

BEACH TEAM REPORT
August 2023
NIO-Buoyant Flake Field Trials at Station ALOHA

Background

The domestication and cultivation of rice as a global staple crop had its origin in China ~10,000 years ago (Normile 1997). More recent advances in agricultural practices and genetic engineering of rice cultivars during and following the 20th century Green Revolution has led to global food security and literally saved humankind from famine. Is it possible that the major by-product of rice cultivation, rice husks, can be used to save our planet from global warming?

In a pioneering paper on the impacts of silicate (Si) fertilizer-facilitated dissolved inorganic nitrogen (N) removal from the polluted waters of Jiaozhou Bay, PRC, Lü et al. (2020) investigated the impacts of rice husk ash (RHA) addition on marine phytoplankton metabolism and nutrient dynamics. They first experimented with different RHA supplements that were prepared from raw, field-collected rice husks as the starting material. The husks were washed, then boiled (both in distilled water) before decarbonization at a range of temperatures (400-800°C). This process produced a Si-rich ash that also contained minimal amounts of phosphorus (Si:P molar ratios ~50:1), but no measurable N. When RHA was added to filtered (sand-leached) seawater (2.5 g of 400°C RHA per 5 L seawater) and incubated in the dark, dissolved reactive (and presumably bioavailable) Si and P were released, and over a 10-day period accumulated to concentrations of ~25 µM and ~0.8 µM above their initial values (Lü et al. 2020). When the same amount of RHA was added to Si-limited, fixed N-enriched Jiaozhou Bay water, there was a rapid removal of Si, P and N, and over time an accumulation of diatoms over dinoflagellates (Lü et al. 2020). The authors concluded that RHA fertilization appeared to be an effective means to improve water quality, reduce the proliferation of toxic dinoflagellates and, possibly, benefit higher trophic levels that rely on diatoms as a food source.

More recently, Shetye et al. (2023) extended the novel concept of Si-fertilization using rice husk-based technology to the open sea, specifically the Si-limited region of the northern Indian Ocean and Arabian Sea. In this pioneering study, a series of laboratory and field experiments were conducted using whole, dried rice husks (not RHA as used by Lü et al. 2020) which remained positively buoyant for an extended period of time when added to seawater. Based on

an elemental analysis using electron dispersive spectroscopy, the rice husks were reported to be primarily SiO₂ with trace amounts of rubidium, chloride and aluminum. When added to seawater samples and incubated in the dark, the rice husks released dissolved Si over time with net accumulations scaling proportionately to the number of rice husks per L of seawater (~12 μM Si per rice husk over a 15-day period; Shetye et al. 2023). No measurable nitrate, nitrite or P was released from the rice husks. When rice husks were added to Si-limited seawater samples and incubated in the light, diatom biomass increased significantly (~10-fold relative to control samples). In discussing the ecological implications of this research, the authors hypothesized that fertilization of Si-limited ocean regions with rice husks could lead to the formation of heavily-silicified diatoms that are more difficult to graze and more likely to be exported to deep waters. This would facilitate an enhanced carbon sequestration via the biological carbon pump and, perhaps, an antidote to global warming (Shetye et al. 2023). They went on to suggest that rice husks produced in countries like India could be dispersed over the open ocean during Si-depleted periods of the year (e.g., during austral summer in the Southern Ocean or during winter in the Arabian Sea) as a countermeasure to greenhouse gas-induced warming of our planet. So, in answer to the rhetorical question raised in paragraph one above, perhaps rice husks can be used to save our planet!

Marine Biomass Regeneration

Several years ago, Sir David King assembled a group of like-minded scholars who were interested in exploring any and all means to improve the health of our planet, with a focus on the open ocean. Through a series of high-level summits, the Marine Biomass Regeneration project emerged with an initial focus on the possible use of buoyant flakes (a product prepared from rice husks) as a fertilizer to stimulate the biological carbon pump and enhance the sequestration of atmospheric carbon dioxide. A proposal is currently pending with the Grantham Foundation.

During the development of our research prospectus, a team of scientists from the National Institute of Oceanography (NIO) in Goa, India (led by Damodar Shenoy and Suhas Suresh) conducted an initial field test of a “brand new bran” as an extension of their previous work using rice husks as Si-fertilizer. To date, neither their novel recipe nor their preliminary field results have been published (to our knowledge). We were fortunate to obtain a bag of new bran from Professor Shenoy to conduct preliminary field experiments at Station ALOHA in the North

Pacific Subtropical Gyre (NPSG) where average, year-round dissolved Si concentrations in the euphotic zone are ~1-2 μM , decreasing to ~0.5 μM during phytoplankton blooms in late summer. According to Damodar Shenoy (email to D. Karl dated 27 June 2023), the novel buoyant flakes are produced from two major local waste streams, rice cultivation and iron ore mining. The preparation is as follows (D. Shenoy, personnel communication).

“The iron ore is first sieved through a 63- μm mesh to obtain a fine powder. The whole rice husk (with seed removed) is used as such. Presently we are using commercial lignin (from Sigma-Aldrich). The lignin is dissolved in water and heated to get the right stickiness (N.B. D. Karl is unsure how stickiness is measured or judged... but he can only imagine). The rice husk is dipped in the hot molten lignin and coated with fine iron ore powder. Then it is dried in the oven at 100 °C. So far, we have made around 5 batches of the buoyant flake, wherein we have kept the same procedure for making the flake so that the results can be compared. The ratio, by weight percentage, is: rice husk (60%), lignin (15%) and iron ore (25%).”

The resultant product is hereafter referred to as NIO-buoyant flake (N-BF) to distinguish it from the previously used RHA (Lü et al. 2020) or whole rice husks (Shetye et al. 2023).

Hawaii Laboratory and Field Experiments

Upon receipt of the N-BF by post from Goa, the BEACH team (Biogeochemical and Ecological Analysis of Complex Habitats; comprised of Karin Björkman, Rhea Foreman, Cathy Garcia, Eric Grabowski, Dave Karl and Payton Schwengel) met to develop a prioritized list of measurements and experiments that we might undertake in a limited period of 2-3 months, and without any dedicated funding. Although the list grew very quickly because we were working from a base knowledge of the composition and expected in situ impact on the N-BF on the NPSG ecosystem, we eventually settled on two major topics: laboratory analyses of N-BF composition and field assessment of impacts of N-BF fertilization on nutrient dynamics, microbial community metabolism and biodiversity. We were also keen to test the working hypothesis (D. Shenoy et al., MBR summit) that the Si- and Fe-enrichments resulting from N-BF fertilization of oligotrophic seawaters would select for nitrogen (N_2)-fixing cyanobacteria (also termed diazotrophs) that would provide excess fixed N (in the form of ammonium) and thereby serve to enhance the efficiency of the biological carbon pump. While there are no known N_2 -fixing diatoms (N_2 fixation is found only in *Bacteria* and *Archaea*) there are several well-studied

symbiotic associations between diatoms and N₂-fixing cyanobacteria that often bloom in fixed N-depleted oligotrophic oceans when there is a sufficient supply of Si, Fe and P. These “diazotrophic diatoms” are known to be a keystone species at Station ALOHA, though there are still major knowledge gaps regarding their biology and ecology, as well as the biogeochemical consequences of the N₂-fixation fueled biological pump.

This report will be divided into two sections: (I) laboratory analyses of the N-BFs and (II) results from field experiments that were conducted on cruises H-341 (March 2023) and H-342 (May 2023) of the Hawaii Ocean Time-series (HOT) program at Station ALOHA (22°45’N, 158°W). For both field expeditions, incubations were initiated at sea and then returned to our shore-based laboratories at C-MORE Hale on the University of Hawai‘i at Mānoa campus to continue the long-term experiments. These extended incubations (up to 45 d) turned out to be critical for the succession of key diatom species and for the selection of N₂-fixing microorganisms, as discussed below.

I. NIO-Buoyant Flake Characterization

When presented with our bag of new bran from Goa, we were at first unsure about how to sample the flakes or to prepare them for field experimentation. We used the Shetye et al. (2023) paper as a guide to the ideal rice husk density (one per L of seawater), but since the N-BF composition was likely to be quite different from the rice husks used previously, this needed to be independently evaluated. We also had no information on N-BF to N-BF variations in mass or the uniformity of the bulk elemental composition. Fortunately, individual N-BFs were large enough to weigh and analyze for total carbon (C), nitrogen (N) and hydrogen (H), so we set out to explore these physical-chemical characteristics.

Experiment #1 Procedure: (1) Randomly select 20 N-BFs. Weigh, dry for 20 hr at 60°C, weigh again.

Results – see Figure 1: The mean (and standard deviation) mass = 8.32 mg (\pm 1.47 mg), mean (and standard deviation) dry mass = 8.01 mg (\pm 1.45 mg). This equates to a water content of 3.73%, assuming that the loss of weight upon heating at 60°C for 20 hr was due to dehydration (rather than, for example, loss of some other volatile component).

Notes and Comments: (1) The ~18% variation in mass/dry mass will add some uncertainty to field experiments using randomly selected N-BFs, especially when used as single flake additions.

Individual N-BFs or pooled samples could be weighed before use in field experiments if greater accuracy is needed (we did not do this – because it represents added time and effort to our already compressed research schedule). (2) Small amounts of brown dust (iron ore) fell off of the N-BFs when picked up with a clean forceps. (3) We attempted to crush/pulverize the N-BF to obtain a bran powder for selected experiments, but this proved to be a “fool’s errand,” at least using a standard laboratory mortar-pestle. Perhaps a good coffee grinder or grain mill would work better. In the end, we concluded that the buoyancy of the flakes was one of the major “selling points” of the N-BF product and saw no reason why we should attempt to pulverize them ahead of use in the lab or field.

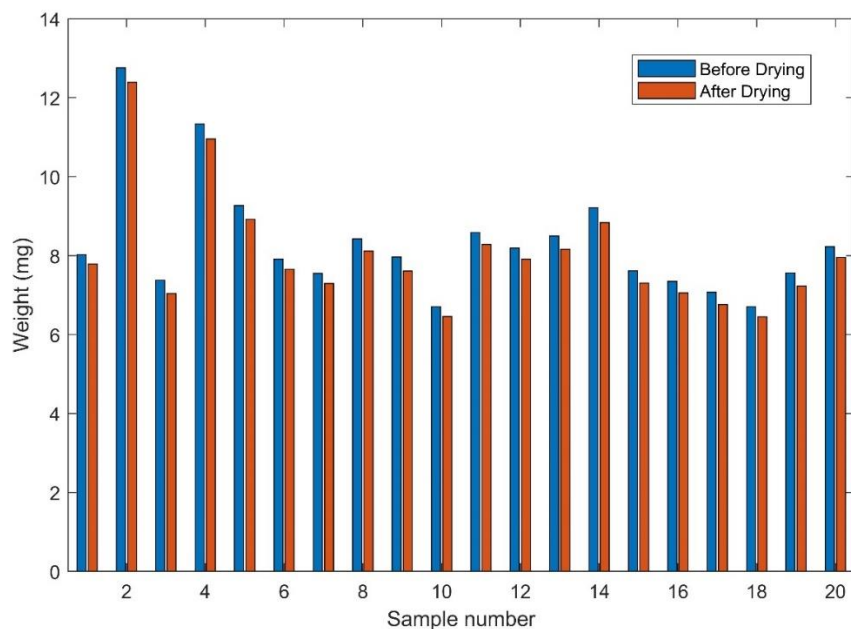


Figure 1. Mass estimates of 20 randomly selected, whole NIO-buoyant flakes before and after drying at 60°C. The mean mass of a single flake is 8.32 ± 1.47 mg before drying and 8.01 ± 1.45 mg after drying. Mean water weight is 3.73% of undried material.

Experiment #2 Procedure: (1) Select 7 N-BFs (at random, as above) for C, N and H analyses. Also, use a pooled sample of ground N-BF (N.B. see comment above on difficulties with preparation of powdered samples) to compare to individual N-BF measurements. All samples were dried at 60°C for 20 hr and analyzed using an Exeter Analytical Elemental Analyzer at a combustion temperature of 1020°C. Acetanilide was used as a standard and the HOT program plankton sample was used as a quality control reference material. Since these C values represent the sum of organic plus inorganic (carbonate) C, we express the values as total C rather than

organic C. However, we have no reason to believe, based on our work or on the previous analyses reported in Shetye et al. (2023), that the rice husks or N-BFs contain significant amounts of carbonate.

Results – see Figure 2 and Table 1: (1) The pooled (crushed/powdered) and whole N-BFs had similar ranges of C, N and H (expressed as % of total mass). (2) The C:N molar ratios were very high (80-100 mol mol⁻¹) compared to average plankton (C:N ~6-7 mol mol⁻¹). (3) The C:H ratios were ~0.6 mol mol⁻¹. If we assume that the average chemical composition of lignin is C₉H_{10.2}O_{3.4} (no N, so C:N of infinity; C:H = 0.88), then the small relative proportion of N contained in the N-BFs and a portion of the total H must derive from the raw rice husk itself or from the iron ore amendment. (4) We also measured the phosphorus (P) content of the buoyant flakes and found it to be negligible (0.01% by weight), but not zero. The ¹³C- and ¹⁵N-isotopic abundances shown in Table 1 were determined on a whole flake and flake powder, respectively.

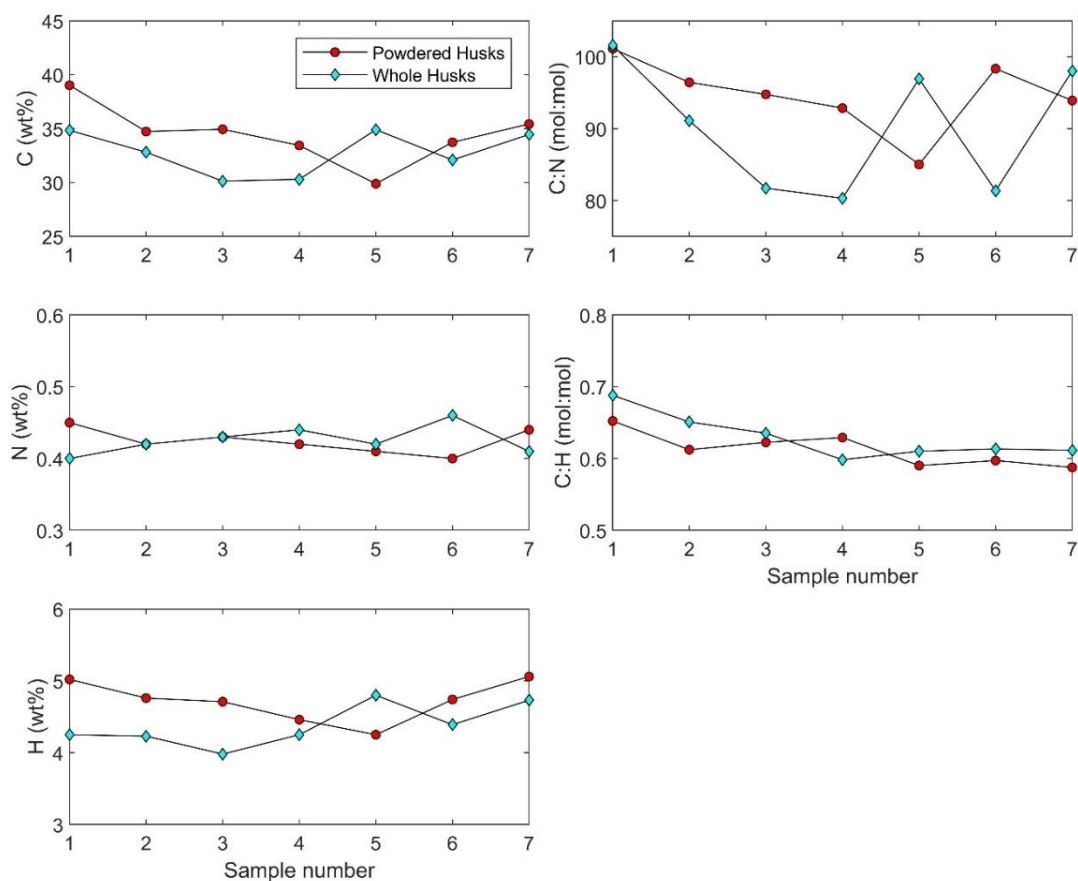


Figure 2. C, N and H contents of 7 whole, randomly selected NIO-buoyant flakes compared to replicate analyses of a pulverized (powdered) sample (expressed as weight percentages), along with the corresponding molar C:N and C:H ratios (also see Table 1 for summaries).

Table 1. Average composition of NIO-buoyant flakes, shown as mean and standard deviation of all powdered and whole flake analyses presented in Figure 2. The P abundance was determined from powdered flake only.

	C (wt %)	N (wt %)	H (wt %)	P (wt %)	$\delta^{13}\text{C}$ (‰ vs. VPDB)	$\delta^{15}\text{N}$ (‰ vs. AIR)
Mean	33.62	0.43	4.55	0.01	-30.4	9.4
1 σ	2.47	0.02	0.33	0.00	n.d.	1.6

II. NIO-Buoyant Flakes at Sea

A series of incubation experiments were conducted at Station ALOHA to evaluate the impacts of N-BF additions on natural microbial community metabolism and nutrient dynamics. Some experiments were completed at sea while others were returned to our shore-based laboratories to provide an analysis of longer-term impacts of N-BF amendments. Some preliminary experiments or measurements obtained on HOT-341 were repeated/refined on HOT-342, while others were conducted on only one of the two field expeditions. Both cruises were part of the long-term HOT project, so there are many ancillary physical, chemical and biogeochemical measurements to help define the environmental conditions during March and May 2023 periods at Station ALOHA. If we are eventually funded by the Grantham Foundation, we will be able to participate in future HOT program cruises on a space available basis (DMK is the founding PI of the HOT project in 1988 and currently serves as the co-PI of a recently funded, 5-year continuation project through the U.S. National Science Foundation). Below is a general description of the field experiments conducted, followed by data summaries, observations and some preliminary interpretations/conclusions.

HOT-341: In the first experiment, surface seawater was collected into a 10-L polycarbonate carboy and 10 N-BFs were added. Subsamples were withdrawn over the first 3-day period for nutrient (nitrate, phosphate, silicate) determinations and measurements of primary production using ^{14}C -bicarbonate as a tracer. Following the cruise, the carboy was returned to shore and incubated in a temperature- and light-controlled room for an additional 42 days and sampled for nutrients, light and epifluorescence microscopy, as well as for rates of N_2 fixation (acetylene reduction [AR] method). When acetylene is added to samples containing actively metabolizing N_2 -fixing microorganisms, the enzyme nitrogenase reduces acetylene gas to ethylene gas, which is then measured using a reduced gas analyzer (Wilson et al. 2012). The AR assay is much more

sensitive than the $^{15}\text{N}_2$ assay though it does require independent information on the ethylene produced: N_2 fixed ratio to extrapolate the measured values to rates of N_2 fixation (see discussion in Wilson et al. 2012). For this report, we present our results as nmol ethylene produced $\text{L}^{-1} \text{hr}^{-1}$ which we believe is a useful relative indicator for N_2 fixation. It should be noted that our AR incubations were short (a few hours) so they do not resolve the diel variability that is known to occur within most diazotrophs. Consequently, our N_2 fixation estimates are probably lower bounds on the actual rates since they would be biased against nighttime fixation. Prior to each sampling period, the carboy was gently mixed with care taken not to disrupt/disaggregate the N-BFs, but some material may have been inadvertently included in the sample. Since these were the first experiments conducted, we took extensive notes and made qualitative assessments of the state of the N-BFs especially for the long-term incubation experiments, and later discussed several possible experimental design improvements. For example, it may be possible to place the N-BFs into a permeable membrane-bound container to exclude any N-BF fragments from contaminating the collected sample while still allowing solute exchanges to occur. This will be especially critical for any future measurements of dissolved and particulate Fe/trace metals which would otherwise be subjected to potential contamination by the iron ore coatings. This “caged” N-BF procedure might also reduce the inevitable biofouling and decomposition of the N-BFs over time, and it would allow for the removal of the N-BFs as a treatment or for the additional of “fresh” N-BFs during an incubation experiment. We need to think more about this, especially if we receive funding to move on to the PERICosm stage of research. The typical volume that was removed during each sampling period was $\sim 0.5 \text{ L}$, so the ratio of N-BF mass-to-seawater volume progressively increased over the duration of the long-term experiment. At the end of the experiment, the remaining volume ($\sim 6\text{-}7 \text{ L}$) was used to measure the concentrations of accumulated suspended particulate C, N, H, P and Si, and to measure the bulk C- and N-isotopic composition of the suspended particulate matter that was produced during the entire incubation period. It is important to note that this is the net yield, not the gross productivity, since particulate matter remineralization is occurring simultaneously. During this final sampling, care was taken to exclude the remaining particulate mass of the buoyant flakes, a portion of which had become negatively buoyant during the extended incubation period. In a separate experiment, we tracked changes in dissolved oxygen (O_2) concentrations in samples amended with 2 rice husks per 125 mL bottle, relative to unamended controls. Replicate samples (triplicate) were

terminated following 24-, 48- and 72-hr of incubation under natural day-night conditions. Most, but not all, of the samples that were collected during these preliminary experiments have been analyzed, and are reported below.

HOT-342: During the second expedition, we repeated the ^{14}C -based primary production and O_2 -based respiration experiments. The latter were expanded to evaluate a dose-response experiment with 7 treatments (0 to 6 rice husks per bottle), in triplicate. Finally, we also evaluated the effect of a phosphate addition (100 nM) on O_2 dynamics. These results are presented and discussed below along with nutrient dynamics and N_2 fixation rates as separate topics with a full understanding that these are inextricably linked impacts of N-BF fertilization of Station ALOHA microbial assemblages.

Results: Microbial community respiration and ^{14}C -based primary production

On H-341 we conducted preliminary experiments of O_2 dynamics using the traditional Winkler technique. The H-341 results documented a net O_2 consumption in N-BF amended bottles (125 mL seawater with 2 N-BFs added per sample), relative to control samples (Figure 3). The net O_2 consumption rates were $\sim 12 \mu\text{mol O}_2 \text{ L}^{-1} \text{ d}^{-1}$ over the initial 3-day period following N-BF fertilization. If these rates continued unabated, the bottles would become anoxic in a period of ~ 19 days. However, it seems unlikely that this would happen because of a simultaneous stimulation of photosynthesis (see below). Nevertheless, the short-term (~ 1 day) effect of N-BF fertilization was to shift the euphotic zone assemblage to a transient net heterotrophic system relative to the unamended 3-day control samples (Figure 3). We interpret this enhancement of O_2 consumption to be the result of either increased bacterial respiration or an inhibition of photosynthesis, or both. We suspect the former since rates of photosynthesis in the control samples (see below) are well below the observed enhancement of O_2 consumption in the N-BF amended samples. Assuming a respiratory quotient (RQ) of 1.0 (1 mol organic C oxidized per 1 mol of O_2 consumed; N.B. RQ at Station ALOHA is unknown and would vary dependent on the oxidation state of the organic matter consumed) the consumption of $12 \mu\text{mol O}_2 \text{ L}^{-1} \text{ d}^{-1}$ would require at least $12 \mu\text{mol organic C}$. We know that the addition of 2 N-BFs per 125 mL sample would add a maximum of 5.6 mg C (8 mg mass per flake x 2 flakes per bottle = 16 mg mass x 34% C [by weight] = 5.4 mg C) or $\sim 450 \mu\text{mol C}$. The C contained in the N-BF product includes hemicellulose, cellulose and lignin (reported to be 75-90% of the total

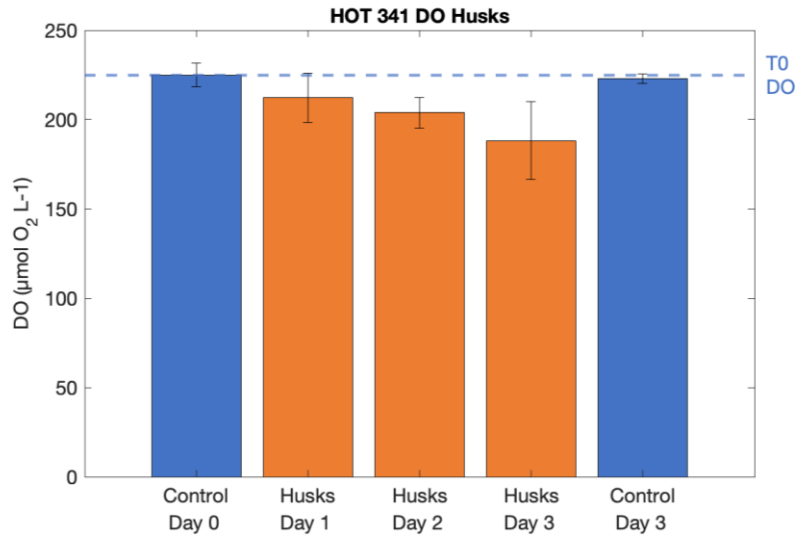


Figure 3. Dissolved oxygen dynamics for Station ALOHA surface seawater collected on H-341 and incubated with 2 N-BFs per 125 mL sample. The data shown are O₂ concentrations (in triplicate; mean±standard deviation) at time 0, and on three successive days of incubation under natural light-dark conditions.

mass of rice husks; Bakar et al. 2016) plus the added lignin used to coat the N-BFs during preparation (15% by weight of N-BF preparations; D. Shenoy, personal communication). Other, unknown, organics may also be present in the iron ore. By comparison, Station ALOHA surface seawater contains approximately 80 μM dissolved organic carbon (DOC) which is mostly uncharacterized. Our current interpretation is that N-BF fertilization adds bioavailable C leading to a transient enhancement of microbial respiration. Because the observed enhancement in respiration was similar each day over the 3-day observation period, we conclude that the additional of oxidizable C must be supplied continuously from the N-BFs in a manner that is similar to the release of Si (see below). Alternatively, the addition of N-BFs may facilitate or enhance the utilization of natural DOC, perhaps via Fe stimulation of bacterial metabolism. Since the N-BFs remained nearly if not entirely intact (by visual inspection) during these short-term respiration experiments, it seems unlikely that solubilization of rice husk-derived organic matter (mostly fiber) was the source of bioavailable DOC. More likely, a portion of the lignin coating or an iron ore-induced priming effect (stimulation of the metabolism of allochthonous DOC) was the cause of the short-term enhancement in heterotroph metabolism. Regardless of the mechanisms, these preliminary data from H-341 clearly documented an enhancement of O₂

consumption relative to controls, so this phenomenon was explored in more detail during the H-342 expedition.

Oxygen dynamics experiments conducted on H-342 confirmed the respiration enhancement following the addition of N-BFs (Figure 4). For this 4-day experiment, samples were exposed to either natural light/dark conditions or were incubated in total darkness. The dark respiration rates over the 4-day period averaged $\sim 8 \mu\text{mol O}_2 \text{ L}^{-1} \text{ d}^{-1}$ a value that is less than we observed during the H-341 experiment (that was conducted under natural light/dark conditions), but still quite large relative to the unamended controls. Furthermore, the net loss of O_2 with added N-BFs in the natural light/dark treatment showed a different pattern of respiration with a relatively large burst of $\sim 17 \mu\text{mol O}_2 \text{ L}^{-1}$ over the initial 24-hr period, followed by a variable net loss over the next 3-day incubation period. This latter pattern is likely a result of the balance between

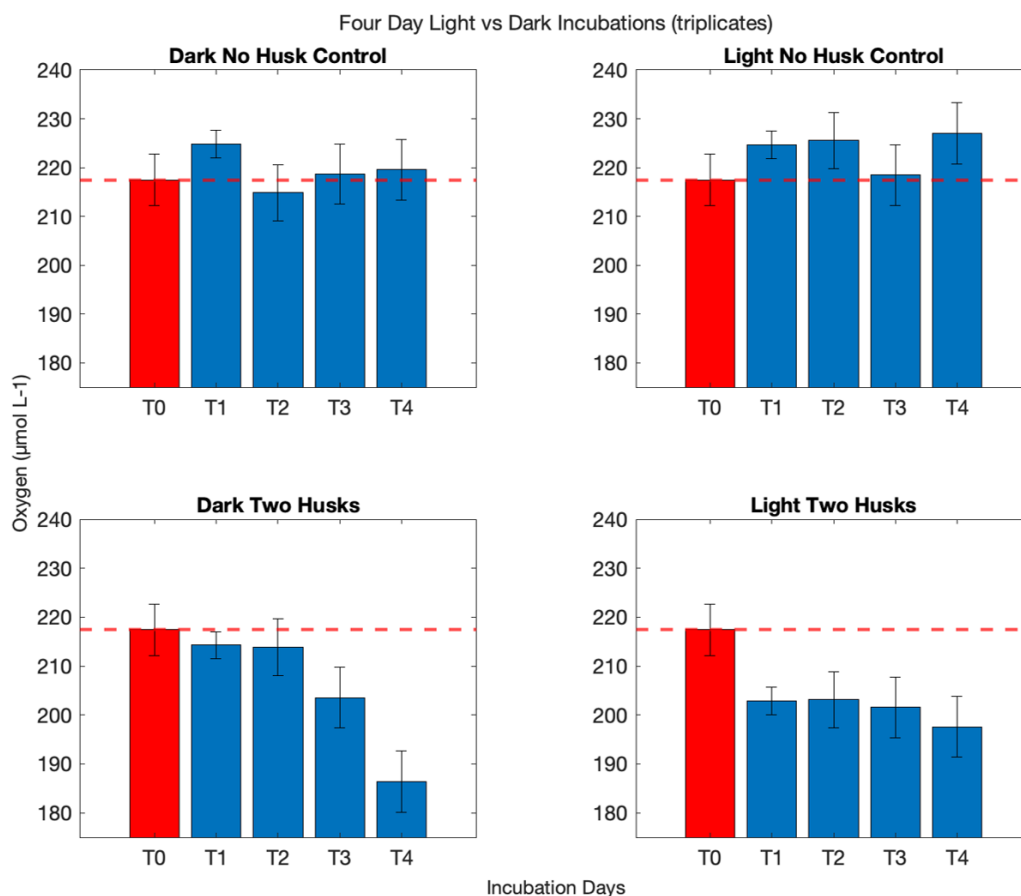


Figure 4. Dissolved oxygen dynamics for Station ALOHA surface seawater collected on H-342 and incubated with 2 N-BFs per 125 mL sample. Shown is a time-course of O_2 concentrations in N-BF amended and control samples incubated under natural light-dark conditions or in total darkness over a 4-day period.

consumption (respiration) and production (photosynthesis), since our experimental design tracked net changes over time. Still the required amount of photosynthesis to counteract the large O₂ consumption is unreasonably large. Alternatively, it is possible that a portion of the enhanced O₂ consumption could be due to “chemical oxygen demand” rather than microbial respiration. This would occur if some component of the N-BF product reacted with dissolved O₂. In order to evaluate this in future experiments, we plan to follow the kinetics (on time scales of minutes to hours) of O₂ consumption following N-BF amendments, and conduct proper low-temperature and poisoned controls to separate the chemical and biological oxygen demands.

On H-342 we also examined the impact of N-BFs by varying the flake # per unit volume from 0-6 flakes per 125 mL bottle (Figure 5). This experiment showed a variable effect after a short-term (1 day) incubation, with negligible (but variable) impact beyond the “2 flake per bottle” treatment. Our preliminary interpretation of this result is that the enhancement (possible addition of Fe to stimulate bacterial respiration) is saturated at 2 flakes per 125 mL and that added Fe beyond that threshold concentration is of little consequence in these short-term

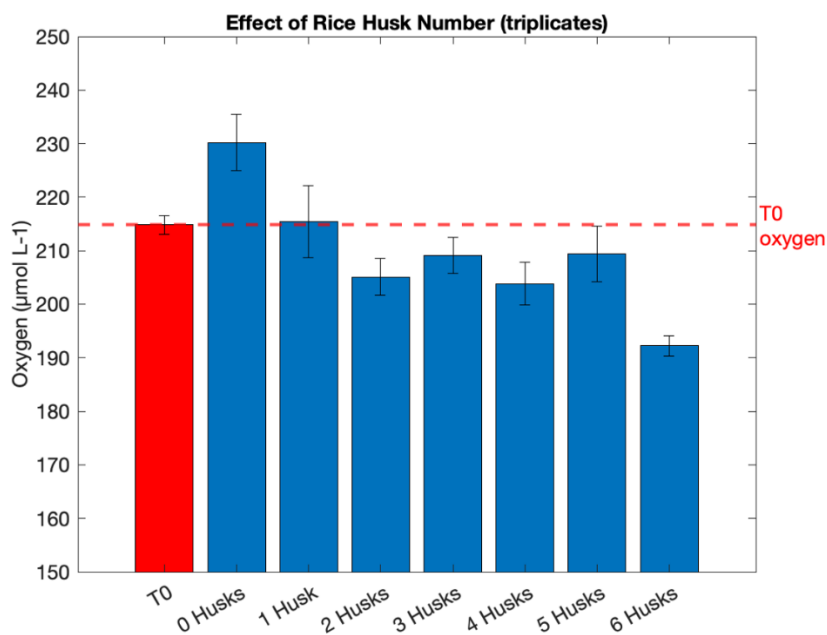


Figure 5. Enhancement of microbial respiration as a function of number of N-BFs (from 0 to 6) added to a 125 mL Station ALOHA surface seawater sample collected on H-342. All samples were incubated for a 1-day natural light-dark period.

exposure studies. The 6 N-BF per 125 mL seawater sample, which would equate to an astronomical 48 flakes L⁻¹, showed an anomalously high net O₂ consumption (>20 μmol O₂ L⁻¹; Figure 5), which may be partly a result of a chemical demand, discussed above.

On H-341, a preliminary shipboard experiment was also conducted to measure the impact of N-BF fertilization on photosynthesis, as measured using ¹⁴C-bicarbonate assimilation in subsamples removed from the 10-L carboy (1 N-BF per L). Primary production approximately doubled in the first day and remained elevated into the second day (this preliminary experiment lasted just 2 days; Figure 6). This short-term stimulation, ~7-10 μg C L⁻¹ d⁻¹, would be expected to produce only ~1 μmol O₂ L⁻¹ d⁻¹ which is not nearly enough to counter the enhanced heterotrophic O₂ consumption discussed above (~10 μmol O₂ L⁻¹ d⁻¹). The impact of N-BF fertilization of photosynthesis was confirmed with a more comprehensive shipboard experiment on the H-342 expedition (Figure 6).

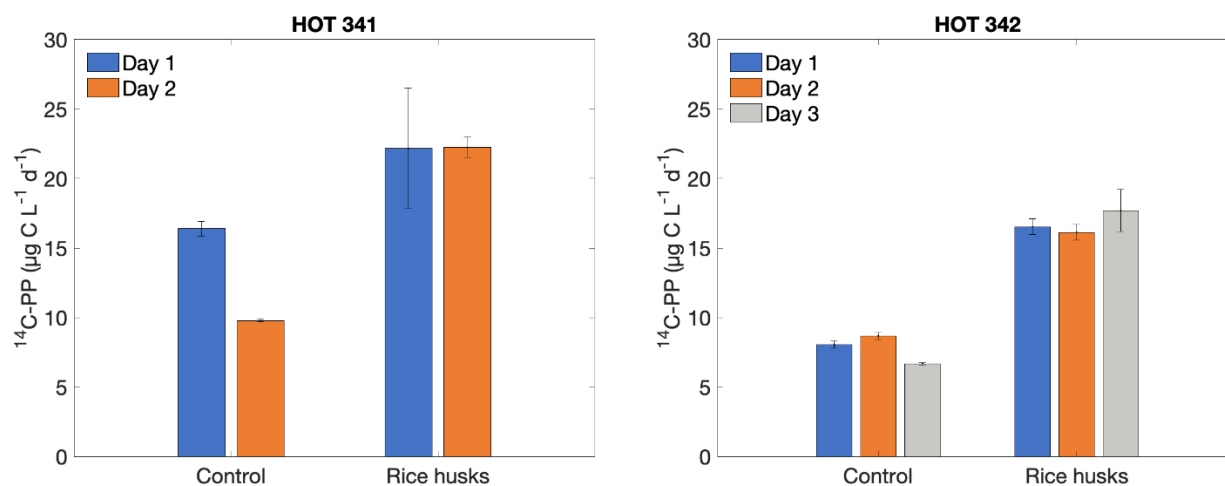


Figure 6. Impact of N-BF addition on ¹⁴C-based primary production over a 2-day (H-341, left) or a 3-day (H-342, right) period.

Results: Nutrient dynamics

As expected, based on the previous research conducted by Lü e al. (2020) and Shetye et al. (2023) using RHA and whole rice husks respectively, the N-BFs reproducibly released dissolved reactive Si over time on both H-341 and H-342 expeditions (Figure 7). For a one N-BF L⁻¹ addition, Si accumulated at an approximately constant rate over the first 2 weeks, then began to level off at a concentration of ~15 μM (in both experiments) after an ~1-month-long incubation. It is important to emphasize that these are net concentrations in live (light-dark) incubations

where both Si uptake and Si remineralization from particulate matter are occurring simultaneously. The “saturation” point of $\sim 15 \mu\text{M}$ Si achieved after a month-long incubation with N-BFs is about an order of magnitude greater than the dissolved Si concentrations in surface seawater at Station ALOHA, and may represent the N-BF amended altered steady-state condition with respect to dissolved Si dynamics. This value is well below the thermodynamic saturation state of amorphous Si so it is probably set by diatom biomass and metabolism rather than N-BF SiO_2 solubility/dissolution. The decline observed for the H-341 incubation at 40-45 d may be the result of the rapid net growth of diazotrophic diatoms (see below), though we have no data (yet) on diatom biomass, species diversity or growth rates.

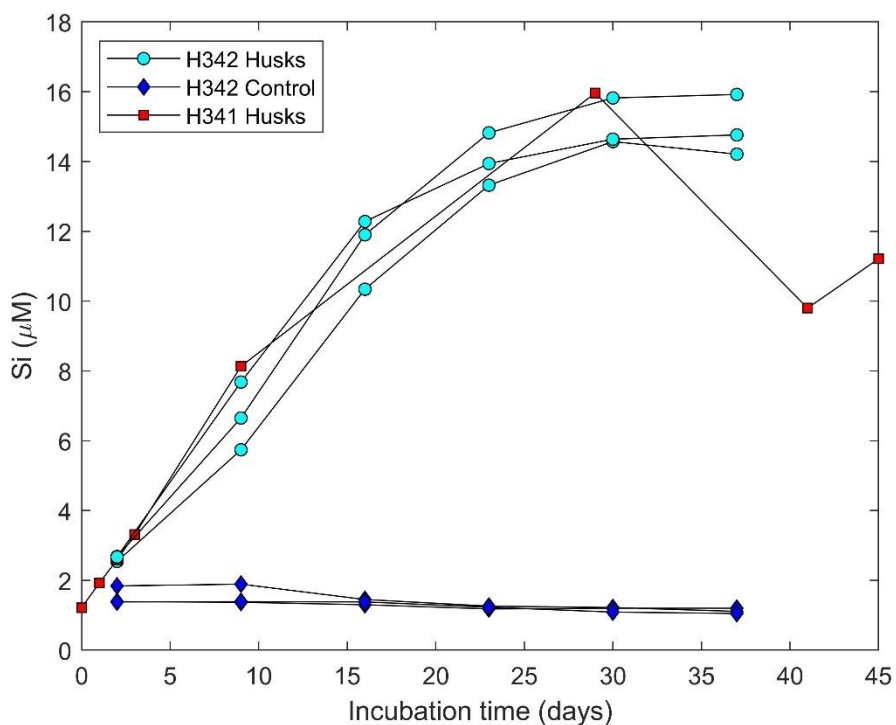


Figure 7. Time-dependent release of dissolved Si in long-term incubation experiments conducted on H-341 and H-342 expeditions. The H-342 experiment also included a long-term control, which showed the expected long-term decrease in Si.

In comparison to the dissolved Si trends, phosphate concentrations generally decreased over extended incubation periods, and there was little to no impact from the added N-BFs (Figure 8). The differences observed for Station ALOHA surface seawater (time zero in Figure 8) between the two expeditions ($\sim 80 \text{ nM}$ for H-341 versus $\sim 160 \text{ nM}$ for H-342) are well within the expected range and month-to-month variability for the NPSG.

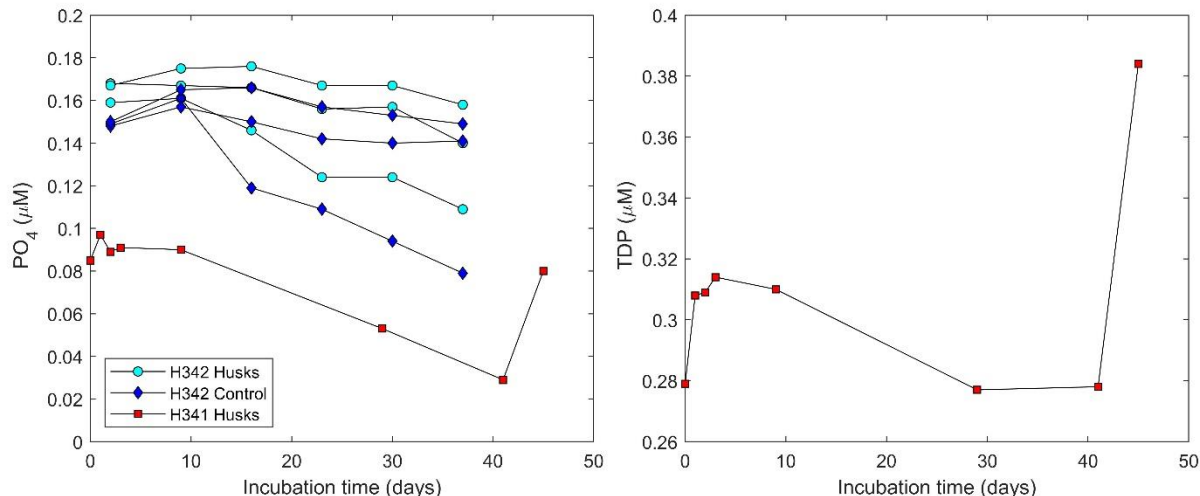


Figure 8. Time dependent release of dissolved phosphate (left panel) and total dissolved phosphorus (TDP, right panel) in long-term incubation experiments conducted on H-341 and H-342 expeditions. The HOT-342 experiment also included a long-term control (no flakes added, blue diamonds).

Variations in the total dissolved P pool (TDP; a measure of dissolved inorganic plus dissolved organic P) were largely controlled by the drawdown of inorganic phosphate over the first month in the H-341 incubation, except for a 29 nM increase in TDP in the first day (Figure 8; no TDP data are available for H-342). Dynamics shifted when the 41-day sampling showed the lowest phosphate concentration measured for the entire experiment (~40 nM; Figure 8), but TDP remained nearly unchanged from day 29, indicating an increase in the concentration of dissolved organic P (DOP) compounds (DOP = TDP – phosphate). From day 29 to day 45, DOP showed a dramatic increase of 80 nM (not plotted), and from day 41 to 45, phosphate also increased by 51 nM (presumably due to remineralization of unknown DOP compounds). Some of this DOP is likely to be from the N-BFs, which we earlier reported contain 0.01% P by weight. So for one N-BF per L seawater, this could represent an additional 26 nM P (e.g., 8 mg mass per N-BF x 0.01% = 0.8 µg P per L = 26 nM). If all of this N-BF associated P was suddenly released into the seawater, it would not be enough to account for any increase in TDP beyond day 1. The source(s) of P in these long-term experiments remains an unresolved issue. We should mention that the dates of greatest DOP increase (days 41 and 45) correspond to the abrupt decreases in dissolved Si (Figure 7), so the two trends may have a common explanation. At present, we have no reason to reject them as “bad data.”

Results: Nitrogen Fixation

Rates of N_2 fixation were measured, near the end (>38 d) of the H-341 long-term incubation and throughout the 37-day H-342 incubation experiment (Figure 9). Rates of AR were variable between expeditions and between separate incubation experiments (different source waters) on H-342 (Figure 9). The AR rates obtained on H-341 were nearly an order of magnitude greater than the maximum rates observed on H-342, but even the much lower rates on H-342 greatly exceeded those measured in the unamended control samples. This provides explicit support for the hypothesized enhancement of N_2 fixation following N-BF fertilization. However, the stimulation of N_2 fixation required >2 weeks for the ecosystem to select for cyanobacteria and N_2 -fixing microorganisms (both free-living cyanobacteria and symbiotic associations with diatoms; see below). The variability between expeditions (with much higher rates on H-341 and between different water samples collected on H-342) is probably due to the ephemeral nature of N_2 -fixing microorganisms at Station ALOHA (Böttjer et al. 2017) and to their competition with other phytoplankton. We hypothesize that the niche space created by N-BF fertilization is due to

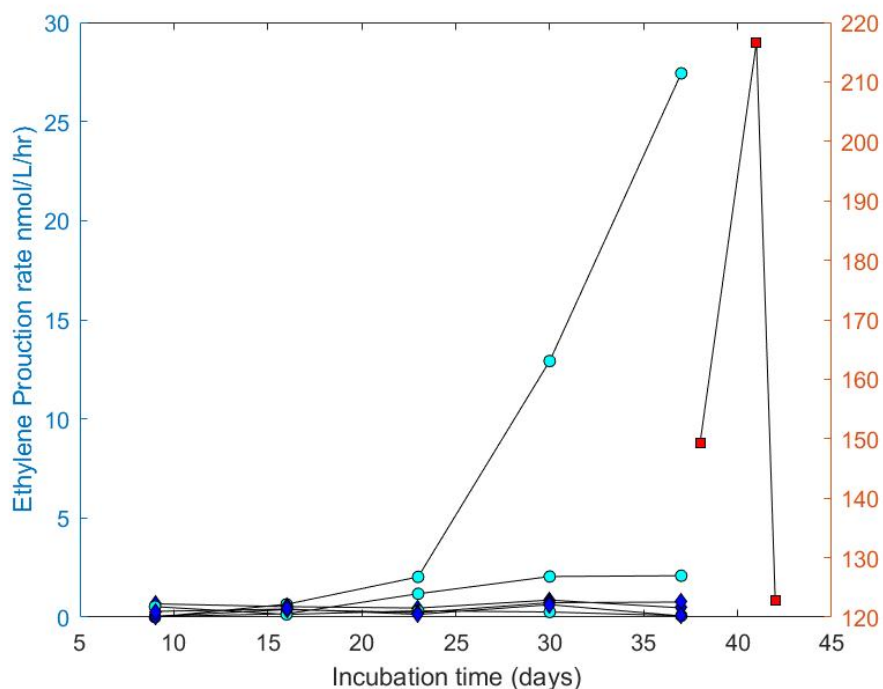


Figure 9. Rates of ethylene production (acetylene reduction to ethylene as a measure of N_2 fixation) for long-term incubation experiments on H-341 and H-342. Legend as in Figure 8. Note that the red symbols for the H-341 experiment are plotted with the righthand y-axis.

the addition of Si and Fe, which favors the initial growth of diatoms followed by selection for diazotrophic diatoms as fixed N supplies are exhausted. In support of this hypothesis, we observed a qualitative transformation in the phytoplankton assemblage from small diatoms to larger (some chain-forming) diatoms, many adorned with ecto- and endosymbiotic (presumably) N₂-fixing cyanobacteria. The former are readily identified using epifluorescence microscopy and chlorophyll autofluorescence, and the latter are identified by phycoerythrin autofluorescence which is a characteristic of cyanobacteria (Figures 10 and 11). For cyanobacteria, we observed both free-living species (filaments of *Trichodesmium* and unicells of *Crocospaera*; Figure 11 and 12) and the commonly observed endosymbiont, *Richelia* (Figure 13). We also observed high concentrations of *Epithemia* (Figure 14), a novel endosymbiont-containing diatom that was recently discovered at Station ALOHA (Schvarcz et al. 2022). The diversity of N₂-fixing microorganisms changed over time which is similar to changes that are observed at different stages of natural blooms of N₂-fixing cyanobacteria in the NPSG. Samples for the analysis of *nifH* gene sequence diversity to identify the various diazotroph species present in our incubation experiments have been sent to our collaborator, Professor Matthew Church (University of Montana), for analysis. Finally, by the end of the long-term incubations (45 days for H-341 and 37 days for H-342), the total particulate C, N and P had increased in samples that were fertilized with N-BFs in contrast to the unamended, control samples (Table 2). In the H-341 incubation which supported the highest rates of N₂ fixation, particulate N increased nearly an order of magnitude from 0.33 to 3.02 μmol N L⁻¹ (Table 2). The particulate Si was 4.81 μmol Si L⁻¹ with a corresponding molar Si:N ratio >1.0 confirming a high proportion of Si-containing diatoms (N.B. the molar Si:N ratio of diatoms varies with medium composition and growth rate but is approximately 1:1 in most laboratory-reared diatom cultures). The results were not as dramatic for the H-342 incubation either in N₂ fixation or accumulation of particulate matter, but in all incubations particulate N scaled on rates of N₂ fixation as they should since there is no other source of fixed N in these incubations. Total particulate P measure at the end of the H-341 long-term incubation was ~100 nmol P L⁻¹ greater than in the original seawater, and since the TDP pool in that incubation was also enriched (rather than being depleted) the source of P to support the production of biomass must have been derived from the N-BF. Previous measurements of dissolved P released from RHA preparations (Lü et al. 2020) appear to be in conflict with our observations of P release from N-BFs. It is possible that the addition of the iron ore in the N-BF

interferes with the colorimetric determination of phosphate and we plan to evaluate that in future laboratory experiments. Another key finding was the unusual elemental stoichiometry of the particulate matter that accumulated in the H-341 experiment. The anomalously high C:P (257:1) and N:P (27:1) molar ratios (relative the canonical Redfield molar ratios of C:P = 106 and N:P = 16) are characteristic of the “P sparing” effect of the growth of N₂-fixing microorganisms under conditions of P-limitation. Finally, the $\delta^{15}\text{N}$ of the total particulate matter for the H-341 sample at the end of the 43-day incubation period was -0.1‰ (relative to air) which is also consistent with 100% of the particulate N being sourced from N₂, as it should be since there are no other major sources of fixed N either in the original seawater sample or in the N-BF amendments.

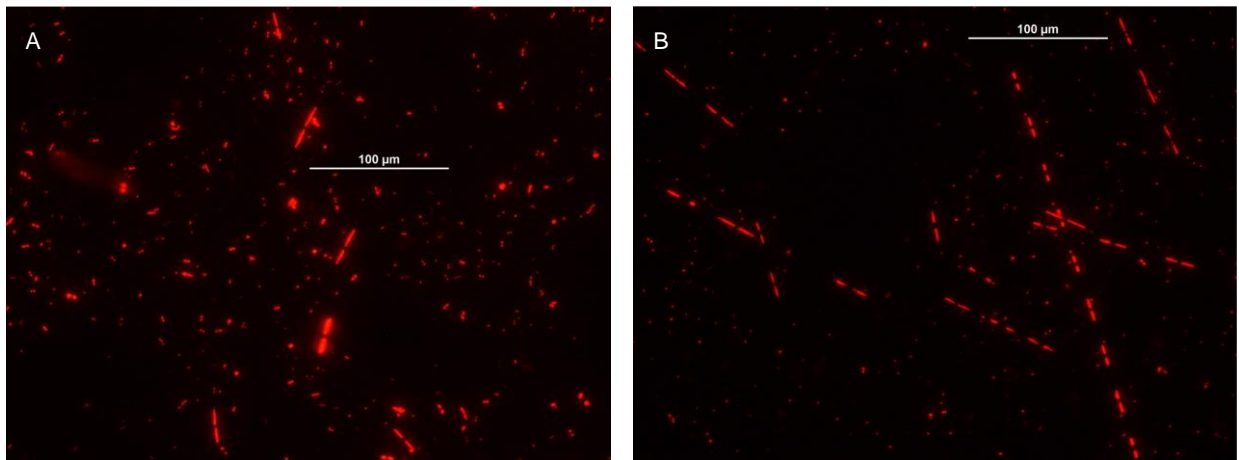


Figure 10. Epifluorescence microscopic images of the long-term incubation experiment showing a qualitative shift from smaller diatoms (A; <100 μm) to larger chain-forming species (B; > a few 100 μm) later in the experiment. The red color depicts autofluorescence of chlorophyll a, the primary photosynthetic pigment in phytoplankton.

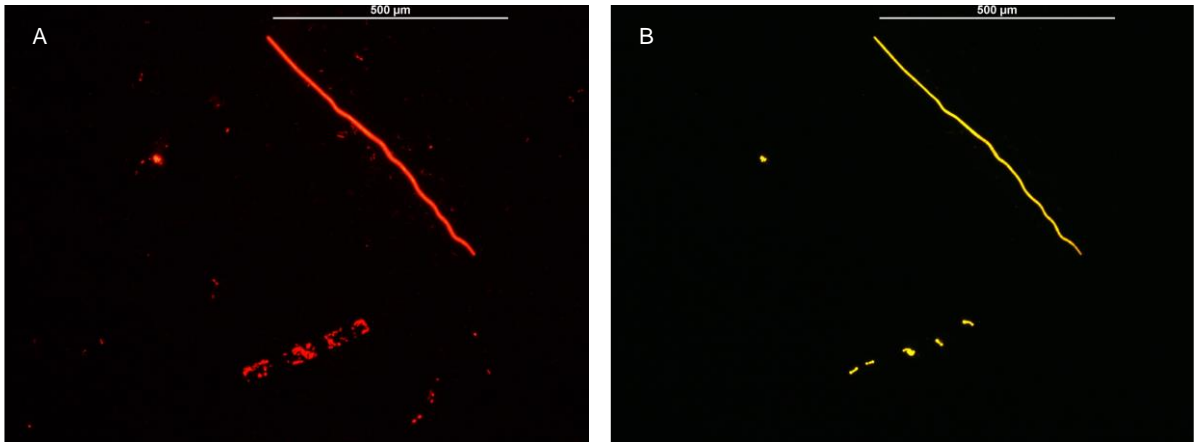


Figure 11. Identical epifluorescence images depicting autofluorescence of chlorophyll (A) and phycoerythrin (B) for a diverse assemblage of N_2 -fixing cyanobacteria during the long-term H-341 incubation experiment. Shown in the upper left of the image is the unicellular species, *Crocospaera*; a long filament of *Trichodesmium* in the upper right; and a chain-forming diazotrophic diatom with *Richelia* as its endosymbiont (lower center).

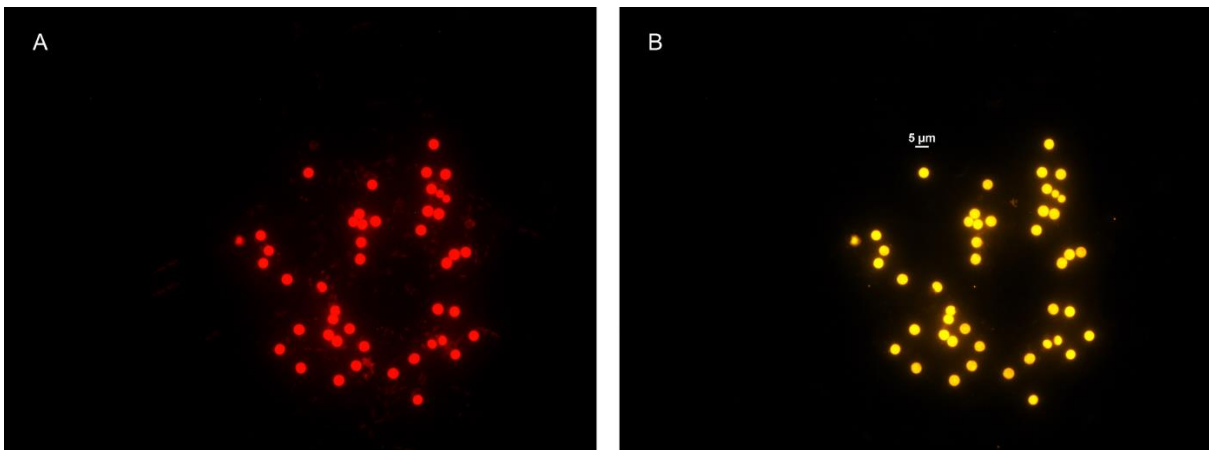


Figure 12. Bloom of *Crocospaera*, during the first two weeks of the H-341 incubation experiment. This small ($\sim 5 \mu\text{m}$) unicellular N_2 -fixing cyanobacterium is common at Station ALOHA. These identical images depict cells illuminated by chlorophyll (A) or phycoerythrin (B) autofluorescence.

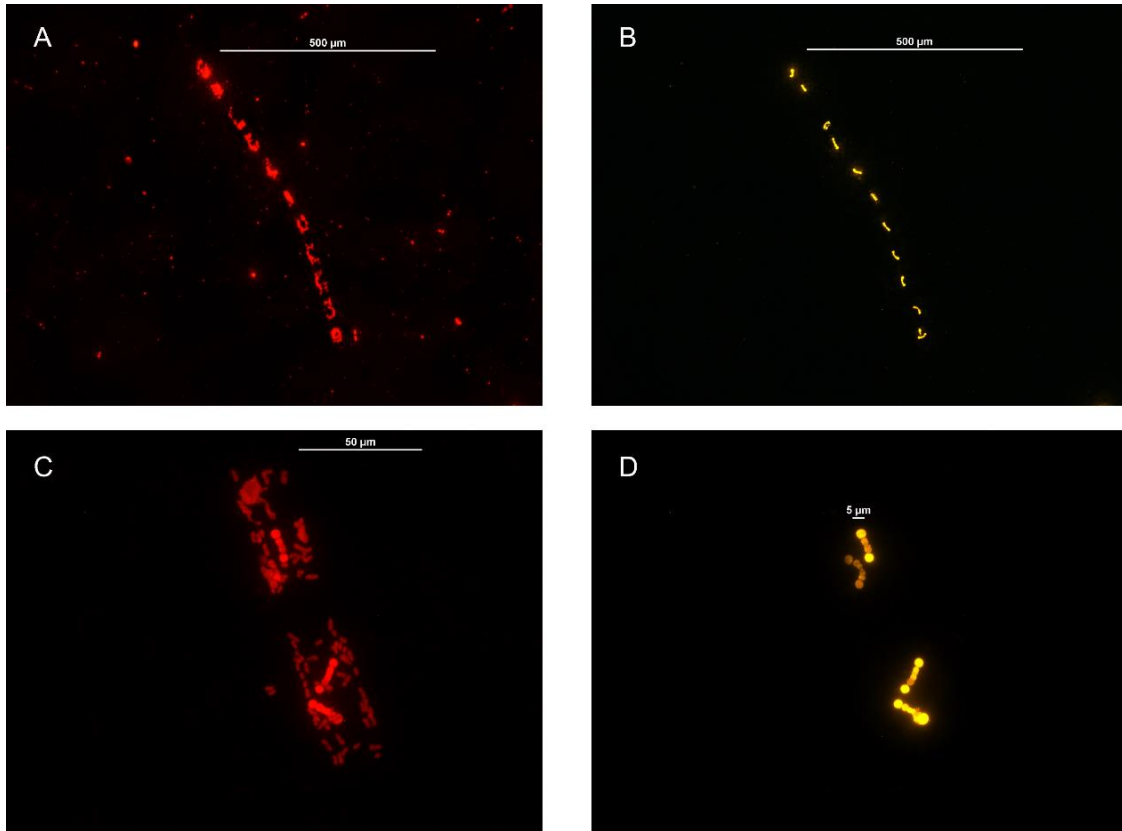


Figure 13. A portion of a long chain of diazotrophic diatoms (most likely *Hemiaulus*) at two different magnifications showing the presence of endosymbiotic, N_2 -fixing *Richelia* cells (typically 2 per diatom cell). Images A/C are using Chlorophyll a autofluorescence and B/D are using phycoerythrin autofluorescence.

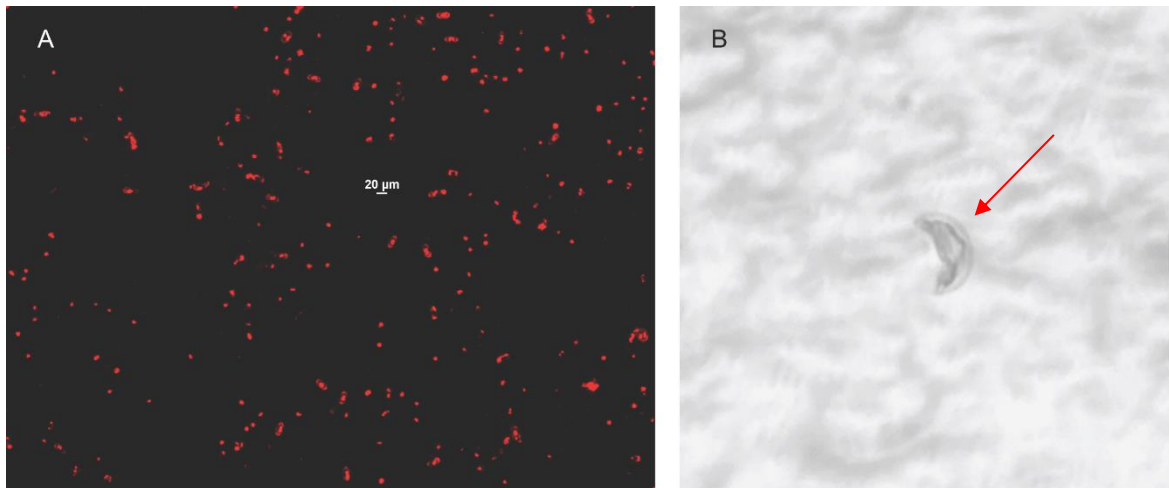


Figure 14. A bloom (nearly unialgal) of the recently described diazotrophic diatom *Epithemia* viewed via chlorophyll a autofluorescence (A) and under higher magnification view under transmitted light (B; red arrow).

Table 2. Particulate matter yields and elemental compositions on the final day of the long term incubations of untreated seawater (“Controls”) and one N-BF L⁻¹ amended seawater (“Flakes”). There was no control incubation for the H341 experiment so the starting seawater before flake addition (T=0) is shown for reference. For the H342 experiments, experiment #1 and #2 were taken from 2 different seawater sampling sources. Harvest (T=final) was at 45 days for H341 and 38 days for H342.

Name	N ($\mu\text{mol/L}$)	C ($\mu\text{mol/L}$)	P (nmol/L)	Si ($\mu\text{mol/L}$)	C:N (mol:mol)	C:P (mol:mol)	N:P (mol:mol)	Si:N (mol:mol)	Ethylene Production (nmol/L/hr)	$\pm 1\sigma$ (nmol/L/hr)
H341 T=0	0.33	2.21	17.5	N/A	6.8	126.5	18.59		N/A	
H341 Flakes T= final	3.02	28.62	111.3	4.81	9.48	257.09	27.13	1.59	122.89	15.99
H342 Control #1 T = final	0.46	6.75	33.4	0.25	14.77	202.23	13.69	0.54	0.76	0.96
H342 Flakes #1 T = final	0.53	6.25	39.7	0.54	11.75	157.58	13.41	1.02	2.09	0.42
H342 Control #2 T = final	0.63	8.34	48.4	0.43	13.21	172.3	13.05	0.68	0.47	0.48
H342 Flakes #2 T = final	1.27	12.65	73	1.42	9.98	173.23	17.36	1.12	27.43	3.56

III. Summary and Conclusions

In summary, for fixed-N starved environments like Station ALOHA, the addition of Si and Fe from the N-BF probably led to severe P limitation which set an upper limit on biomass production and eventual C sequestration via the biological C pump. We have previously reported that a concentration of ~50-60 nM P was the threshold between P-limitation below that value and Fe-limitation above it (Letelier et al. 2019). By this assessment conditions on both H-341 and H-342, with P concentrations >60 nM, were most likely Fe-limited. The addition of N-BF added Fe and Si, led to a stimulation of primary production and bacterial respiration, net consumption of P, and an exacerbation of N-limitation. The latter selected for N₂-fixation by free-living cyanobacteria and diazotrophic diatoms, the biomass of which was controlled by P as concentrations were drawn down below 60 nM in the extended H-341 experiments which exhibited elevated rates of N₂ fixation. The production of P-depleted (elevated C:P and N:P ratios) biomass, relative to the expected Redfield ratio of nutrient-saturated microorganisms, is consistent with a N₂-fixation controlled ecosystem by the end of our ~40-day experiments. Future studies will explore the long-term metabolic and biogeochemical impacts of N-BF plus P amendments, possibly leading to a revised recipe for the next generation of N-BFs.

One option is to add bioavailable P (as phosphate) to the N-BF product or to add phosphate at the same time as N-BF fertilizer (but the phosphate spike would likely have a different behavior and half-life in seawater than the buoyant flakes and soon become decoupled). From an ecological and carbon sequestration perspective, the optimal delivery of P would be as a time-release matching the kinetics of Si and Fe to sustain the proliferation of N₂-fixing microorganisms, especially large diazotrophic diatoms. Incorporation of P in a new NIO-Hawaii rice husk product (possibly dubbed NIO-HI-BF+P) would require laboratory tests to define/refine the chemistry and field tests to evaluate the biogeochemical impacts of Si-Fe-P fertilization, much like we are reporting here for the current N-BF product. We have devised a ‘conceptual plan’ to achieve the desired outcome using authigenically produced brucite (Mg(OH)₂) as the P-carrier. For the past 30 years or so we have used authigenic brucite formation for the quantitative determination of dissolved P in seawater using the “MAGIC” method (MAGnesium Induced Coprecipitation; Karl and Tien 1992). We propose to develop a modified MAGIC technique using seawater collected from Station ALOHA. Prior to the addition of NaOH (required for the coprecipitation process), we would add reagent grade

phosphate to the seawater (surface seawater at ALOHA contains about 100 nM P) to ensure that the brucite formed has the highest weight percent P that is possible (maximum adsorption). Authigenic brucite formation from seawater is preferred to adsorption onto commercially available brucite if the goal is to maximize the P content due to the greater adsorption capacity (surface area) of the authigenically produced mineral. Following precipitation, the brucite-P is collected (filtration, centrifugation or settling), washed, dried at 60°C and pulverized to a fine powder that is similar in size (<63 μm) to the iron ore powder used to prepare the N-BFs. The brucite-P could be mixed with the iron ore prior to use or used separately to coat the rice husks after they are “dipped into hot molten lignin” (Damodar Shenoy, discussed above). Either way, the relative proportions of iron ore-to-brucite-P would need to be adjusted to optimize the simultaneous release of Si-Fe-P in a stoichiometric ratio that sustains N_2 fixation. An added benefit of using brucite (as opposed to some other time release process) is the bonus of carbon dioxide sequestration by alkalinity enhancement since brucite is an alkaline mineral. Indeed, field trials using brucite fertilization of seawater as a means to sequester carbon dioxide are already underway in Cornwall, UK and Halifax, Canada. These efforts are part of the much larger vision of Planetary Technologies, Inc. for carbon dioxide removal through alkalinity enhancement (*planetarytech.com*). If the MBR project is approved for funding by the Grantham Foundation, we could explore both the possible formulation of a novel NIO-HI-BF+P product and, perhaps, establish a future collaboration with like-minded scientists at Planetary Technologies, including my former VERTEX collaborator and long-term (since the 1980s) colleague, Greg Rau who is the co-founder and current Chief Technology Officer at Planetary Technologies, Inc. Given the current state of our planet, there is no time to spare.

IV. General Observations and Conclusions

The Goa-supplied N-BF “worked as advertised.” They provided a time-release supply of bioavailable Si (and presumably Fe, which was not measured in these preliminary experiments) to sustain and enhance the growth of large (including chain-forming) diatoms, stimulated primary production and net community production following a brief period of net heterotrophy immediately following the addition of N-BFs (up to several days), and most importantly created a chemical environment (Si plus Fe) that was conducive for the proliferation of N_2 -fixing microorganisms that further sustained enhanced primary production and particle production, and

– presumably – carbon export. In our (limited) experience with the N-BFs, here are a few observations of their “behavior” in seawater during the shipboard and laboratory incubation experiments: (1) During the at-sea experiments, often the buoyant flakes get stuck to the side of the carboy above the water line during normal sea motions. This was hard to avoid, and difficult to repair. (2) In the smaller volume (125 mL) dissolved oxygen experiments that lasted at most 4 days, most of the N-BFs remained buoyant but a few sank when we tried to remove them prior to chemical fixation of the sample. (3) In the two long-term experiments, approximately half of the N-BFs were still buoyant at the end of the H-341 experiment but only about 25% at the end of the H-342 experiment. Many of the N-BFs that had become negatively buoyant showed signs of disaggregation/dissolution. This difference between experiments may have been a result of the more frequent sampling during the H-342 experiment (human disruption). (4) Several buoyant flakes were sampled at the end of the H-342 experiment and extracted for total particulate ATP to assess the degree of biofouling (total microbial biomass) that accumulated during the 45-day incubation period. While these results showed significant biofouling by microorganisms, chemical interference of firefly bioluminescence (the method used to quantify particulate ATP) from the N-BFs precludes anything but a qualitative assessment. We do not know whether biofouling accelerates N-BF degradation during long-term experiments or helps to hold the N-BFs together via the formation of exopolymers. Future experiments will focus on the dynamics of the biological carbon pump and on the inextricably linked biogeochemical cycles of Si-Fe-P. Pending future funding from the Grantham Foundation, more detailed laboratory- and field-based studies of the N-BF technology is almost guaranteed to lead to a more comprehensive understanding of the potential for Fe controlled carbon sequestration in ocean systems.

References

- Bakar, R. A., R. Yahya, and S. N. Gan. 2016. Production of high purity amorphous silica from rice husk. *Procedia Chemistry* **19**, 189-195.
- Böttjer, D., J. E. Dore, D. M. Karl, R. M. Letelier, C. Mahaffey, S. T. Wilson, J. Zehr, and M. J. Church. 2017. Temporal variability of nitrogen fixation and particulate nitrogen export at Station ALOHA. *Limnology and Oceanography* **62**, 200-216.
- Karl, D. M., and G. Tien. 1992. MAGIC: A sensitive and precise method for measuring dissolved phosphorus in aquatic environments. *Limnology and Oceanography* **37**, 105-116.

Letelier, R. M., K. M. Björkman, M. J. Church, D. S. Hamilton, N. M. Mahowald, R. A. Scanza, N. Schneider, A. E. White, and D. M. Karl. 2019. Climate-driven oscillation of phosphorus and iron limitation in the North Pacific Subtropical Gyre. *Proceedings of the National Academy of Sciences USA* **116**, 12720-12728.

Lü, J.-J., G.-T. Zhang, and Z.-X. Zhao. 2020. Seawater silicate fertilizer facilitated nitrogen removal via diatom proliferation. *Marine Pollution Bulletin* **157**, 111331.

Normile, D. 1997. Yangtze seen as earliest rice site. *Science* **275**, 309.

Schvarcz, C. R., S. T. Wilson, M. Caffin, R. Stancheva, K. A. Turk-Kubo, A. E. White, D. M. Karl, J. P. Zehr, and G. F. Steward. 2022. Overlooked and widespread pennate diatom-diazotroph symbioses in the sea. *Nature Communications* **13**, 799.

Shetye, S., A. Pratihary, D. Shenoy, S. Kurian, M. Gauns, H. Uskaikar, B. Naik, K. Nandakumar, and S. Borker. 2023. Rice husk as a potential source of silicate to oceanic phytoplankton. *Science of the Total Environment* **879**, 162941.

Wilson, S. T., D. Böttjer, M. J. Church, and D. M. Karl. 2012. Comparative assessment of nitrogen fixation methodologies, conducted in the oligotrophic North Pacific Ocean. *Applied and Environmental Microbiology* **78**, 6515-6523.