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Nitrogen fixation associated with the decomposition of the giant kelp *Macrocystis pyrifera*

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ABSTRACT

The seaweed *Macrocystis pyrifera* plays an important role in the coastal zone as a result of its global distribution, high productivity and detrital export, and the high diversity of the ecological communities that it supports. Growth of the giant kelp *Macrocystis*, like other seaweeds, is often limited by the availability of fixed nitrogen. Several other macroalgal species have been reported to harbor microbial symbionts that fix ubiquitous dissolved N_2, converting it into bioavailable forms, and potentially making a significant contribution to algal nutrition. Our investigation shows that N_2 fixation on living *Macrocystis* (mean 0.17 μmol g^{-1} d^{-1}) was more than 10 times greater than that associated with all but one of the 14 other macroalgal species we studied. However, this rate is insufficient to contribute significantly to *Macrocystis* growth. Instead, we found much higher rates of N_2 fixation (up to 23 μmol g^{-1} d^{-1}) associated with detrital or decomposing *Macrocystis*. Decomposition-related N_2 fixation on *Macrocystis* is stimulated both by anaerobic conditions and amendment with mannitol or phosphate, but is inhibited by light, and is therefore, likely a heterotrophic process in contrast to the cyanobacterial N_2 fixation reported for *Codium* and *Sargassum*. Using O_2 microsensors, we show that anaerobic conditions around the *Macrocystis* blade are unlikely to develop in attached plants. Nitrogen fixation may be important in kelp rafts and wrack, where it could contribute as much as a 4-fold increase in the availability of nitrogen to support microbial and invertebrate communities.

1. Introduction

Seaweeds, or marine macroalgae, are macroscopic multicellular photoautotrophs that are largely benthic, and play a significant role in structuring many coastal marine communities (reviewed in Graham et al., 2007). Although macroalgae only occupy ca. 5% of the global coastal benthic habitat, they make a disproportionate contribution to coastal carbon (C) cycling (Duarte et al., 2005). Macroalgae account for 62% of the global coastal gross primary production (5180 Tg C y^{-1}), and 41% of the global coastal respiration (ca. 7300 Tg C y^{-1}) budgets (Duarte et al., 2005). Their high net community (or export) production (2220 Tg C y^{-1}) combined with the long turnover time of macrophyte carbon (ca. 1 y) relative to planktonic carbon (ca. 2 weeks) makes macroalgae significant contributors of detrital material to the coastal zone (Smith, 1981).

Growing largely in shallow waters, marine macroalgal production is often limited by nitrate availability during summer thermal stratification (Zimmerman and Robertson, 1985; Pedersen and Borum, 1996; Brzezinski et al., 2013). However, nitrate uptake may be insufficient to support growth, and macroalgae may in addition rely on ammonium regenerated in the benthos or excreted by epizooids, for their nitrogen (N) requirements (Hepburn and Hurd, 2005; Fram et al., 2008). Another potential source of N to macroalgae is prokaryotic dinitrogen (N_2) fixation, or diazotrophy, the reduction of atmospheric N_2 to organic N (reviewed in Howarth et al., 1988). Nitrogen fixation is carried out by diverse species of cyanobacteria and heterotrophic prokaryotes (e.g., Hamersley et al., 2011) and is important not only to their own nutrition, but as a significant source of new N supporting marine communities. The key enzyme responsible for this reaction, nitrogenase, is inactivated by oxygen (O_2), so these prokaryotes must live in anaerobic environments or employ specialized cellular structures (e.g., heterocysts), metabolic processes, or temporal segregation to protect nitrogenase from O_2 exposure (Paerl et al., 1995). Although N_2 fixation plays an important role in the marine N cycle, its contribution to coastal N cycling is typically low relative to terrigenous, benthic,

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and upwelling sources of N (reviewed in Sohm et al., 2011; Howarth et al., 2011).

A number of macroalgal species have been found to harbor N_2-fixing symbionts (e.g. Head and Carpenter, 1975), whereas other species have been reported to have no associated N_2 fixation (Stewart, 1971; Gerard et al., 1990 this study). Nitrogen fixation associated with macroalgae has been attributed to both cyanobacteria (e.g. Gerard et al., 1990) and heterotrophic bacteria (Head and Carpenter, 1975) living on both macroalgal surfaces (epibionts) or within macroalgal tissues (endobionts). Macroalgae with associated diazotrophy include species of the genera *Caulerpa, Dictyota, Enteromorpha, Gracilaria*, *Halimeda, Laurencia, Microdictyon,* and *Padina* (Capone et al., 1977; Bohlool and Wiebe, 1978; Penhale and Capone, 1981; Chisholm et al., 1996; Singh et al., 2011), but N_2 fixation associated with the genera *Sargassum* and *Codium* is best described. Nitrogen fixation has been found associated with three species of *Sargassum*: *S. natans*, *S. fluitans*, and *S. filipendula*, from the Sargasso Sea and Gulf Stream (Carpenter, 1972; Phlips et al., 1986), and has been attributed to the common and densely colonizing cyanobacterial epibionts *Dichothrix fucicola* and *Calothrix* spp. (Carpenter, 1972; Phlips et al., 1986). Nitrogen fixation was estimated to be capable of supplying up to 143% of the growth needs of *Sargassum* (Phlips et al., 1986).

Nitrogen fixation associated with *Codium decorticatum*, *C. fragile*, and *C. adhaerens* has been most closely linked to cyanobacteria of the genera *Calothrix* and *Microcoleus*, but three other cyanobacterial taxa have been implicated as well (Dromgoole et al., 1978; Rosenberg and Paerl, 1981; Gerard et al., 1990). In contrast to these reports of cyanobacterial N_2-fixing symbionts on *Codium*, Head and Carpenter (1975) reported that N_2 fixation on *C. fragile* was stimulated by glucose additions and that no symbiotic cyanobacteria were observed; instead they attributed the N_2 fixation to a heterotrophic *Azotobacter* isolate. Diazotrophs associated with *Codium* have been estimated to be capable of providing from 5% to 50% of its N requirements (Head and Carpenter, 1975; Rosenberg and Paerl, 1981; Gerard et al., 1990).

Among the macroalgae, the kelps (brown algae of the order *Laminariales*) have an outsize ecological significance because they form dense beds or "forests" that support diverse marine ecosystems (reviewed in Graham et al., 2007). Of all the kelps, the canopy-forming giant kelp *Macrocystis* is the largest, most widely distributed, the most productive, and supports the most complex ecosystem. It is also the largest of all the macroalgae. The primary range of *Macrocystis* (currently considered to be monospecific, but species names are still used to refer to the distinct growth forms) extends from the Alexander Archipelago of Alaska to the Baja Peninsula of Mexico, and from northern Peru to Patagonia. From there, its range extends across the sub-Antarctic Islands and the southern tips of Africa, Australia, and New Zealand, from about 35° to 60° S latitude. *Macrocystis* is an attached macroalga, but as a result of its growth form (fronds may grow to 45 m in length), it may grow in waters up to 60 m in depth. The tropical gap in the distribution of *Macrocystis* may be a result of low nutrient availability and high temperature.

Despite the importance of *Macrocystis pyrifera* and other kelps, we are aware of no published investigations of possible associated N_2 fixation. In this study, we detail the results of our investigation into N_2 fixation associated with *M. pyrifera* from the west coast of the United States. We focused our research on *M. pyrifera* from the Southern California Bight, where summer thermal stratification of the water column is strong and the growth of giant kelp is limited by nitrogen availability (Zimmerman and Robertson, 1985; Brzezinski et al., 2013).

2. Material and methods

2.1. Field work

Macroalgal samples for N_2 fixation measurements were collected primarily in Fisherman's Cove (Catalina Island, California, USA: 33.445°N, 118.486°W) and Malaga Cove (Palos Verdes, California: 33.798°N, 118.407°W) (Supplementary Table 1). Additional algal samples were collected in Friday Harbor (Friday Harbor, Washington: 48.545°N, 123.012°W), off Red White, and Blue Beach (Santa Cruz, California: 39.976°N, 122.143°W), and in San Simeon Bay (San Simeon, California, 35.642°N, 121.191°W) (see Supplementary Table 1 for sample locations and dates). The blades (leaf-like thalli) were used for all experiments on *M. pyrifera* (assays of stipes and pneumatocysts were negative for N_2 fixation – data not shown). "Live" samples were collected from benthos-attached plants within 1 m of the sea surface. "Detrital" samples were from detached plant parts collected either from the intertidal zone (wrack) or found floating in nearshore waters (rafts). "Mature" and "senescent" kelp samples were collected from live plants. Mature blades were healthy non-apical tissue with little or no damage (holes, tears), few visible epibionts, and a bright green color. Senescent blades had decomposing tips, edges, and holes in the blades, were heavily colonized by the bryozoan *Membranipora membranacea*, and were a pale olive-green color.

2.2. Nitrogen fixation assays

Nitrogen fixation was assayed as nitrogenase enzyme activity via the acetylene reduction approach (Hardy et al., 1968). Additional assays of N_2 fixation using $^{15}N_2$ as a tracer are described in Section 2.6. For measurement of N_2 fixation activity associated with foliose macroalgal thalli, 3–6 weighed disks (1.5-cm diam.) of thallus or blade tissue were placed in 14-ml serum vials with 10 ml of filtered (0.2-μm membrane) seawater. For non-foliose algal tissues, weighed pieces (ca. 1 g wet wt.) were used. From 3 to 5 replicate vials were assayed per collection. To reduce variability between vials during the *M. pyrifera* incubations, disks were subsampled from random locations throughout blades, and each disk in each vial was subsampled from a different kelp blade. One milliliter of acetylene (C_2H_2) was injected into the headspace, and equilibrated by shaking. The vials were incubated at in situ temperatures.

The evolution of ethylene (C_2H_4) by nitrogenase and consumption of O_2 were calculated from temporal changes in the concentration of these gasses in the vial headspace determined by gas chromatography (ethylene: flame ionization detection and O_2: electron capture detection; Shimazdu, Columbia, USA). Gas measurements were typically made 4 times during the first 24 h and then daily for 4–7 days. These longer incubation times enabled the measurement of both the initial N_2 fixation rate and the higher rates usually (85%) observed after ca. 24 h.

Gas production and consumption rates were determined on a mass basis (i.e. including dissolved gas) by linear regression and ethylene production was converted into N_2 fixation rates by dividing by a factor of 3 (Capone, 1993), then converted to moles of fixed N (rather than of N_2 fixed) by multiplying by 2. Although, our own calibration of the acetylene reduction approach by $^{15}N_2$ tracer yielded a factor of 3.3 ($r^2 = 0.91$; Section 3.5), we chose to use the factor of 3 for consistency with previous reports of macroalgal N_2 fixation. Dissolved gas concentrations in the seawater incubation medium were calculated from the partial pressure of the gas in the headspace and the corresponding Bunsen coefficient (Weiss, 1970; Breitbarth et al., 2004). A subsample of algal disks were dried (60 °C to constant weight) and weighed, and N_2 fixation rates were calculated on a dry weight basis.

Control incubations of *Macrocystis* samples without added acetylene were made ($n = 10$, in duplicate or triplicate), including mature and senescent samples from both Catalina and Palos Verdes), to determine any background ethylene production. Mean ethylene production was (±standard deviation) 0.0001 ± 0.0010 μmol C_2H_4 g^{-1} d^{-1}, corresponding to an apparent N_2 fixation rate of 0.00007 ± 0.00067 μmol N g^{-1} d^{-1}, below our limit of detection. Filtered seawater samples without macroalgae but with added acetylene ($n = 6$) were also analyzed as controls; ethylene production in these experiments averaged (±standard deviation) -0.00005 ± 0.00002 μmol C_2H_4 d^{-1} $bottle^{-1}$, with the standard deviation representing about 4% of the mean initial ethylene production associated with kelp.

2.3. *In situ decomposition experiment*

Nitrogen fixation was measured on *Macrocystis* kelp blades undergoing in situ decomposition in mesh bags for 24 days. Mature, senescent, and detrital blades (ca. 270 g wet wt.) were each incubated in 5 replicate 1-mm mesh bags which were suspended in seawater from a dock adjacent to the Catalina kelp forests where they were collected. At six sampling times over the course of 24 days, the bags were weighed wet (after draining excess seawater), and 18 disks of blade tissue were removed at random locations within randomly selected blades for triplicate determination of N_2 fixation as described in Section 2.2. On days 0, 9, and 24, triplicate subsamples were analyzed for N content as described in Section 2.6.

2.4. *Oxygen microenvironment around* Macrocystis

The dissolved O_2 microenvironment around kelp blades was measured with an Clark-type O_2 microsensor with a tip width of 10 μm (Unisense, Aarhus, Denmark). Kelp blades were affixed to a seawater agar substrate within a benchtop flow system which recirculated seawater over the kelp surface. The water flow rate was adjusted to 2 cm s^{-1} using neutrally-buoyant beads (ca. 0.8 mm diam., Cospheric, Santa Barbara, USA). The seawater was held at 15 °C by heat exchange with a refrigerated bath. Light experiments were conducted at 28 μmol photons m^{-2} s^{-1}. Dark experiments were conducted under an opaque hood. The microelectrode was manipulated using a micromanipulator (Unisense) and the position of the microelectrode with respect to the kelp surface was determined by microstereoscopy.

2.5. *Experimental manipulations of incubation conditions*

Nitrogen fixation associated with *M. pyrifera* blades was measured under variety of experimental conditions to elucidate the environmental conditions supportive of kelp-associated N_2 fixation. Each experimental measurement was determined as the mean of 3–5 replicate incubations as described in Section 2.2; the numbers of such measurements for each experimental treatment are indicated in the corresponding figures. Anaerobic incubations were carried out using N_2-sparged seawater. Light incubations were conducted at ca. 25 μmol photons m^{-2} s^{-1}, sufficient to maintain a constant O_2 level within the incubation bottles via kelp photosynthesis. In some experiments, incubation bottles were shaken at 50 cycles min^{-1} on an orbital shaker. Paired (control:treatment) incubations with nutrient amendments were made with glucose (100 μmol l^{-1}), mannitol (100 μmol l^{-1}), Fe^{3+} (50 nmol l^{-1}), or PO_4^{3-} (0.5 μmol l^{-1}).

2.6. *Nitrogen fixation measured by* $^{15}N_2$ *tracer*

The incorporation of fixed N_2 into *Macrocystis* blade and epibiont biomass was also measured on 6 March and 20 July 2006 in incubations with $^{15}N_2$ (98%, Sigma–Aldrich) as a stable isotopic tracer. For the $^{15}N_2$ incubations, pieces (ca. 80 cm^2 each) of both mature and senescent kelp blades were incubated in triplicate with 2 ml $^{15}N_2$ in sealed 600-ml flasks containing 500 ml of filtered seawater. Control incubations were identical, but without added $^{15}N_2$. The flasks were shaken and kept on a 12-h light/dark cycle during a 3-day incubation. At the end of the experiment the entire kelp sample was dried at 60 °C, ground, and analyzed for C, N, and ^{15}N content by Dumas combustion and isotope ratio mass spectrometry (SerCon Integra, Crewe, UK; University of California, Davis Stable Isotope Facility). Treatment results were calculated relative to the final kelp ^{15}N content of control (no added $^{15}N_2$) incubations. We evaluated the maximum increase in measured N_2 fixation rates resulting from the potential contamination of $^{15}N_2$ gas with ^{15}N-labeled inorganic N, and found that such contamination could have contributed no more than 3.2% to the measured rates (Dabundo et al., 2014), At the same time, we measured N_2 fixation with the acetylene reduction assay (Section 2.2) on subsamples of the same kelp material.

3. Results

3.1. *Nitrogen fixation associated with 15 macroalgal species*

We measured N_2 fixation associated with disks or pieces of the thalli of 15 species of macroalgae collected along the west coast of the United States (Supplementary Table 1), with most samples collected along the coast of central and southern California. Initial N_2 fixation rates (acetylene reduction) were ≤0.011 μmol N dry g^{-1} d^{-1} in all 10 of the macroalgal species collected north of Point Concepción, including the kelp *M. integrifolia* (Supplementary Table 2), as well as in 4 of the 6 species collected south of Point Concepción. In contrast, mean initial N_2 fixation rates associated with the remaining two southern macroalgal species were (±standard error) 0.150 ± 0.073 μmol N g^{-1} d^{-1} (*Sargassum muticum*) and 0.177 ± 0.074 μmol N g^{-1} d^{-1} (*M. Pyrifera*) (Supplementary Table 2). We focused our study on *M. pyrifera*, and assayed 185 samples over a 7-year period from two sites in Southern California: Catalina Island and Palos Verdes.

3.2. *Nitrogen fixation in decomposing* Macrocystis

During 25 days of *Macrocystis* decomposition in 1-mm mesh bags submerged in situ in seawater, initially senescent blades lost biomass the most rapidly, followed by kelp detritus (Fig. 1A). Initially mature, healthy kelp blades lost mass the most slowly, though by 25 days, less than 15% remained of all three kelp types. Oxygen consumption by decomposing kelp peaked at 9 days for all kelp types. The highest respiration rates were associated with senescent and mature kelp (Fig. 1B). The initial rates of N_2 fixation associated with subsamples of decomposing kelp removed from the bags over the course of the experiment increased for all kelp types during the experiment (Fig. 1C). Senescent kelp had the highest rates throughout, followed by detritus. Nitrogen fixation associated with mature kelp blades remained low for the first 17 days, after which rates increased to near those found on senescent kelp. By the end of the experiment, N_2 fixation rates associated with senescent, mature, and detrital kelp were 22.3 ± 2.0, 23.9 ± 4.6, and 17.4 ± 2.2 μmol g^{-1} d^{-1}, respectively. The N_2 fixation rates measured at the end of this experiment were two orders of magnitude higher than the mean initial rates (0.177 μmol N g^{-1} d^{-1}) associated with freshly collected *M. pyrifera* that we measured. The N content of the

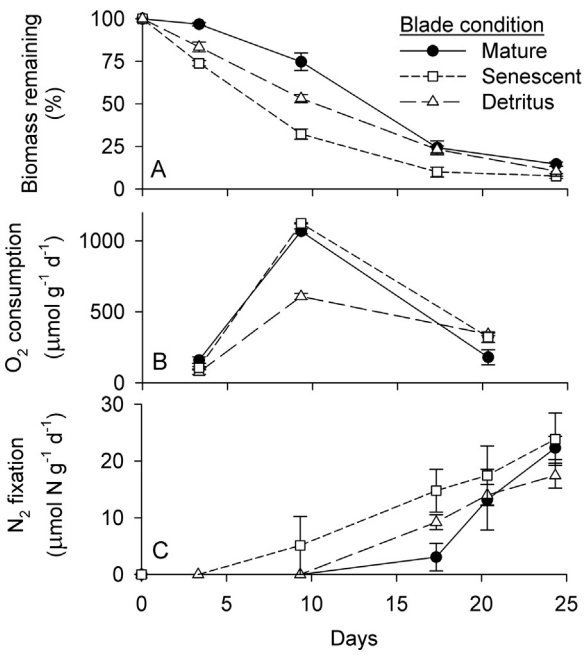

Fig. 1. Degradation of *M. pyrifera* in situ in 1-mm mesh bags containing initially mature, senescent, or detrital kelp blades ($n = 5$). A. Loss of biomass over time. B. Change in dark O_2 respiration rate. C. Increase in N_2 fixation associated with kelp.

decomposing kelp increased by 42% from 1.2 ± 0.1% to 1.7 ± 0.1% after 9 days of in situ decomposition, and remained at that level until the end of the experiment.

3.3. Rapid-phase nitrogen fixation and dissolved oxygen at the blade surface

In long N_2 fixation incubations of *Macrocystis* blade disks, after a "lag" of hours or days (median 26 h for live kelp), ethylene production indicative of nitrogenase activity entered a much more rapid phase in 85% of the samples assayed (Supplementary Fig. 1). Experimental manipulations of the incubation conditions showed that this lag period could be shortened by three incubation conditions supporting anaerobic conditions around the kelp disks: a) darkness (which inhibited oxygenic photosynthesis relative to light; Fig. 2A), b) still (unshaken) incubations (which promoted the development of an anaerobic microzone around the blade during dark incubations by increasing the diffusion distance for O_2 compared to shaken incubations, where water turbulence decreased the thickness of the diffusive boundary layer around kelp blades; Fig. 2B), and c) initially anaerobic relative to aerobic incubations (Fig. 2C). When vials were shaken (keeping the dissolved O_2 concentration at the blade disk surface near that of the bulk solution), the rapid N_2 fixation rates did not begin until dissolved O_2 had fallen below 14 µmol l^{-1}, or ca. 5% of saturation values (Fig. 2D). In contrast, when vials were kept still, allowing the development of an O_2-depleted microzone around the kelp blades, the threshold for rapid N_2 fixation began at a much higher bulk O_2 concentration of 120 µmol l^{-1} (ca. 40% saturation; Fig. 2D).

Microsensor measurements of dissolved O_2 in the microzone surrounding a kelp blade confirmed that in the light, O_2 supersaturation developed in the diffusive boundary layer around the kelp blade (Supplementary Fig. 2A). In the dark, with aerobic water was flowing over the kelp blade, the diffusive boundary layer remained oxygenated (ca. 75 µmol l^{-1}, or 27% of saturation) (Supplementary Fig. 2A). However, without water movement over the kelp blade, dissolved O_2 levels eventually fell below 10% of saturatation (Supplementary Fig. 2B).

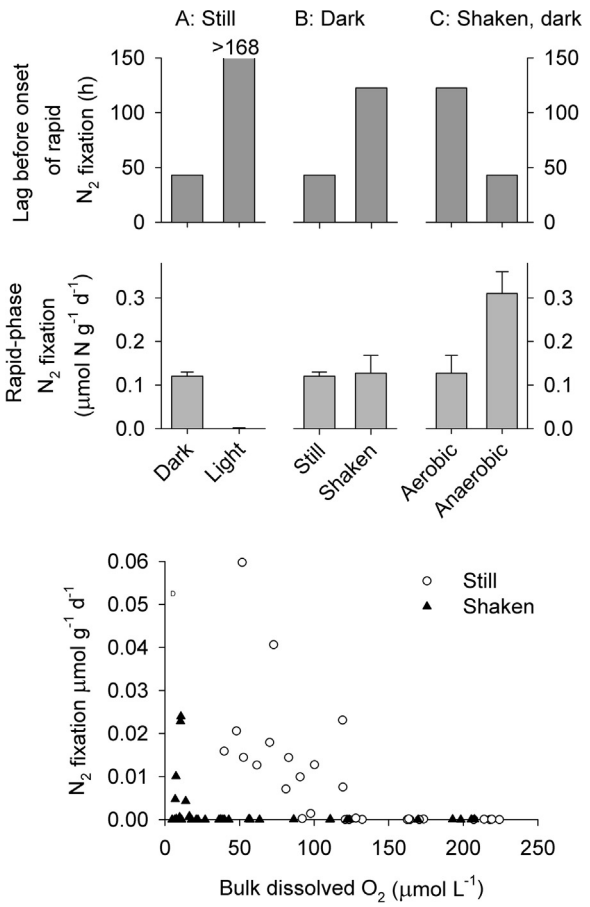

Fig. 2. Effects of experimental manipulations of the dissolved O_2 regime around incubated *M. pyrifera* blade disks on associated N_2 fixation. Initially measured rates of N_2 fixation were usually (85%) followed by a transition to a rapid N_2 fixation phase after a lag period. A. In still water, the rapid N_2 fixation phase developed more quickly in the dark relative to the light (did not develop during the 168-h incubation), likely because of inhibition of N_2 fixation by photosynthetically-produced O_2 (top and bottom panels). B. In dark (respiration only) incubations, shaking lengthened the lag period relative to still incubations (top panel), likely because anaerobic conditions at the blade surface, supportive of N_2 fixation, developed more quickly in diffusion-limited still water. C. In dark, shaken incubations, incubations in initially-aerobic water took longer to begin the rapid N_2 fixation phase than those incubated anaerobically (top panel), and developed lower N_2 fixation rates afterward (bottom panel). Error bars are standard errors ($n = 5$). D. Relationship between mean bulk O_2 concentrations and N_2 fixation rates (by difference in measurements taken ca. 1 d apart) during dark N_2 fixation assays. Rapid N_2 fixation rates only developed when bulk O_2 levels were <14 µmol l^{-1} in shaken incubations, or <120 µmol l^{-1} in still incubations.

3.4. Conditions supporting N_2 fixation on Macrocystis

The condition of the kelp samples was an important determinant of associated N_2 fixation. Detrital kelp (both wrack and rafts detached from the holdfast) had significantly higher associated initial N_2 fixation rates, lower lag times, and higher rapid-phase N_2 fixation rates than live kelp (attached to sea floor; Fig. 3A). Similarly, senescent kelp samples, which showed signs of decomposition, had higher initial N_2 fixation rates than mature blades, which were undecomposed and of a greener color (see Section 2.1 for full description; Fig. 3B). N_2 fixation associated with the kelp was significantly stimulated by dark incubation conditions; incubation under the light lengthened the lag time before the onset of rapid-phase N_2 fixation (Fig. 3C).

Experimental amendation of kelp with the substrates mannitol, iron, or phosphate increased mean and median N_2 fixation rates by large margins relative to paired controls, though these increases were not stastistically significant ($p > 0.05$; Fig. 4A,C). The

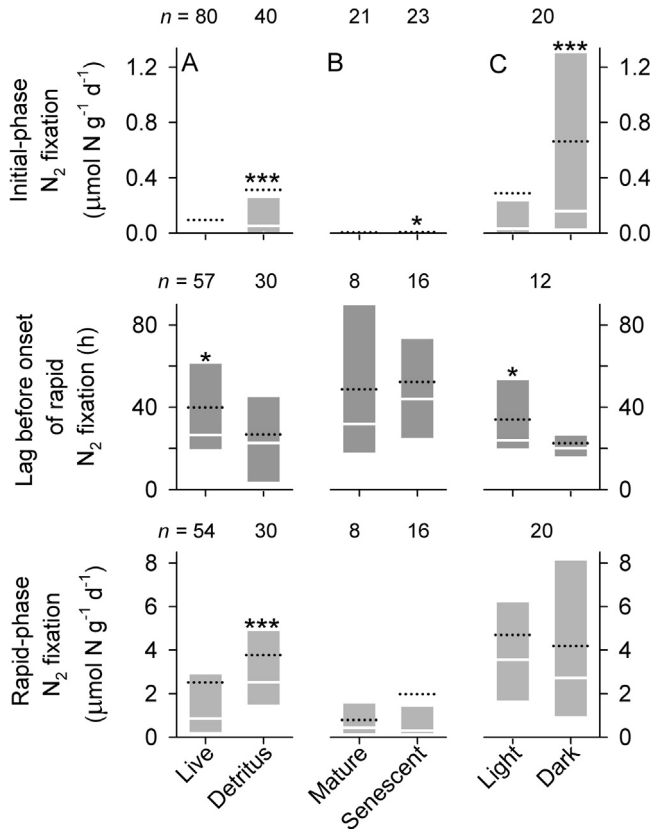

Fig. 3. Distributions of N_2 fixation rates associated with different conditions of the kelp sample. Initially measured rates of N_2 fixation were usually (85%) followed by a transition to a rapid N_2 fixation phase after a lag period. The number of measurements is indicated above each box plot; each measurement is the mean of three to five replicate incubations. A. Live kelp blades include rates from both mature and senescent material collected from benthos-attached plants. Detritus was kelp collected either on the shore or drifting. B. Mature and senescent kelp blades were both "live" and are described in Section 2.1. C. Light and dark experiments were paired (simultaneous measurement of light and dark N_2 fixation on the same kelp material) and combine results from both live and detrital kelp. Box plots show distributions from the 25th to the 75th percentile. Dashed lines indicate the mean, white lines indicate the median. Single and triple asterisks indicate significantly larger measurements at the $p < 0.05$ and $p < 0.001$ level, respectively. Statistical tests: panels A and B: two-sample one-tailed Mann–Whitney; panel C: one-sample one-tailed Mann–Whitney on arithmetical difference between treatments.

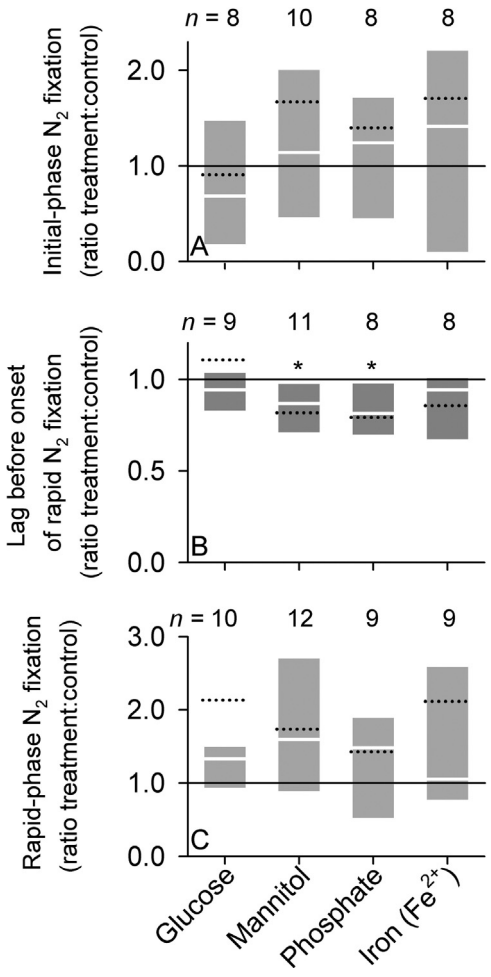

Fig. 4. Effect of four biochemical amendments on N_2 fixation rates associated with kelp (data from experiments on mature, senescent, and detrital samples were combined; see Section 2.5 for experiment description). Initially measured rates of N_2 fixation were usually (85%) followed by a transition to a rapid N_2 fixation phase after a lag period. The numbers of paired experiments (treatment vs. control) are indicated above each box plot; each measurement (treatment and control) was the mean of three replicate incubations. Box plots show distributions of the treatment:control ratios from the 25th to the 75th percentile. Dashed lines indicate the mean, white lines the median. Asterisks show significance at the $p < 0.05$ level (paired one-tailed Mann–Whitney test on log-transformed treatment:control ratios).

effect of mannitol was greater than that of glucose. Similarly, the amendments tended to decrease the lag time before the onset of rapid-phase N_2 fixation rates; the effect was significant at the $p < 0.05$ level for both mannitol and phosphate (Fig. 4B).

3.5. $^{15}N_2$ fixation experiment

Incubating *Macrocystis* blades with $^{15}N_2$ resulted in the ^{15}N enrichment of the organic N of the kelp and its associated epibionts relative to controls. N_2 fixation measured by $^{15}N_2$ assimilation and by acetylene reduction yeilded similar results in three of the four incubations (Supplementary Fig. 3).

4. Discussion

Macroalgae are known to have close associations with symbiotic microbes (reviewed in Hollants et al., 2013). The structure of the microbial communities associated with marcroalgae differs from that of the surrounding water, and is determined by both algal cell wall composition and secondary metabolites. Antimicrobial compounds produced by macroalgae deter pathogens, grazers and biofouling. Chemical signaling by macroalgae promotes colonization by beneficial microbes, which can play roles in macroalgal nutrition, morphogenesis, and life cycles. A number of macroalgal species are known to harbor N_2 fixing bacteria.

4.1. Identity of kelp-associated diazotrophs

Of the nine studies of macroalgal-associated diazotrophy that we reviewed, all but one attributed diazotrophy to photosynthetic cyanobacteria. Cyanobacteria have adaptations to sustain diazotrophy in aerobic environments, and may be the most important diazotrophic symbiont on living, photosynthesizing macroalgae. The one report associating heterotrophic diazotrophy with macroalgae found the bacteria endosymbiotically embedded in non-photosynthesizing macroalgal tissue where O_2 levels were likely lowered (Head and Carpenter, 1975). Although N_2 fixation associated with cyanobacterial symbionts of macroalgae was stimulated by light, in our study the initial N_2 fixation associated with *M. pyrifera* was inhibited by light (Fig. 3C) and stimulated by mannitol (Fig. 4), consistent with the hypothesis that heterotrophic bacteria could have been responsible. A recent 16S rRNA sequencing of the epimicrobiome of *M. pyrifera* shows that bacterial communities on

the kelp surface differ from those of the surrounding water, and change seasonally (Michelou et al., 2013). Although, no functional genes were amplified, the bacterial taxa identified include many groups known to include heterotrophic diazotrophs.

4.2. Oxygen limitation of N_2 fixation

Oxygen was found to limit N_2 fixation activity on *M. pyrifera*, consistent with the hypothesis of heterotrophic diazotrophy (Fig. 2). We measured much higher rates of N_2 fixation under anaerobic than under aerobic conditions (Fig. 2D). Anaerobic conditions in the incubation vials also reduced the lag period before rapid-phase N_2 fixation occurred and induced higher rapid-phase N_2 fixation rates (Fig. 2C).

However, it appears unlikely that living *M. pyrifera* kelp blades could be exposed to anaerobic conditions in situ. Our microsensor measurements showed that even in the dark, and at low water flow velocities (2 cm s^{-1}), O_2 levels at the blade surface remained above 75 μmol l^{-1}, or 27% of O_2 saturation (Supplementary Fig. 2A), much higher than the levels required to induce rapid-phase N_2 fixation in our incubations (Fig. 2D). Dissolved O_2 levels only decreased to <10% saturation (i.e. <30 μmol l^{-1}) with no water movement (Supplementary Fig. 2B), when the supply of O_2 to the blade surface was reduced as a result of a longer diffusion path. Although water flow rates in *M. pyrifera* forests <2 cm s^{-1} have been measured in sheltered locations within large forests (Hurd et al., 1996), it appears unlikely that strongly anaerobic conditions can be found around living kelp blades in situ. In contrast, anaerobic conditions would appear likely to develop around kelp detritus: rafts or wrack (Section 4.5).

4.3. No likely role for N_2 fixation in Macrocystis nutrition

Previous studies examining a possible role for macroalga-associated N_2 fixation in algal nutrition have shown that N_2 fixation was potentially capable of supplying up to 50% (*Codium*, Gerard et al., 1990) or 143% (*Sargassum*, Phlips et al., 1986) of macroalgal N requirements. However, an attempt to quantify the transfer of added $^{15}N_2$ to *Codium* biomass suggested that the fate of much of the fixed N may not be macroalgal assimilation, but instead may be cycling through the epibiotic community or lost via exudation (Gerard et al., 1990). In the present study, the apparently greater N_2 fixation associated with *M. pyrifera* in the oligotrophic waters south of Point Concepción, California, relative to samples collected farther north (where nitrate is more available in surface waters; Palacios et al., 2013), and relative to other macroalgal species, suggests the possibility of a specific adaption of *M. pyrifera* and its epimicrobiome to N limitation in these oligotrophic waters (Supplementary Table 2). However, the initial N_2 fixation rates we measured associated with southern Californian *M. pyrifera* populations (which we take as representative of in situ rates) averaged only 0.17 μmol N g^{-1} d^{-1}, or about 6% of the gross N assimilation by *M. pyrifera* (2.6 μmol N g^{-1} d^{-1}; modeled in a nearby *Macrocystis* forest during the May to August period when most of our samples were collected; Fram et al., 2008). Eighty seven percent of our measurements of initial kelp-associated N_2 fixation were capable of contributing less than 10% of this rate of kelp N assimilation (Fram et al., 2008). The initial N_2 fixation rates we measured on *M. pyrifera* were also at the low end of the range previously reported for other macroalgal species (Supplementary Table 3). It therefore seems unlikely that N_2 fixation is a major contributor to the nutrition of living *M. pyrifera*, even in the oligotrophic waters of the Southern California Bight.

4.4. Nitrogen fixation and Macrocystis decomposition

The highest N_2 fixation rates we measured were associated with kelp decomposing in mesh bags, where rates were two orders of magnitude higher than initial rates measured on live kelp (Fig. 1, Supplementary Table 2). In addition, both initial and rapid-phase N_2 fixation rates were higher in detritus and senescent kelp blades, where decomposition of the kelp blade was visible (Fig. 3). An association of high N_2 fixation rates with decomposition or senescence has also been noted for *Sargassum* by Hanson (1977) and for *Codium* by Phlips et al. (1986). Nitrogen fixation has also been associated with the decomposition of other aquatic plants, including mangroves (Woitchik et al., 1997) and saltmarsh grasses (Newell et al., 1992). Nitrogen fixation in decomposing kelp could be ecologically significant for microbial and invertebrate communities that are supported by decomposing kelp, such as wrack and kelp rafts (reviewed in Thiel and Gutow, 2005). Kelp rafts are composed of masses of floating detached plants and in the southern California Bight they average ca. 50 kg in weight and may persist for up to 110 days (Hobday, 2000). About half of the productivity of Southern California *M. pyrifera* forests is exported in rafts (Gerard, 1976). Kelp rafts are an important dispersal route for marine invertebrates and algae (Hobday, 2000; Thiel and Gutow, 2005), and a nutrient subsidy to the intertidal zone as wrack (Dugan et al., 2011). Within rafts, water flow through the densely massed and decomposing kelp is likely greatly reduced, and could lead to anaerobic conditions supporting higher N_2 fixation rates. The possibility that N_2 fixation could be ecologically significant in rafts of *Sargassum* was also suggested in Carpenter's (1972) original paper on macroalgal N_2 fixation. At the mean rapid N_2 fixation rates we measured for detrital kelp (ca. 2.9 μmol g^{-1} d^{-1}), and over an 80-day raft lifetime, N_2 fixation could potentially contribute an increase of ca. 47% above the initial ca. 1.2% N content of the kelp tissue. Similarly, we found a 42% increase in kelp N content during in situ decomposition. At the higher N_2 fixation rates measured at the end of our in situ decomposition experiment, N_2 fixation could contribute up to a 4-fold increase in N availability from kelp. Since, the C:N ratio of live kelp is high (mean 44.2, this study; data not shown), epibiotic N_2 fixation could potentially improve the nutritional quality of the kelp substrate for herbivores and detritivores (Smith and Foreman 1984).

4.5. Microbial community development in decomposing Macrocystis

Although initially anaerobic conditions around the kelp reduced the lag period before rapid N_2 fixation rates were measured, they did not eliminate it (Fig. 2C), suggesting that in addition to anaerobic conditions, microbial growth, shifts in community composition, or changing availability of macroalgal carbon to support bacterial respiration may also play a role in inducing the higher N_2 fixation rates. Studies of the variation in community structure across individual samples of the green seaweed *Ulva australis* showed, that although epibiotic microbial communities differed strongly in phylogenetic structure between individuals, the similarity in functional composition was high (Burke et al., 2011). The functional community structure of symbionts of living macroalgae is controlled by host physiology and biochemistry, but host physiology will likely have a diminished role in structuring the microbial communities of senescing or decomposing macroalgae. As the physiological responses of the host alga breakdown, the developing microbial community structure is likely to be determined by both stochastic and selective mechanisms of community assembly (Burke et al., 2011). The high proportion (85%) of kelp samples that developed rapid N_2 fixation rates after a median 26-h incubation suggest selective growth of diazotrophic microbes under conditions of low O_2

and kelp decomposition. The high C:N ratio of the kelp substrate (44.2) could be one of the factors selecting for diazotrophic growth. Diazotrophy associated with macroalgae may be photoautotrophic or heterotrophic, epibacterial or endobacterial, and may contribute to macroalgal growth or be associated with decomposition. Our study suggests a significant role for diazotrophs in the decomposition of *Macrocystis* biomass.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquabot.2015.05.003

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