Whatman, UK) before adding 10% (v/v) HCl and scintillation cocktail and measuring radioactivity as indicated in Fig.1.

Fig. 3. Cell abundance (cells ml^{-1}, including standard deviation) for small (Plast-S) and large (Plast-L) plastidic protists, at the start and end of the incubations at selected stations sampled during SOLAS and AMT20 cruises AMT20. Dashed line corresponds to the unity line.



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365 Fig. 1

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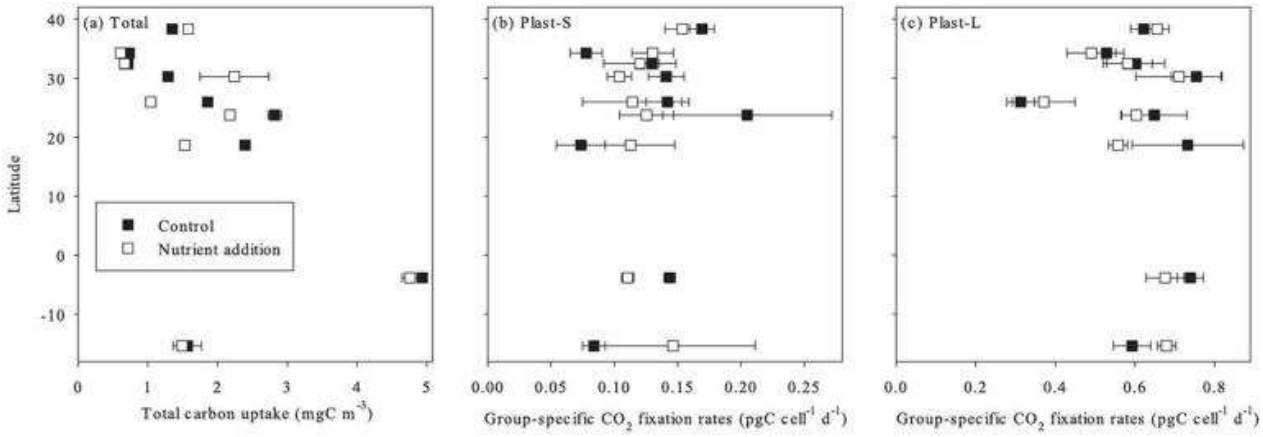

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368 Fig. 2.

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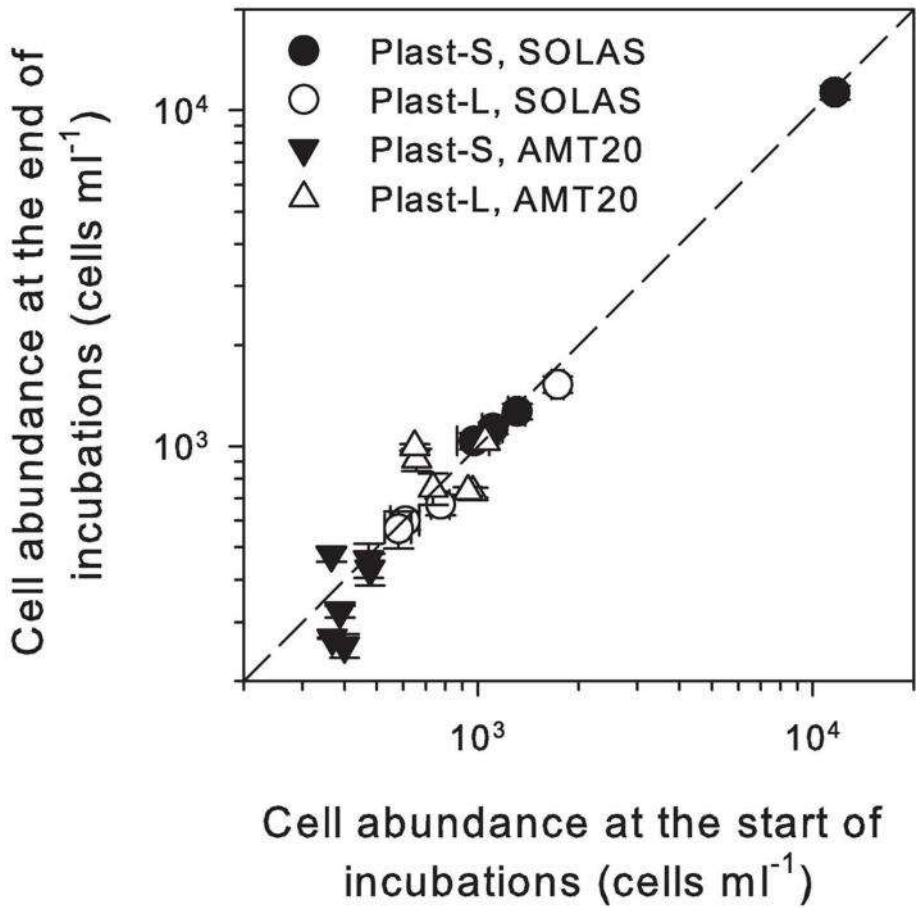

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371 Fig. 3

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