Crocosphaera as a major consumer of fixed nitrogen despite its capability of nitrogen fixation

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Abstract

Crocosphaera watsonii (hereafter *Crocosphaera*) is a key nitrogen (N) fixer in the ocean, but its ability to consume combined N sources is still unclear. Using *in situ* microcosm incubations with an ecological model, we show that *Crocosphaera* has high competitive capability both under low and moderately high combined N concentrations. In field incubations, *Crocosphaera* accounted for the highest consumption of ammonium and nitrate, followed by pico-eukaryotes. The model analysis shows that cells have a high ammonium uptake rate (~7 mol N (mol N)$^{-1}$ d^{-1} at the maximum), which allows them to compete against pico-eukaryotes and non-diazotrophic cyanobacteria when combined N is sufficiently available. Even when combined N is depleted, their capability of nitrogen fixation allows higher growth rates compared to potential competitors. These results suggest the high fitness of *Crocosphaera* in combined N limiting, oligotrophic oceans, and thus heightens its potential significance in its ecosystem and in biogeochemical cycling.

Introduction

Marine phytoplankton contribute about one half of the global net primary production and play a key role in regulating global biogeochemical cycles (1). Since phytoplankton are biochemically, metabolically, and ecologically diverse (2-4), understanding the contribution of different phytoplankton groups to ecosystem functioning is central to the precise estimation of the global carbon (C) and nitrogen (N) budget and in predicting the biogeochemical impact of future environmental changes (5).

In the oligotrophic subtropical gyres, combined N (defined as N covalently bonded to one or more elements other than N (6)) limits primary production and controls planktonic community composition (7-10). Therefore, N_2 fixing microorganisms (diazotrophs) are important as a source of combined N in oligotrophic ecosystems (11, 12). In the subtropic oligotrophic ocean, the unicellular diazotroph, *Crocosphaera watsonii* (2.5 – 6 μm), is widely distributed (10, 13-16) in addition to pico-sized (<3 μm) cyanobacteria (e.g., *Prochlorococcus* and *Synechococcus*) and pico-eukaryotes (17-19). Recent studies reveal *Crocosphaera watsonii*'s ability to assimilate dissolved inorganic nitrogen (DIN), such as ammonium (NH_4^+) and nitrate (NO_3^-), at a nanomolar level and keep fixing N_2 (20, 21). Model results indicate using DIN enables *Crocosphaera* to increase their abundance and expand their niche (22). These studies proposed that unicellular diazotrophs can be competitors with non-diazotrophic phytoplankton for combined N. However, how *Crocosphaera* competes for combined N is poorly evaluated. In this study, we combine an *in situ* microcosm experiment with N addition at the nanomolar level and model (23) to evaluate the competitiveness of *Crocosphaera* in a N limiting environment.

Results

71 **Summary of the experiment.** We carried out five nitrogen (N) and phosphorus (P)-addition
72 bioassays (M1 to M5) at a station in the subtropical Northwestern Pacific (12ºN, 135ºE) from
73 6 to 25 June 2008 during the MR08-02 cruise on the R/V *MIRAI*. Nutrient concentrations
74 initially were less than 36 nM for ammonium (NH_4^+), 7 nM for nitrate plus nitrite (NO_3^- +
75 NO_2^-) and 64 nM for phosphorus (PO_4^{3-}) (24). The physical and biological parameters at the
76 initial condition of the experiments are described in (24). Hydrography and biochemistry at the
77 station are described in (25). Although we performed pre-filtration with a 1 µm-filter to
78 eliminate the effect of grazing, water samples contained plankton with up to ~5 µm in size.
79
80 **Nutrient uptake and fate of enriched DIN.** For 3 days of incubation, the phytoplankton
81 community consumed NH_4^+ entirely at the end, while NO_3^- was not always consumed
82 completely (Fig. 1, Fig. S1). Estimated biomass explains about half of consumed combined N
83 sources (Figs. 1, 2A), possibly due to luxury uptake (26, 27).
84

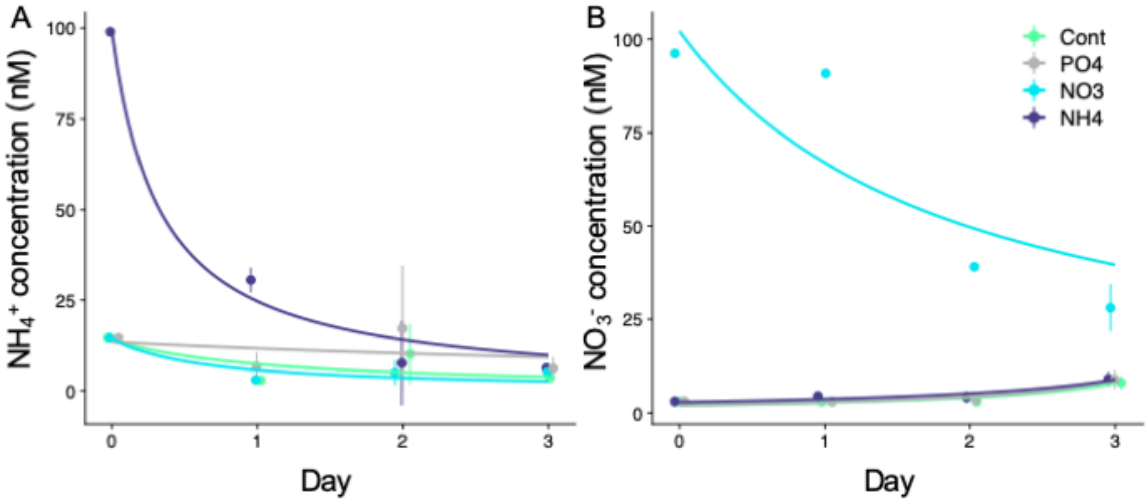

Fig. 1 Temporal change in NH_4^+ and NO_3^- concentrations of Ex. M3. (A) NH_4^+ concentration in the NH_4^+ treatment exponentially decreased during the experiment down to the detection limit of 6 nM on day 3. (B) NO_3^- concentrations in the NO_3^- treatment exponentially decreased during the experiment, but enriched NO_3^- was not always entirely consumed. Error

bar shows a standard deviation of triplicate. Temporal change in Urea-N concentration is shown in Fig. S2.

The greatest portion of estimated C and N in biomass were found in *Crocosphaera* (39-93% in all N addition incubations) followed by pico-eukaryotes (5-55% in N addition incubations) (Fig. 2A, Fig. S3). Although the origin of water mass changed from oligotrophic-water to mixed-water between experiments (Exs.) M1-M3 and M4-M5 (25), with more *Crocosphaera* in cell density at the latter environment (Table S1), the dominance of *Crocosphaera* as a C and N biomass was observed from all the experiments. N derived from N_2 fixation was not always sufficient to support the N demand of *Crocosphaera*, especially in N amendment (Fig. S4). Estimated N_2 fixation supported 0.5 – 12.7% of N demand of *Crocosphaera* in control and 0.5 – 11.6% in NH_4^+ treatment (Fig. S4), suggesting that *Crocosphaera* consumed amended N sources. Assimilation of combined nitrogen (NH_4^+ and NO_3^-), together with N_2 fixation by *Crocosphaera,* has been reported earlier (20, 21). Although enriched 100 nM NH_4^+ was completely consumed (< 6 nM; detection limit, on day 3), increases in N-biomass of non-diazotrophs for 3 days were limited to up to 58 nmol L^{-1}, again suggesting *Crocosphaera* took up combined nitrogen.

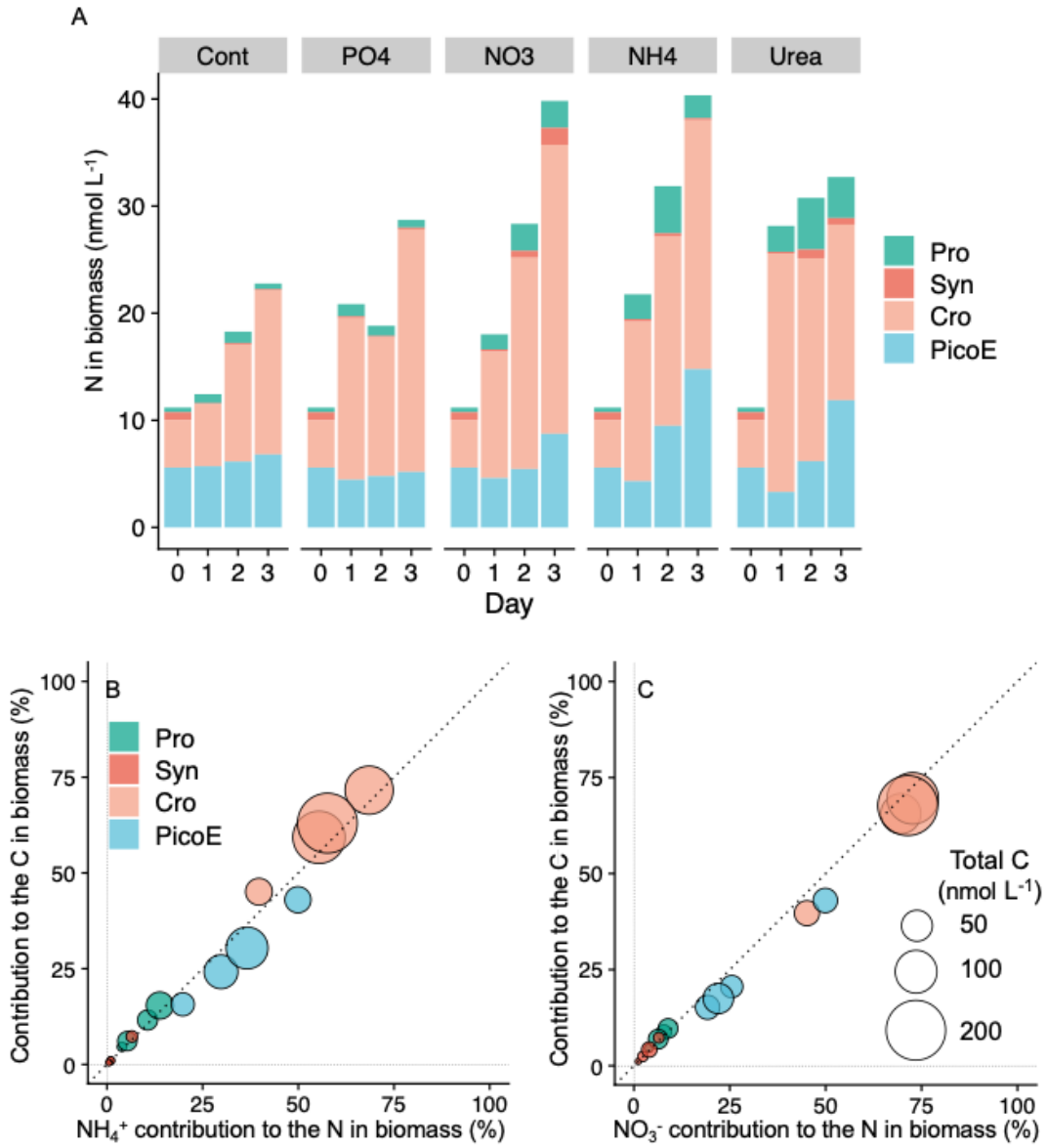

Fig. 2 (A) N in biomass in each treatment and its contribution of each phytoplankton group of experiment M3. (B) Contribution to total carbon C in biomass as a function of the contribution of NH_4^+ - N biomass for each phytoplankton group. (C) Contribution to total carbon C in biomass as a function of the contribution of NO_3^- - N biomass for each phytoplankton group. Each circle shows data from a different day, and the size of the dots represents the total C in biomass (nmol C L^{-1}). Pro; *Prochlorococcus*, Syn; *Synechococcus,* Cro; *Crocosphaera*, PicoE; pico-eukaryotes.

Model analysis of the data. To quantitatively interpret the observed data, we used a simple model of the cellular growth, which is based on the uptake of NH_4^+ and NO_3^- (see Methods).

We used the data from experiment M3 since it shows the clearest trends with low initial nutrient concentrations. The model captured the overall trend of the transition of cellular N (Fig. 3) based on the available nutrient (Fig. S5). The parameterization of the model reveals high rates of N uptake by *Crocosphaera*. Especially, we used about 7 (mol N (mol N)$^{-1}$ d^{-1}) for maximum NH_4^+ uptake to represent the data, which shows high combined-N uptake compared to other phytoplankton. Specifically, such parameterization was needed to reproduce the rapid growth of *Crocosphaera* under NH_4^+ added case between day 0 and day 1. The predicted maximum NO_3^- uptake rate for *Crocosphaera* is also higher than for other phytoplankton, which is supported by *Crocosphaera*'s faster growth with NO_3^- addition.

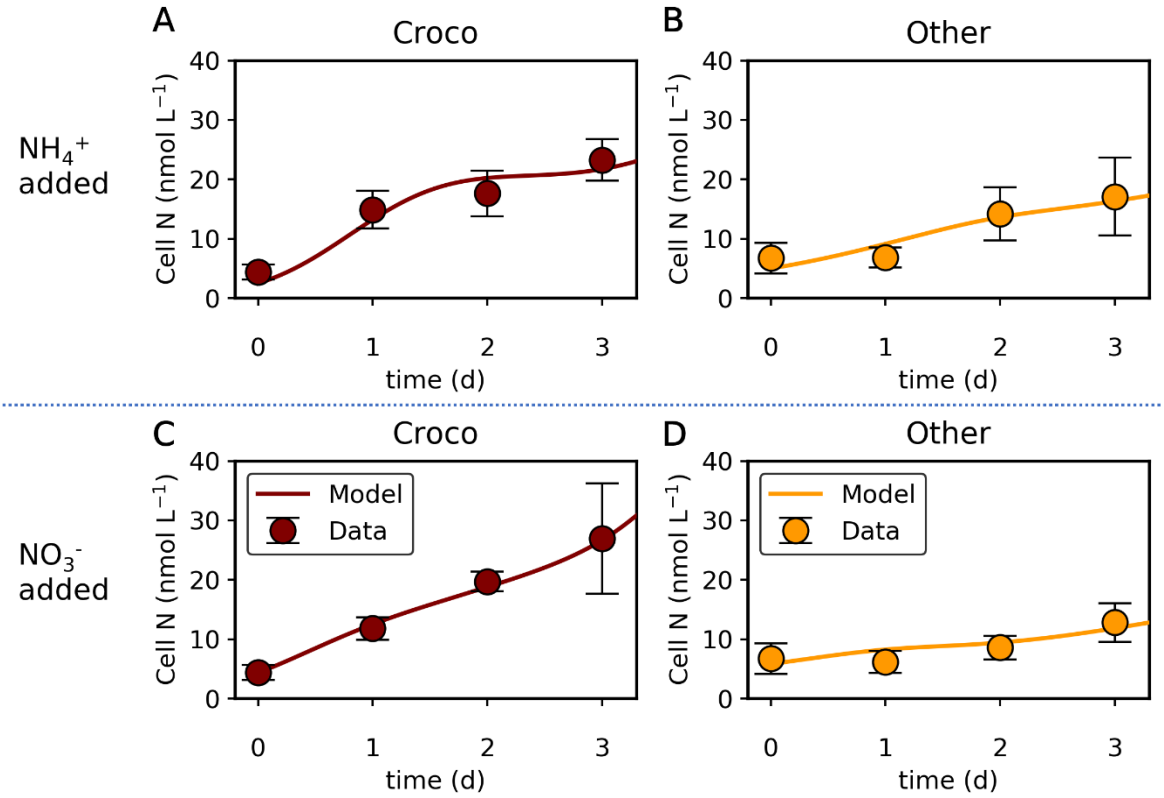

Fig. 3 Simulated transition of cellular N with nutrient addition compared with data. (A)(B) NH_4^+ added case. (C)(D) NO_3^- added case. Croco: *Crocosphaera*. Other: other phytoplankton. Data are from experiment M3.

115 To test the competitiveness of *Crocosphaera*, we simulated a simple ecological
116 situation. Here, we simulate zooplankton with Kill the Winner Theory (KTW) (28), which is
117 based on a commonly observed active prey-switching behavior of zooplankton (29-31). The
118 result shows the high competitiveness of *Crocosphaera* both under high and low nutrient
119 concentrations. Under high nutrient concentration, *Crocosphaera* may dominate other
120 phytoplankton due to the high rate of nutrient uptake (Fig. 4A, S6B). However, under
121 extremely low nutrient conditions (NH_4^+ and NO_3^- are both at 1 nmol L^{-1}), *Crocosphaera* is
122 slightly outcompeted (Fig. 4B, S6B). This is due to the relatively high half-saturation constant
123 for NH_4^+, which is manifested by the sudden decrease in growth rate with a drop in NH_4^+ under
124 NH_4^+ addition (Fig. 3A, S5A). However, this relationship flips if we consider the effect of N_2
125 fixation, which maintains their growth rates at a higher level rather than relying on external N
126 under N depletion (Fig. 4C, S6C). These results suggest that possession of nitrogenase (an
127 enzyme complex involved in N_2 fixation) allows for *Crocosphaera's* survival under low
128 nutrient environments.
129

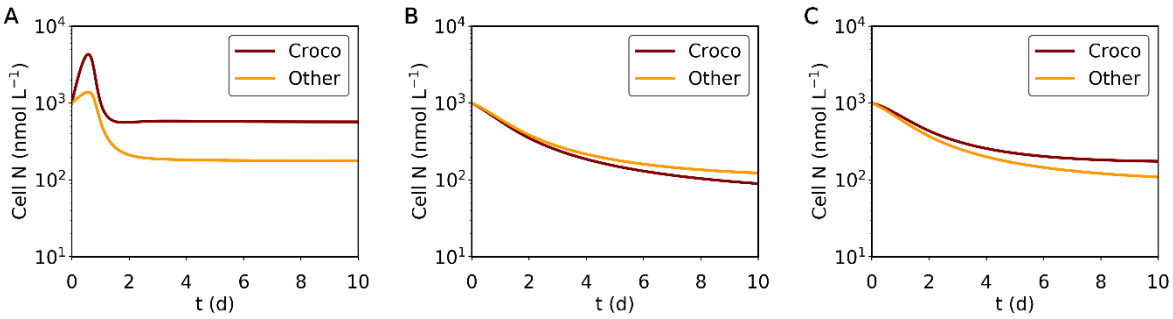

Fig. 4 Simulated transition of cellular N in a simple ecosystem model for three different scenarios. (A) The concentrations for NH_4^+ and NO_3^- are both 100 nmol L^{-1} (B)(C) The concentrations for NH_4^+ and NO_3^- are both 1 nmol L^{-1}. In only (C) *Crocosphaera* may acquire N via N_2 fixation; in (A) and (B) the effect of N_2 fixation is neglected. Croco: *Crocosphaera*. Other: other phytoplankton. Parameters are based on NH_4^+ added case.

130

131 **Discussion**

Our study shows high uptake of N by *Crocosphaera* under relatively high N concentration. The results counter the general image of *Crocosphaera* since it is mostly known as a diazotroph and is considered to be a provider of N to the environment. Rather, our result supports more recent studies, where *Crocosphaera* does not increase the productivity of other phytoplankton (32) or even compete with other species over combined N (22). Surprisingly, our study even shows higher maximum uptake rates of NH_4^+ and NO_3^-, which allow its dominance just by uptake of combined N. When nitrogen concentration is extremely low, they could be outcompeted in N uptake, but their N_2 fixation allows maintaining *Crocosphaera* biomass at a certain level, which can still be higher than those of non-diazotrophic phytoplankton. This high consumption of NO_3^- may differ from UCYN-A (15, 33-35), which keeps fixing nitrogen under high NO_3^- availability (36, 37), leading to their unique niche acquisition. These results suggest that *Crocosphaera* has high competitiveness under both low and high nutrients.

Despite that, we generally do not observe the oligotrophic ocean completely dominated by *Crocosphaera*. One reason might be the grazing selection. *Crocosphaera* is a unicellular cyanobacterium a few microns to 6 μm in diameter (38), and its tight coupling with predators is reported recently (39). The new production of *Crocosphaera* is estimated to support up to 400% of C demand of the main grazers, and the grazing rates of the main predator *Protoperidinium* were found to be nearly equivalent to growth rates of *Crocosphaera* (39). On the other hand, its potential competitor, *Trichodesmium*, a major N_2 fixer in the ocean, is reported to produce a toxin (40-42), and creates large colonies of ~10^4 cells (43), potentially protecting themselves from grazing. Another reason might be the growth limitation by other nutrients such as P and Fe. Although there are some reports that *Crocosphaera* shows adaptation for low P and low Fe, their relative fitness to such low P or low Fe environments compared to other organisms has not been quantified. Since having nitrogenase enzymes require a high concentration of Fe, non-nitrogen fixers, such as *Prochlorococcus* and

157 *Synechococcus*, may have lower Fe requirements and are more adapted to Fe depletion. Also,
158 *Crocosphaera* does not seem to fully utilize sulpholipid, which would save P use, as opposed
159 to other cyanobacteria, such as *Synechococcus* (44, 45), and thus may not compete strongly
160 under P limitation.

161 At the same time, it is largely possible that *Crocosphaera* dominates at some regions
162 in the oligotrophic ocean given its high competitiveness under N limitation, which is the
163 characteristic of the oligotrophic ocean (7, 46). For example, a study of flow cytometry shows
164 a high abundance of *Crocosphaera*-like cells in a wide region of the North Pacific (47), where
165 the abundance of *Trichodesmium* seems limited (48). Also, a recent study shows multiple gene
166 copies of *Trichodesmium* (up to ~700 gene copied per cell) (49), which would overestimate
167 their abundance (50). Given these factors and our analysis showing their high fitness to both
168 low and high nitrogen concentration, it is possible that we are still underestimating the relative
169 abundance and role of *Crocosphaera* in global biogeochemical cycling.

170

171 **Materials and methods**

172 **Experimental setup and sample collection.** The dataset presented herein originates from an
173 experimental setup described in (24). Briefly, we carried out five macro-nutrient (N and P)-
174 addition bioassays (M1 to M5) using natural phytoplankton assemblages collected at a station
175 in the subtropical Northwestern Pacific (12ºN, 135ºE) from 6-25 June 2008 during the MR08-
176 02 cruise on the R/V *MIRAI*. For macro-nutrient bioassays, we distributed pre-filtered seawater
177 from 10 m depth into 4-liter polycarbonate bottles. We performed three treatments with 100
178 nM addition of N as $NaNO_3$, NH_4Cl, or urea, and one treatment with 10 nM of NaH_2PO_4. Our
179 control was without nutrient addition. Bottles were incubated on deck for three days with daily
180 sample harvest in flow-through seawater tanks covered with a neutral density screen to
181 attenuate light intensity to 50% of its corresponding surface value.

Macro-nutrient and iron concentrations. Concentrations of NO_3^-+NO_2^- (N + N), NH_4^+, Soluble Reactive Phosphorus (SRP), and urea were measured using a high-sensitivity colorimetric approach with an AutoAnalyzer II (Technicon) and Liquid Waveguide Capillary Cells (World Precision Instruments, USA) as outlined (51). We analyzed urea concentrations using the diacetyl monoxime method (52). Detection limits of NO_3^- + NO_2^-, NH_4^+, and SRP were 3, 6, and 3 nM, respectively.

Flow cytometry. Flow-cytometry (FCM) identified *Prochlorococcus*, *Synechococcus*, pico-eukaryotes, and *Crocosphaera* based on cell size and chlorophyll- or phycoerythrin-fluorescence. Aliquots of 4.5 mL were preserved in glutaraldehyde (1% final concentration), flash-frozen in liquid N_2, and stored at -80 ºC until analysis on land by flow cytometry (PAS-Ⅲ, Partec, GmbH, Münster, Germany) equipped with a 488 nm argon-ion excitation laser (100 mW). We recorded forward- and side-angle scatter (FSC and SSC), red fluorescence (>630 nm, FL3), and orange fluorescence (570–610 nm, FL2). FloMax® (Partec, GmbH, Münster, Germany) distinguished *Synechococcus*, *Prochlorococcus*, *Crocosphaera*, and pico-eukaryotes based on their auto-fluorescence properties and their size.

Gene analysis. We collected DNA samples from each treatment of the Fe addition bioassay and collected aliquots of 0.5 to 1.0 L of sample on 0.2 μm SUPOR® polyethersulfone membrane filters, which we then placed in sterile tubes containing glass beads, frozen in liquid N_2, and stored at -80ºC until further analysis. DNA was extracted according to (53) to determine the abundance of *Crocosphaera watsonii* by quantitative PCR (qPCR) using a 5' nuclease assay as described in (54).

206 Quantitative PCR showed that cell densities of FCM-identified *Crocosphaera* were
207 significantly, positively correlated with *nif*H gene copies used to quantify the proportion of
208 *Crocosphaera*, indicating that *nifH* abundance accounted for 68% of the variation in FCM-
209 identified *Crocosphaera* (r^2 = 0.463, n = 48, p=0.001, Pearson Product Moment correlation).
210 Therefore, this study treated FCM-identified *Crocosphaera* as diazotroph *Crocosphaera*. Cell
211 abundance estimated by qPCR was 0.63 ± 0.23 fold lower than those measured by FCM.
212
213 **Nitrogen fixation.** To measure *in situ* N_2 fixation activity, we used the acetylene reduction
214 assay of (55, 56). We dispensed a total of 550 milliliter bioassay samples into 1200 mL HCl–
215 rinsed glass PETG bottles with 6 replicates and sealed with butyl rubber stoppers. Aliquots of
216 120 mL of acetylene (99.9999% (v:v), Kouatsu Gas Kogyo, Japan) were injected through the
217 stopper by replacing the same volume of headspace. After 24 h in the on-deck flow-through
218 seawater tanks, we analyzed ethylene concentrations by converting the ethylene to fixed
219 nitrogen with a molar ratio of 4:1 (57).
220
221 **Cellular C and N estimation.** We used a conversion factor of 235 fg Cμm^{-3} for
222 *Prochlorococcus*, *Synechococcus* and *Crocosphaera* (58) to estimate cellular carbon content.
223 For picoeukaryotes, we represented cell volume by converting it into carbon per cell, using a
224 modified Strathmann equation (58, 59):
225 $$logC(pg/cell) = 0.94 \times logVol(\mu m^3) - 0.6.$$
226 Then, using an earlier reported C:N ratio (C:N ratio = 9.1 for *Prochlorococcus*, 8.6 for
227 *Synechococcus*, 8.7 for *Crocosphaera*, 6.6 for picoeukaryotes), we converted the cellular C
228 content into cellular N (21, 60, 61).
229

Statistical analysis. Phytoplankton cell densities of each bioassay were first compared between treatments using repeated measurements Analysis of Variance (RM-ANOVA) with nutrient treatments as a between-subjects factor (5 levels) and time (4 levels) as the within-subjects factor. Treatment effects were considered significant if $p < 0.05$. Then, means between five treatments were compared by post hoc Turkey test (n = 3 replicates per treatment throughout, degrees of freedom = 40).

Quantitative model of microbial growth. To quantitatively analyze the fitness of *Crocosphaera* under N limiting conditions, we ran two simulations. One was to represent the incubation experiment to extract parameters manually and the other was the simple ecosystem model to simulate their competitiveness under different nutrient concentrations and scenarios. The list of parameters and used values are in Table S2 and S3, respectively.

Simulation of the incubation experiment. We used the following equations for the growth of phytoplankton to represent the field incubation experiment:

$$\frac{dN_i}{dt} = \mu_i N_i - m_i N_i \qquad \text{[eq. 1]}$$

where N_i (nmol L^{-1}) is the cellular nitrogen concentration of phytoplankton i (i = Cro, Oth: *Crocosphaera* and other phytoplankton, respectively) per volume water, t (d) is time, μ_i (d^{-1}) is the growth rate of phytoplankton i, and m_i (d^{-1}) is a mortality rate of phytoplankton i.

To represent the growth of *Crocosphaera* and other phytoplankton, we used simple growth equations based on the sum of Monod kinetics (62) for each nutrient:

$$\mu_i = V_{Max,i}^{NH4} \frac{[NH_4^+]}{[NH_4^+] + K_i^{NH4}} + V_{Max,i}^{NO3} \frac{[NO_3^-]}{[NO_3^-] + K_i^{NO3}} \qquad \text{[eq. 2]}$$

$V_{Max,i}^{NH4}$ and $V_{Max,i}^{NO3}$ (d^{-1}) are the maximum uptake rate of phytoplankton for NH_4^+ and NO_3^- respectively, $[j]$ (nmol L^{-1}) is the concentration of nutrient j (j=NO_3^-, NH_4^+), and K_i^{NH4} and K_i^{NO3}

252 (nmol L^{-1}) are half-saturation constant of nutrient for phytoplankton i respectively. We used
253 the data-fitted quadratic curve of nutrient concentrations (Fig. S5).
254
255 *Simple ecosystem simulation*. To simulate the simple ecosystem situation, we introduced the
256 grazing by zooplankton:

$$\frac{dN_i}{dt} = \mu_i N_i - G_i N_i \quad \text{[eq. 3]}$$

$$\frac{dN_{Zoo}}{dt} = (G_{Cro} + G_{Oth})N_{Zoo} - m_{Zoo}N_{Zoo}^2 \quad \text{[eq. 4]}$$

257 where G_i (d^{-1}) is the grazing rate of phytoplankton *i* by zooplankton, N_{Zoo} (nmol L^{-1}) is the
258 nitrogen concentration in zooplankton per volume water, and M_{Zoo} (d^{-2}) is a quadratic
259 mortality rate of zooplankton. When we allow nitrogen fixation, we used μ_{Cro} = 0.31 (d^{-1}) (a
260 typical growth rate under diazotrophic conditions (63), if the computation based on [eq.2]
261 yields a value below 0.31 (d^{-1}).
262 For G_i we have applied the KTW method:

$$G_i = G_{max}\left(\frac{N_i^2}{N_{Cro}^2 + N_{Oth}^2}\right)\left(\frac{(X_{Cro} + X_{Oth})^2}{(X_{Cro} + X_{Oth})^2 + K_G^2}\right) \quad \text{[eq. 5]}$$

263 where G_{max} (d^{-1}) is the maximum grazing rate and K_G (nmol L^{-1}) is grazing half-saturation.
264 This equation reflects the commonly observed prey-switching behavior of zooplankton (29-
265 31), which stabilizes ecosystems (64, 65).
266
267 **Code availability**
268 The model developed in this paper has been uploaded in GitHub/Zenodo and is freely available
269 at https://zenodo.org/record/5095790 (DOI: 10.5281/zenodo.5095790).
270
271 **Author contributions**

T.Masuda, K.F and S.T designed the *in situ* microcosm experiments, T.Masuda. T.K, T.S, S.K, and T.Matsui carried out the experiment and analyzed data supervised by K.F, S.T and K.S. T.Masuda and K.I shaped the concept of the study with the supervision of O.P. K.I. and G.A. developed and ran the model. T.Masuda and K.I. wrote the original draft with substantial input from all the authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table S1. *In situ* nitrogen fixation rate at 10 m depth and cell density of *Crocosphaera* in the incubation bottle at initial. For all data, means are shown with ± standard deviation for triplicate samples.

Ex.	Date In 2008	*In situ* N_2 fixation rate (nmolN $L^{-1}d^{-1}$)	*In situ* N_2 fixation rate <10μm (nmolN $L^{-1}d^{-1}$)	*Crocosphaera* at initial (cells mL^{-1})
M1	6 June	1.33 ± 1.81	2.75 ± 4.68	32 ± 62
M2	10 June	2.37 ± 0.59	0.66 ± 0.96	270 ± 225
M3	14 June	0.19 ± 2.06	0.28 ± 2.40	126 ± 32
M4	18 June	6.65 ± 2.52	2.37 ± 0.77	1513 ± 684
M5	22 June	4.94 ± 1.38	4.75 ± 1.72	306 ± 112

Table S2 Used symboles, units and definitions in the quantitative model

Symbol	Unit	Definition
i	n.a.	i = Cro, Oth
j	n.a.	$j = NO_3^-, NH_4^+$
N_i	nmol L^{-1}	Cellular nitrogen concentration of phytoplankton i per volume water
t	d	Time
μ_i	d^{-1}	Growth rate of phytoplankton *i*
m_i	d^{-1}	Mortality rate of phytoplankton *i*
$V_{Max,i}^{j}$	d^{-1}	Maximum uptake rate of nutrient *j* by phytoplankton i
$[j]$	nmol L^{-1}	Concentration of nutrient *j*
K_i^j	nmol L^{-1}	Half saturation constant of nutrient *j*
G_i	d^{-1}	Grazing rate of phytoplankton *i*
N_{Zoo}	nmol L^{-1}	Nitrogen concentration in zooplankton per volume water
m_{Zoo}	d^{-2}	Quadratic mortality rate of zooplankton
G_{max}	d^{-1}	Maximum grazing rate
K_G	nmol L^{-1}	Grazing half saturation

Table S3 Values used for parameters

Parameter	Unit	Value
m_i	d^{-1}	0.4
For NH_4^+ added case		
N_{Cro}	nmol L^{-1}	2.50*
N_{Oth}	nmol L^{-1}	5.00*
$V_{Max,Cro}^{NH4}$	d^{-1}	6.6
$V_{Max,Cro}^{NO3}$	d^{-1}	2.8
$V_{Max,Oth}^{NH4}$	d^{-1}	1.1
$V_{Max,Oth}^{NO3}$	d^{-1}	1.8
K_{Cro}^{NH4}	nmol L^{-1}	140
K_{Cro}^{NO3}	nmol L^{-1}	80
K_{Oth}^{NH4}	nmol L^{-1}	6
K_{Oth}^{NO3}	nmol L^{-1}	500
For NO_3^- added case		
N_{Cro}	nmol L^{-1}	4.36*
N_{Oth}	nmol L^{-1}	5.83*s
$V_{Max,Cro}^{NH4}$	d^{-1}	8
$V_{Max,Cro}^{NO3}$	d^{-1}	1.3
$V_{Max,Oth}^{NH4}$	d^{-1}	0.9
$V_{Max,Oth}^{NO3}$	d^{-1}	0.5
K_{Cro}^{NH4}	nmol L^{-1}	70
K_{Cro}^{NO3}	nmol L^{-1}	90
K_{Oth}^{NH4}	nmol L^{-1}	2
K_{Oth}^{NO3}	nmol L^{-1}	700
Ecosystem simulation		
N_{Cro}	nmol L^{-1}	1000*
N_{Oth}	nmol L^{-1}	1000*
N_{Zoo}	nmol L^{-1}	1000*
m_{Zoo}	d^{-2}	0.01[#]
G_{max}	d^{-1}	7.5
K_G	d^{-1}	500[#]

458 * Initial value. [#]Value from Inomura et al (2019).

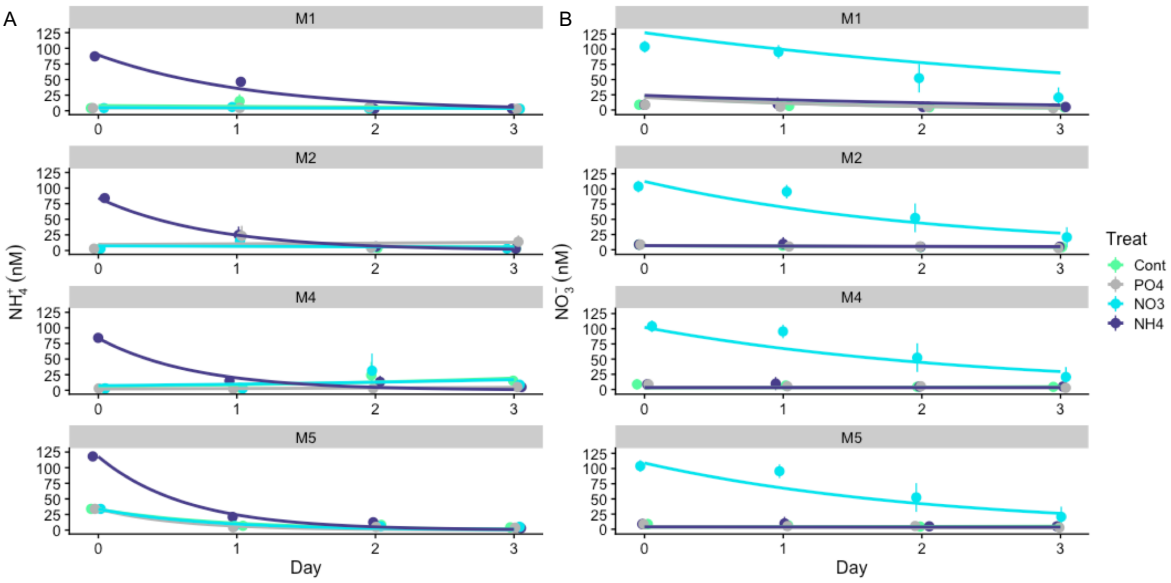

Fig. S1. Temporal change in NH_4^+ and NO_3^- concentrations of Ex. M1, M2, M4 and M5. (A) NH_4^+ concentration in the NH_4^+ treatment exponentially decreased during the experiment down to the detection limit of 6 nM on day 3. (B) NO_3^- concentrations in the NO_3^- treatment exponentially decreased during the experiment but enriched NO_3^- was not always entirely consumed. Error bar shows a standard deviation of triplicate.

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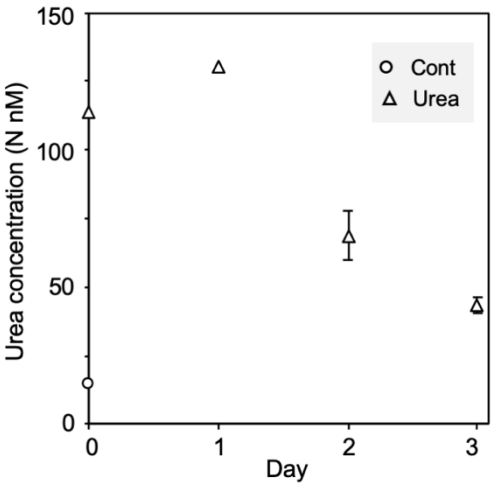

Fig. S2 Temporal change in Urea-N concentration. Concentration in control was measured only at the initial. Error bar shows the standard deviation of triplicate samples.

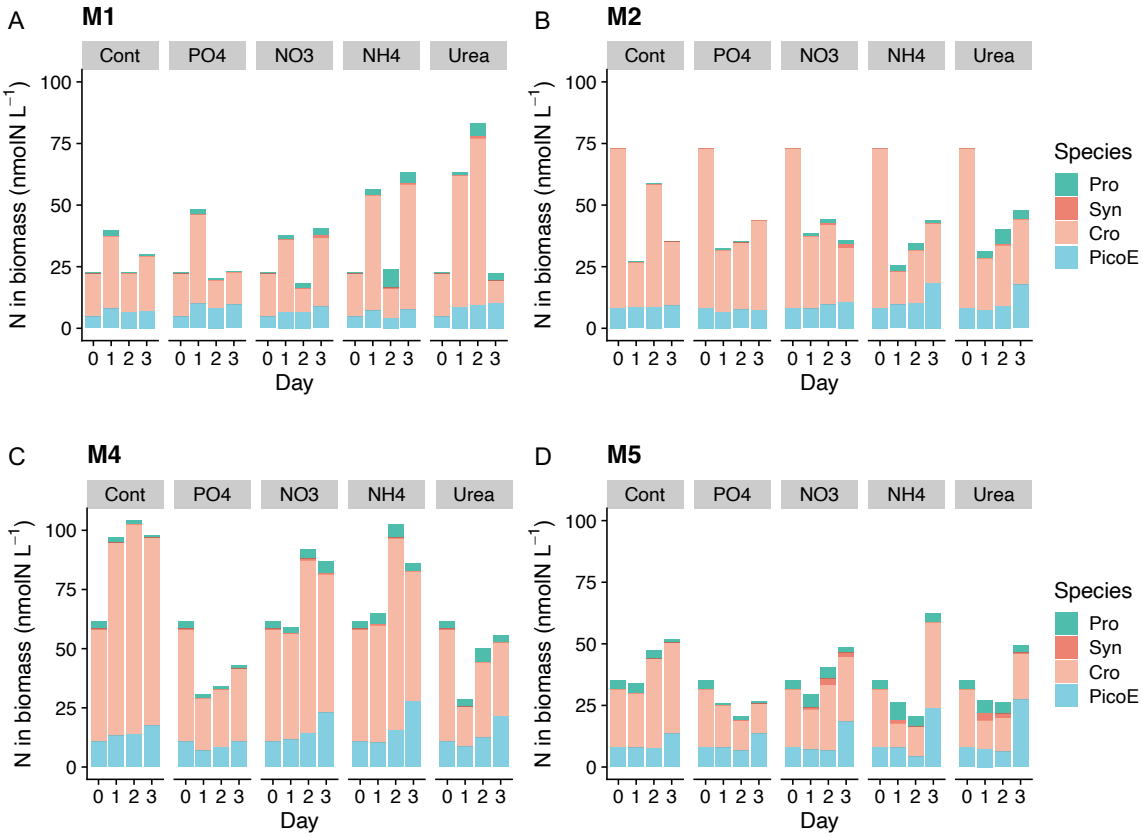

Fig. S3 N in biomass in each treatment and its contribution of each phytoplankton group of experiment M1, M2, M4 and M5. Pro; *Prochlorococcus*, Syn; *Synechococcus,* Cro; *Crocosphaera*, PicoE; pico-eukaryotes.

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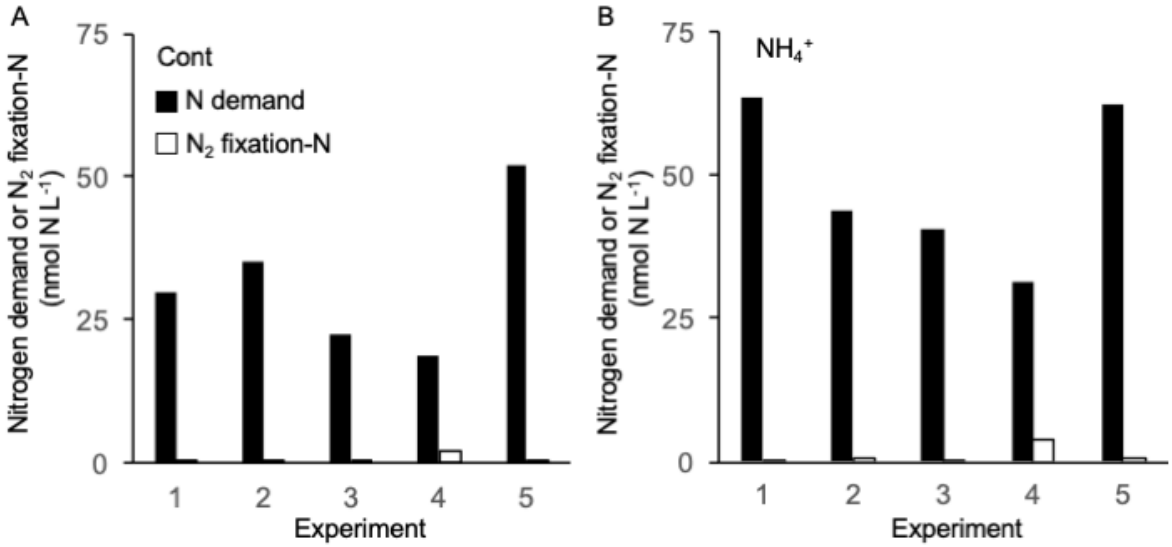

Fig. S4 Nitrogen demand and N derived from N_2 fixation in Control (A) and NH_4^+ treatment (B) for each experiment (M1-M5). Nitrogen demand is N in biomass in 3 days. N_2 fixation rate was estimated from the reported maximum cellular N_2 fixation rate 1.12 fmol N mol cell^{-1} day^{-1} (valued obtained in day 3 in Fe + N treatment of Fe3 (Masuda et al Pre print)) and cell density.

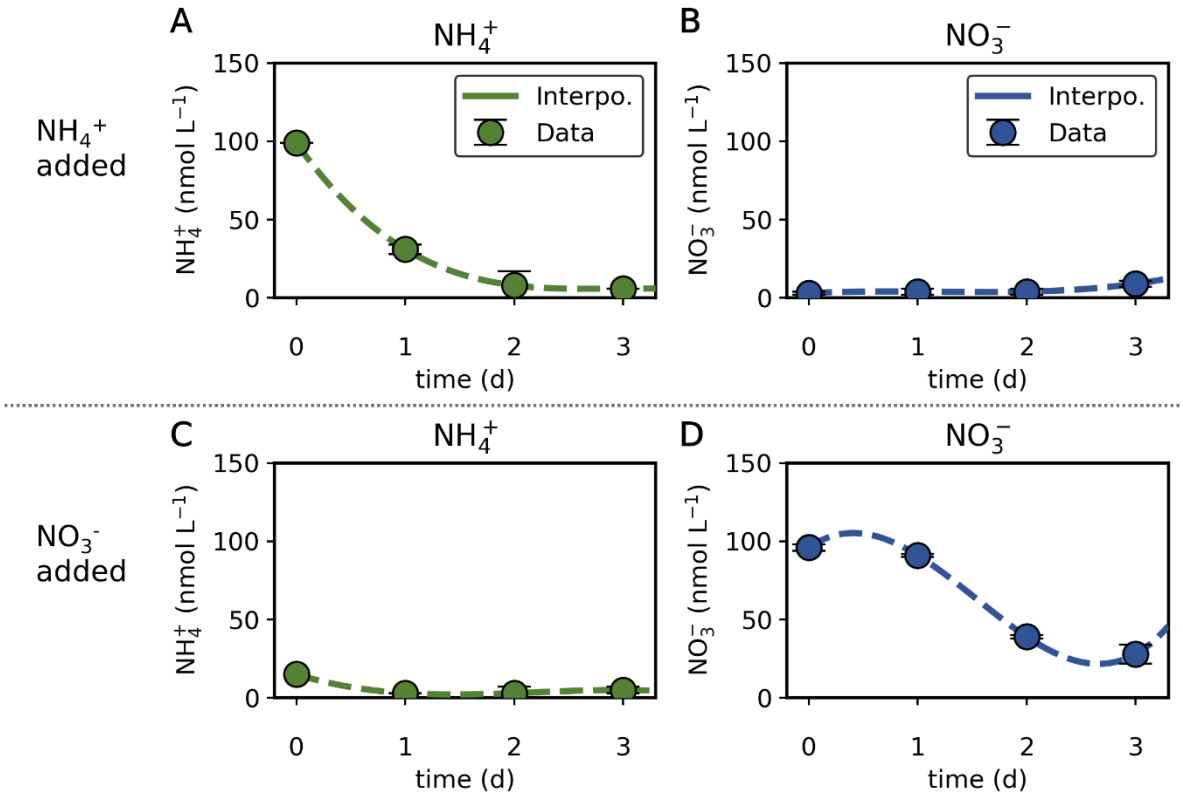

Fig. S5 Measured NH_4^+ and NO_3^- concentrations for NH_4^+ and NO_3^- added cases. Dashed lines show quadratic interpolation. Data are from experiment M3.

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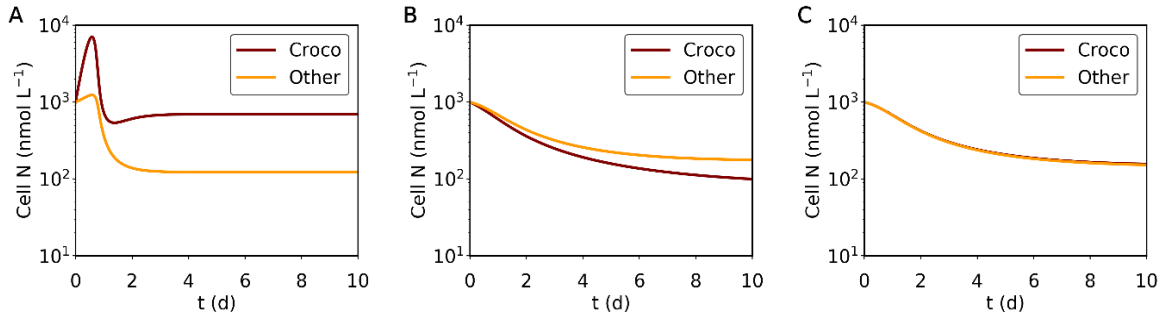

Fig. S6 Simulated transition of cellular N in a simple ecosystem model for three different scenarios. (A) The concentrations for NH_4^+ and NO_3^- are both 100 nmol L^{-1}. (B)(C) The concentrations for NH_4^+ and NO_3^- are both 1 nmol L^{-1}. In only (C) *Crocosphaera* may acquire N via N_2 fixation. Croco: *Crocosphaera*. Other: other phytoplankton. Parameters are based on NO_3^- added case.

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