Supplementary information for "NanoSIMS single cell analyses reveal the contrasting nitrogen sources for small phytoplankton"

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CTD characteristics

SBE 911plus CTD (conductivity, temperature, and pressure sensors), photosynthetically available radiation sensor (PAR, Biospherical/Licor), and fluorometer (WET Labs ECO-AFL/FL) calibrated with Chl *a* standards.

Nutrient analyses

Samples for Chl a were collected on GFF filters and determined using high performance liquid chromatography at NASA Goddard Space flight Center following the procedures of Van Heukelem *et al.* (1) and further described in Hooker *et al.* (2). Samples for the determination of NO_3^-+NO_2^- (hereafter referred as to NO_3^-) and urea were collected unfiltered from Niskin bottles in 20 or 50 ml polyethylene bottles and stored at -20°C until analysis. Back in the laboratory, the samples were thawed at room temperature and analyzed colorimetrically for the determination of NO_3^- on an analytical segmented flow analyzer (detection limit = 2 nmol L^{-1}), according to the protocol of Raimbault *et al.* (3). Urea concentrations were determined colorimetrically using the diacetylmonoxime method using a 10 cm long cuvette according to Mulvenna and Savidge (4) (detection limit = 20 nmol N L^{-1}). NH_4^+ samples were collected and directly analyzed aboard the ship using the method of Holmes *et al.* (5) (detection limit = 3 nmol L^{-1}).

Isotope ratio mass spectrometry

The samples, filtered onto GF/F filters after the incubations, were dried back onshore. The C and N content and isotopic composition were measured using an isotope ratio mass spectrometer coupled to an elemental analyzer (EA-IRMS, delta V, Thermo Finningan). Both C and N content and isotope ratios were corrected using blanks.

Cell concentration and flow cytometry analysis

In order to improve sorting efficiency, the cells were concentrated ~~after the~~ following incubations~~. For this purpose,~~ by filtering the bottle contents ~~whereas filtered~~ onto a 47 mm, 0.2 µm pore size polycarbonate membrane. Just before the filter went dry, the filtration was stopped, and the filter was placed in a 5 ml polypropylene tube filled with a solution of paraformaldehyde (1.6 % v/v) diluted in 0.2 µm filtered seawater. The tube was vortexed to dislodge ~~the~~ cells from the filter and left for at least 1 h in darkness at 4°C before being flash~~-~~frozen and stored in liquid nitrogen until cytometry cell sorting. A seawater sample from the same bottle was collected in a 2 mL tube prior to the concentration step, using the same preservation and storage procedures for the flow cytometry determination of cell abundance.

The sheath fluid consisted of a sodium chloride solution filtered in-line through a 0.22 µm Sterivex™ filter unit. *Prochlorococcus*, *Synechococcus*, and PPE were discriminated in unstained samples based on their chlorophyll (red) fluorescence and forward scatter (size) signatures. The high phycoerythrin (orange) signal in *Synechococcus* was used to distinguish them from *Prochlorococcus* and PPE. Using a forward scatter detector with the small particle option and focusing a 488 plus a 457 nm (200 and 300 mW solid state, respectively) laser into the same pinhole greatly improved the resolution of dim surface *Prochlorococcus* group from background noise (6)~~(Duhamel et al., 2014)~~. Reference beads (Fluoresbrite, YG, 1-µm) were added to each sample to maintain proper alignment and focus of the instrument. For cell enumeration, cytograms were analyzed using the FCS Express 6 Flow Cytometry Software (De Novo Software, CA, US). For cell sorting, the "1.0 drop pure" sort mode was selected to insure purity of the sorted groups.

Correction of the uptake rates from the stimulation due to nutrient additions

The average N additions during the incubations represented 38% (range 8–93%) of the initial pool, leading to a potential nutrient stimulation in N depleted waters. To correct the uptake from this stimulation, the following correction was applied according to Rees et al. 1999:

$$uptake\ corrected = \frac{original\ uptake}{\frac{N_{incub}}{Ks + N_{incub}} * \frac{Ks + N_A}{N_A}}$$

N_{incub} is the final concentration in N (ambient + tracer). N_A represents the ambient concentration and K_s the half saturation parameter assuming Michaelis-Menten kinetics. When N_A was not detected, it was considered to equal the detection limit. For NO_3^- and NH_4^+, K_s was estimated from two kinetics experiments performed in ~~the~~ NPSG surface waters (near station 1 and 2), with N additions ranging from 5 nmol L^{-1} to 500 nmol L^{-1}. The K_s values were retrieved using the Hanes-Woolf method and assuming Michaelis-Menten kinetics, which led to constants of 15 and 18 nmol L^{-1} for NO_3^- and NH_4^+ respectively, in good agreement with previous reports from open ocean studies (7–9). For urea, a K_s of 20 nmol L^{-1} was used according to Sahlsten (8). The corrections factors reduced N uptake rates of 56%, 17% and 5% on average for NO_3^-, NH_4^+ and urea, respectively. Note that the correction was applied similarly to the different plankton groups, assuming similar kinetic constants between the different populations.

The uncoupling between C and N urea uptakes

Despite relatively high contribution of N-urea to the N uptake for all the groups investigated, no C-urea uptake was measured in this study. Assuming that the hydrolysis of urea into CO_2 and NH_4^+ is the main metabolic pathway for urea assimilation, the absence of ^{13}C-urea enrichment might be explained by the isotopic dilution of the $^{13}CO_2$ urea produced (<20 nmol L^{-1}) within the pool of extra or intracellular inorganic C (~2 mmol L^{-1}). Thus, while urea appears as a significant source of N for marine plankton, it may not represent a significant source of C and cannot be used as a relevant proxy of heterotrophic activity (~~*sensus*~~ *i.e.* the uptake of organic C for growth). The few studies which have investigated the uptake of ^{13}C and ^{15}N urea simultaneously report either insignificant or low C urea

uptake compared to N urea uptake (10–12). However, Bradley *et al.* (13) measured significant ^{13}C-urea enrichments at the total community level (including photosynthetic and heterotrophic cells), while photosynthetic cells sorted from the same incubation did not show significant ^{13}C-enrichment. This would argue for a distinct urea metabolic pathway in heterotrophic bacteria that might be able to directly use C-derived urea.

The coupling between specific C fixation and specific N uptake

C:N specific uptake ratios were one average close to one for all the groups investigated, indicating a balanced incorporation of C and N during daylight periods. One could have expected over-incorporation of C during daylight to balance the measured night N uptake (Table 1, Fig 4) and night C respiration. Indeed, if the night N uptake rates are included (only available for surface at station 1 and 3), the C:N specific ratios drop from 0.96 to 0.42 on average (data not shown). This suggests that either the C sources are underestimated or the N sources are overestimated. A potential missing C source is osmo-heterotrophy, i.e. the uptake of organic sources of C. Here, we show that C-urea is not significantly used both at the community and group levels. However, a large range of C rich organic molecules are present in the oceanic surface waters (e.g. glucose, free amino acids), which have been shown to represent a potential additional source of C for small plankton (6,14,15)*. Alternatively, N uptake can be overestimated due to the short incubation times, as a significant fraction of the N uptake may not be metabolized by the cells within the timespan of the incubation but stored and potentially released on a longer timescale* (16,17)*, in particular in N depleted environments* (18–22)*. Such unbalances are a common feature in literature* (23–26) *and point out that efforts to characterize the coupling of C and N in phytoplankton nutrition are still needed.*

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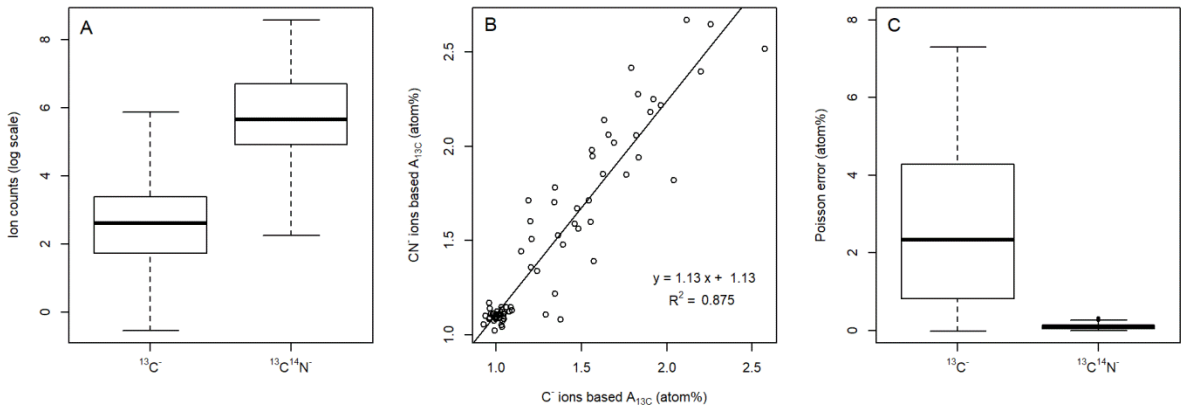

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168 Fig S1~~2~~. (a) Boxplot of the average $^{13}C^-$ and $^{13}C^{14}N^-$ ion counts detected in the cells analyzed (note the
169 log scale). (b) Scatter plot of the A_{13C} calculated either from the $^{12}C^-$ and $^{13}C^-$ ions or from the $^{13}C^{14}N^-$
170 and $^{12}C^{14}N^-$ ions. Each dot represents the average cellular A_{13C} analyzed for each group in each assay.
171 (c) Boxplot of the Poisson error associated ~~to~~ with the A_{13C} computed using C^- ions and using CN^-
172 ions.

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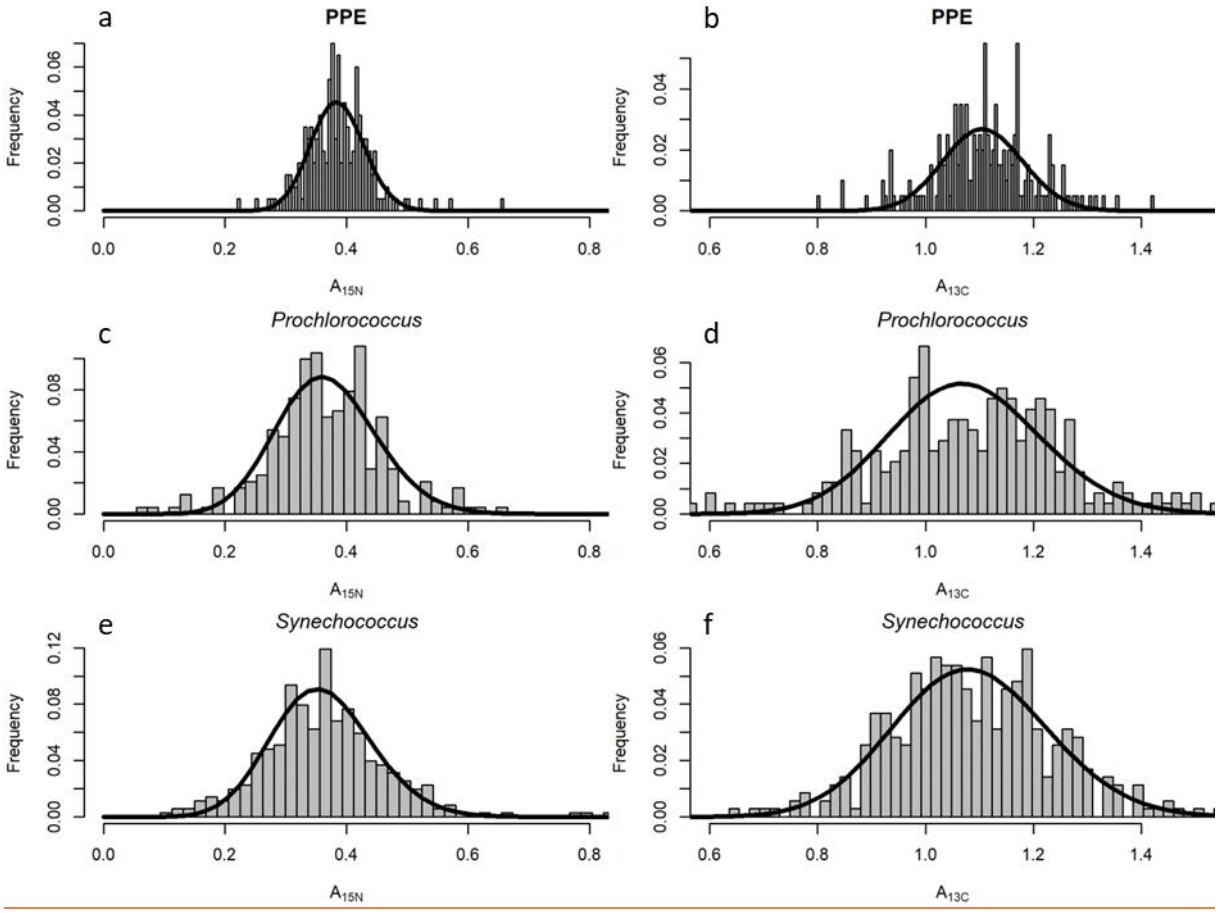

Fig S2. Distribution of the measured A_{15N} (a,c,e) and A_{13C} (b,d,f) of PPE, *Prochlorococcus*, and *Synechococcus* cells without addition of isotopic tracer (grey bars), with the modeled Poisson distribution superimposed (black line) and parametrized with the measured mean A_{15N} or A_{13C} and mean ions count per cell of each population ($\lambda = A_{group} * N_{CN^-}$).

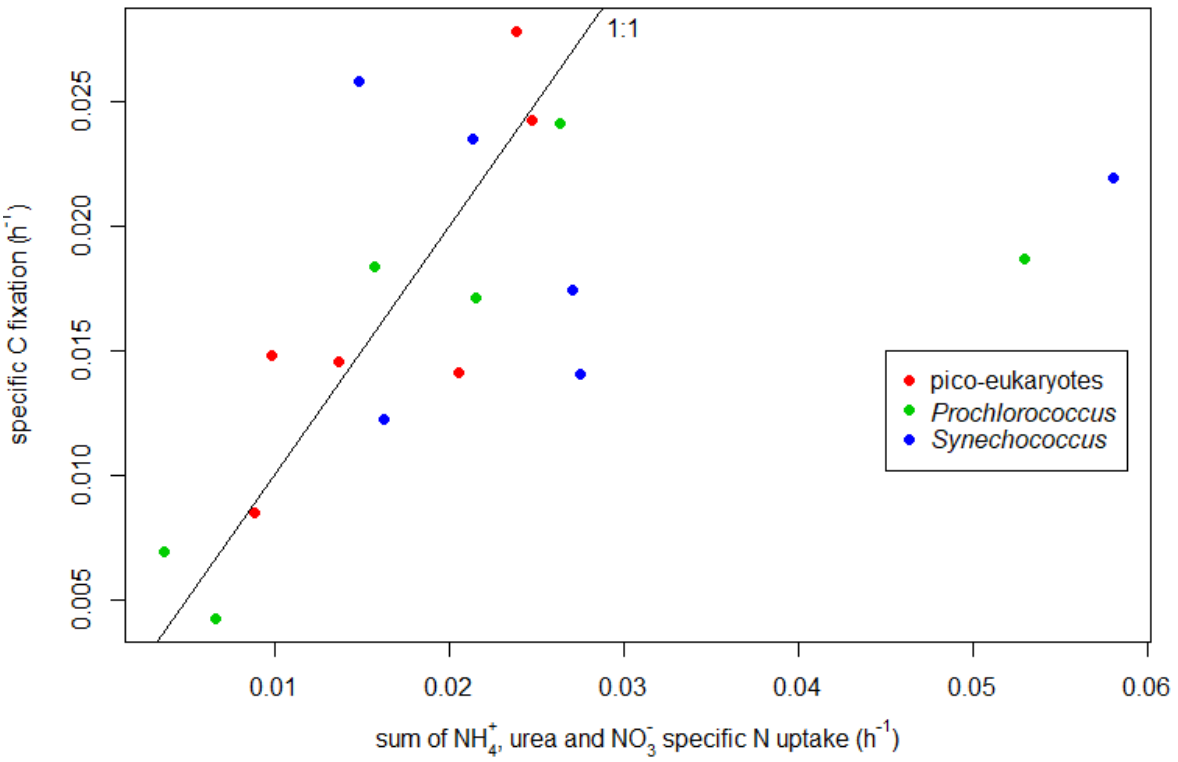

Fig. S3. Scatter plot of the specific C fixation as a function of the sum of NH_4^+, urea and NO_3^- specific N uptake from daylight incubations. The dark line represents the 1:1 unity line.

Table S1. Contribution of PPE, *Prochlorococcus*, and *Synechococcus* to total community C fixation, and NO_3^-, NH_4^+, and urea uptake (in %).

		Depth (m)	Contribution to total community C fixation (%)	Contribution to total community NO_3^- uptake (%)	Contribution to total community NH_4^+ uptake (%)	Contribution to total community urea uptake (%)
PPE	Station 1	5	6.8	4.4	2.6	0.9
		125	20.2	13.9	16.9	8.2
	Station 2	5	15.1	36.2	7.2	2.0
		110	12.5	11.5	11.2	8.8
	Station 3	5	39.3	10.7	36.5	41.0
		47	40.5	19.5	41.0	31.1
Prochlorococcus	Station 1	5	57.4	79.0	92.6	34.7
		125	40.3	1.2	57.2	17.6
	Station 2	5	33.9	19.3	38.5	17.5
		110	28.1	1.2	50.8	28.2
	Station 3	5	0.1	<0.1	0.2	0.1
		47	0.1	<0.1	0.2	0.1
Synechococcus	Station 1	5	2.4	10.3	2.3	1.2
		125	0.8	<0.1	0.7	0.5
	Station 2	5	2.6	6.5	1.7	2.0
		110	2.1	0.3	3.1	2.7
	Station 3	5	18.6	0.3	26.0	11.1
		47	10.1	<0.1	17.1	12.6

Table S2. ^{13}C isotopic abundance ($A^{13}C$, atom%), ^{15}N isotopic abundance ($A^{15}N$, atom%), number of cells analyzed and C-based and N-based metabolic heterogeneity in each assay and plankton group. Metabolic heterogeneities with a Poisson dispersion representing more than 50% of the coefficient of variation of the groups are not presented (see material and method section for further details) and are referred to as ND (not determined).

Station	Depth	Period of the day	Tracers	Group	Number of cells analyzed	A_{13C}	A_{15N}	^{13}C-metabolic heterogeneity	^{15}N-metabolic heterogeneity
1	5	day	$^{15}NH_4^+$, $H^{13}CO_3^-$	PPE	122	1.938±0.522	1.179±0.534	0.65	0.65
				Prochlorococcus	115	1.728±0.246	3.357±1.067	0.40	0.35
				Synechococcus	443	2.131±0.212	2.969±0.344	0.21	0.13
			$^{15}NO_3^-$, $H^{13}CO_3^-$	PPE	127	1.443±0.237	1.007±0.933	0.74	1.44
				Prochlorococcus	307	1.984±0.274	1.555±1.783	0.33	1.47
				Synechococcus	90	1.836±0.331	5.796±2.783	0.46	0.49
			^{13}C-^{15}N-urea	PPE	84	1.115±0.035	1.313±0.527	ND	0.55
				Prochlorococcus	112	1.086±0.039	4.187±2.997	ND	0.76
				Synechococcus	314	1.048±0.145	5.155±3.628	ND	0.73
	5	night	$^{15}NH_4^+$, $H^{13}CO_3^-$	PPE	12	1.130±0.074	1.105±0.495	ND	0.66
				Prochlorococcus	245	1.171±0.054	2.965±1.370	ND	0.52
				Synechococcus	404	1.121±0.146	2.882±0.416	ND	0.16
			$^{15}NO_3^-$, $H^{13}CO_3^-$	PPE	70	1.143±0.066	0.992±0.892	ND	1.41
				Prochlorococcus	275	1.102±0.072	0.637±0.437	ND	1.59
				Synechococcus	310	1.118±0.048	2.545±0.732	ND	0.33
			^{13}C-^{15}N-urea	PPE	91	1.101±0.151	1.386±0.753	ND	0.73
				Prochlorococcus	12	1.078±0.174	3.005±2.318	ND	0.86
				Synechococcus	468	1.115±0.058	7.921±2.077	ND	0.26
	125	day	$^{15}NH_4^+$, $H^{13}CO_3^-$	PPE	82	1.342±0.159	0.751±0.298	0.72	0.76
				Prochlorococcus	1040	1.943±0.328	1.304±0.345	0.39	0.36
				Synechococcus	95	1.842±0.169	1.186±0.318	0.25	0.38
			$^{15}NO_3^-$, $H^{13}CO_3^-$	PPE	76	1.534±0.208	0.991±0.866	0.52	1.37
				Prochlorococcus	206	1.712±0.358	0.404±0.116	0.61	2.65
				Synechococcus	186	2.204±0.291	0.400±0.091	0.27	ND
			^{13}C-^{15}N-urea	PPE	27	1.072±0.081	0.876±0.218	ND	0.42

					Prochlorococcus	349	1.085±0.065	1.162±0.505	ND	0.63
					Synechococcus	291	1.109±0.038	2.043±0.655	ND	0.38
	2	5	day	$^{15}NH_4^+$, $H^{13}CO_3^-$	PPE	124	1.613±0.237	1.527±0.578	ND	0.49
					Prochlorococcus	35	1.586±0.282	2.581±1.157	ND	0.51
					Synechococcus	182	1.490±0.370	1.731±0.560	ND	0.40
				$^{15}NO_3^-$, $H^{13}CO_3^-$	PPE	98	1.584±0.227	3.430±3.261	0.51	1.03
					Prochlorococcus	129	1.779±0.279	0.951±1.002	0.44	1.69
					Synechococcus	563	1.668±0.207	3.063±1.507	0.39	0.55
				^{13}C-^{15}N-urea	PPE	202	1.032±0.101	1.297±0.732	ND	0.78
					Prochlorococcus	72	1.119±0.173	3.222±1.516	ND	0.52
					Synechococcus	379	1.019±0.089	4.900±2.087	ND	0.44
				Control (no isotope addition)	PPE	148	1.107±0.084	0.386±0.056	ND	ND
					Prochlorococcus	184	1.071±0.178	0.374±0.096	ND	ND
					Synechococcus	254	1.089±0.136	0.362±0.099	ND	ND
		110	day	$^{15}NH_4^+$, $H^{13}CO_3^-$	PPE	178	1.850±0.265	1.949±0.724	0.37	0.45
					Prochlorococcus	316	2.677±0.322	4.133±0.686	0.21	0.18
					Synechococcus	329	2.255±0.212	3.187±0.467	0.20	0.16
				$^{15}NO_3^-$, $H^{13}CO_3^-$	PPE	153	2.022±0.304	3.748±3.166	0.35	0.91
					Prochlorococcus	182	2.423±0.477	0.544±0.240	0.37	1.26
					Synechococcus	394	2.048±0.292	0.879±0.327	0.28	0.61
				^{13}C-^{15}N-urea	PPE	173	1.103±0.103	3.055±1.195	ND	0.43
					Prochlorococcus	190	1.088±0.124	4.906±1.632	ND	0.35
					Synechococcus	110	1.048±0.136	5.691±1.318	ND	0.24
	3	5	day	$^{15}NH_4^+$, $H^{13}CO_3^-$	PPE	98	2.391±0.457	0.764±0.187	0.37	0.46
					Prochlorococcus	210	1.515±0.383	0.455±0.080	0.97	ND
					Synechococcus	335	2.507±0.275	0.826±0.086	0.20	0.18
				$^{15}NO_3^-$, $H^{13}CO_3^-$	PPE	161	2.619±0.545	2.617±2.167	0.37	0.94
					Prochlorococcus	78	1.387±0.320	0.558±0.164	1.20	0.82
					Synechococcus	219	2.278±0.505	0.466±0.137	0.45	1.28
				^{13}C-^{15}N-urea	PPE	101	1.150±0.063	1.174±0.388	ND	0.48
					Prochlorococcus	160	1.088±0.105	0.456±0.130	ND	1.26

				Synechococcus	282	1.093±0.056	0.728±0.242	ND	0.66
		night	$^{15}NH_4^+$, $H^{13}CO_3^-$	PPE	55	1.099±0.028	0.899±0.259	ND	0.48
				Prochlorococcus	161	1.120±0.127	0.731±0.278	ND	0.73
				Synechococcus	501	1.088±0.154	1.049±0.339	ND	0.49
			$^{15}NO_3^-$, $H^{13}CO_3^-$	PPE	111	1.149±0.064	2.235±1.279	ND	0.67
				Prochlorococcus	166	1.121±0.092	0.511±0.292	ND	1.97
				Synechococcus	364	1.146±0.030	0.456±0.059	ND	0.61
			^{13}C-^{15}N-urea	PPE	81	1.092±0.094	1.470±0.832	ND	0.74
				Prochlorococcus	260	1.086±0.120	0.492±0.131	ND	ND
				Synechococcus	513	1.102±0.029	1.132±0.308	ND	0.40
	45	day	$^{15}NH_4^+$, $H^{13}CO_3^-$	PPE	139	2.202±0.334	0.831±0.345	0.31	0.73
				Prochlorococcus	63	1.251±0.251	0.504±0.120	1.75	0.75
				Synechococcus	281	1.573±0.078	0.690±0.051	0.18	0.14
			$^{15}NO_3^-$, $H^{13}CO_3^-$	PPE	140	2.050±0.453	0.774±0.461	0.50	1.12
				Prochlorococcus	306	1.350±0.332	0.400±0.120	1.32	ND
				Synechococcus	253	1.714±0.166	0.359±0.067	0.26	ND
			^{13}C-^{15}N-urea	PPE	219	1.099±0.134	1.273±0.666	ND	0.73
				Prochlorococcus	348	1.094±0.089	0.577±0.247	ND	1.15
				Synechococcus	375	1.099±0.038	1.001± 0.224	ND	0.35

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