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Oxygen-Poor Microzones as Potential Sites of Microbial N_2 Fixation in Nitrogen-Depleted Aerobic Marine Waters

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The nitrogen-deficient coastal waters of North Carolina contain suspended bacteria potentially able to fix N_2. Bioassays aimed at identifying environmental factors controlling the development and proliferation of N_2 fixation showed that dissolved organic carbon (as simple sugars and sugar alcohols) and particulate organic carbon (derived from *Spartina alterniflora*) additions elicited and enhanced N_2 fixation (nitrogenase activity) in these waters. Nitrogenase activity occurred in samples containing flocculent, mucilage-covered bacterial aggregates. Cyanobacterium-bacterium aggregates also revealed N_2 fixation. In all cases bacterial N_2 fixation occurred in association with surficial microenvironments or microzones. Since nitrogenase is oxygen labile, we hypothesized that the aggregates themselves protected their constituent microbes from O_2. Microelectrode O_2 profiles revealed that aggregates had lower internal O_2 tensions than surrounding waters. Tetrazolium salt (2,3,5-triphenyl-3-tetrazolium chloride) reduction revealed that patchy zones existed both within microbes and extracellularly in the mucilage surrounding microbes where free O_2 was excluded. Triphenyltetrazolium chloride reduction also strongly inhibited nitrogenase activity. These findings suggest that N_2 fixation is mediated by the availability of the appropriate types of reduced microzones. Organic carbon enrichment appears to serve as an energy and structural source for aggregate formation, both of which were required for eliciting N_2 fixation responses of these waters.

Oxygenated marine and freshwater systems, as well as aerobic soils, are often nitrogen depleted (6, 13, 31). Potentially, biological N_2 fixation could help alleviate nitrogen-limited primary and secondary production in such systems. However, among aquatic and terrestrial N_2-fixing microorganisms the enzyme complex responsible for the reduction of dinitrogen to ammonia, nitrogenase, is highly sensitive to and readily inactivated by molecular oxygen (8, 28).

Some filamentous cyanobacteria have adapted to this dilemma by forming biochemically and structurally differentiated cells termed heterocysts (45). Heterocysts, which lack photosytem II and therefore produce no oxygen (12), possess photoreducing capabilities and make use of reduced carbon compounds derived from neighboring vegetative cells to support N_2 fixation, while maintaining oxygen-free intracellular conditions (14, 38). However, N_2-fixing nonheterocystous cyanobacteria and other diazotrophic eubacteria have developed alternative ways of fixing N_2 under aerobic conditions. Symbioses, for example, such as the legume root nodule-*Rhizobium* sp., actinorhizal, lichen, *Azolla* sp., *Anabaena* sp., sponge-cyanobacteria, sea urchin-bacteria, and termite-bacterial gut flora associations exemplify biotic adaptations in which host plants or animals provide localized oxygen-poor microhabitats, or microzones, occupied by N_2-fixing endosymbionts (28, 39). Numerous asymbiotic N_2-fixing procaryotes must find other means of protecting nitrogenase under ambient aerobic conditions. It has been shown that the heterotrophic soil and aquatic bacterial genus *Azotobacter* assures compatibility between N_2 fixation and ambient oxygenated conditions by maintaining high endogenous respiration rates (11, 29, 32). Some marine and freshwater filamentous cyanobacteria (*Trichodesmium* and *Microcoleus* species) form aggregates (trichomes) supporting internal O_2-reduced microzones where N_2 fixation is thought to be concentrated (2, 4, 9, 24). There is also evidence that some organisms may cope with oxygen either by producing large quantities of nitrogenase so that some active enzyme remains available or by converting the active nitrogenase to a nonactive form which is insensitive to oxygen inactivation (15, 36). Another mechanism of bacterial O_2 protection may exist in which electrons under aerobic conditions are diverted from nitrogenase to flavodoxin to react directly with oxygen, thus inactivating nitrogenase by blocking electron flow to it (33, 46). Several cyanobacterial species have been found to separate temporally O_2-evolving photosynthesis from O_2-sensitive N_2 fixation by conducting N_2 fixation mainly during darkness (27, 35). In a recent study (20), it has been shown that a marine coccoid unicellular cyanobacterium (*Synechococcus* spp.) was able to alternately conduct O_2-evolving photosynthesis and N_2 fixation during continuous illumination. Apparently, photosynthetic O_2 evolution was greatly reduced during N_2-fixing periods, thereby facilitating the metabolic compatibility of these processes.

Recent studies have shown that aquatic and terrestrial asymbiotic microorganisms are capable of fixing N_2 in association with suspended and benthic organic detritus (5, 21), planktonic and benthic microbial mats (2, 19; L. J. Stal, Ph.D. thesis, University of Oldenburg, Oldenburg, Federal Republic of Germany, 1985), and a wide range of organically enriched soils (41–43). In such habitats patchily distributed, nutrient-enriched microenvironments or microzones occur (21, 22). It has been suggested that localized enrichment by metabolizable organic matter in such microzones provides both a heterotrophic energy source for powering N_2 fixation and a source of oxidizable substrates promoting localized oxygen removal (21, 22). The use of tetrazolium salts as redox indicators has revealed the presence of oxygen-depleted microzones as potentially suitable habitats for marine and freshwater N_2-fixing microorganisms (22, 24). Direct experimental evidence actually linking low oxygen concentrations and oxygen gradients to N_2 fixation poten-

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tials in such microzones has remained elusive. However, the advent of microelectrode technology has facilitated identification of oxygen-depleted microzones. We present results of assays for nitrogenase activity (NA) done on both detrital aggregates formed in response to the addition of dissolved and particulate organic matter to seawater and naturally occurring cyanobacterial aggregates. Aggregate-associated oxygen gradients, measured with microelectrodes and tetrazolium salt reduction, are also shown. The physiological and ecological impacts of marine microzone formation on N_2 fixation potentials are considered.

MATERIALS AND METHODS

Sampling locations and sources of N_2-fixing microzones. The Atlantic coastal waters of North Carolina typify full-salinity, nitrogen-depleted habitats. Previous nutrient addition bioassays have confirmed nitrogen-limited phytoplankton growth conditions on a year-round basis (22). In addition, nutrient analyses conducted by several investigators (40, 44) have consistently revealed depletion of inorganic nitrogen (NH_4^+, NO_2^- and NO_3^-) throughout much of the year, especially during spring-summer phytoplankton growth periods. On the basis of this information, we chose to examine detrital aggregate microzone formation and resultant impacts of N_2 fixation potentials in these waters.

Three aggregate types were examined. These were: (i) aggregates formed in response to dissolved organic carbon (DOC) additions, as the simple sugar maltose and sugar alcohol mannitol, to full-salinity seawater; (ii) aggregates formed in response to particulate organic carbon, as sterilized *Spartina alterniflora* particles, alone or in combination with DOC; and (iii) naturally occurring cyanobacterium-bacterium flocculant aggregates which can be found in both benthic (mudflats, lagoons, sandy beaches) and planktonic habitats. We examined flocs which were found floating in an experimental seawater pond located on the shore of Bogue Sound, N.C. These mucilaginous aggregates were found to contain predominantly nonheterocystous *Oscillatoria* sp. filaments and associated bacteria.

The choice of *S. alterniflora* as a source of particulate matter was based on its abundance as a marsh grass in North Carolina coastal waters and estuaries. Decomposing *Spartina* particles are a common source of particulate organic carbon in these waters. Previous experiments (H. W. Paerl, K. Crocker, and L. E. Prufert, Limnol. Oceanogr., in press) showed maltose and mannitol to be effective carbohydrates capable of stimulating and supporting naturally occurring heterotrophic N_2-fixing bacteria. Accordingly, we focused on mechanisms by which enrichment with such DOC compounds enhanced N_2 fixation potentials.

Aggregate types 1 and 2 formed in response to DOC and particulate organic carbon additions to seawater which were made during a series of experiments designed to assay for factors limiting N_2 fixation in coastal waters (L. E. Prufert and H. W. Paerl, manuscript in preparation). Full-salinity surface seawater samples were collected from Beaufort Inlet (connected to the Atlantic Ocean). Subsamples of 60 ml were dispensed, in triplicate, into sterile 125-ml Erlenmeyer flasks having additions of 11 mM maltose for type 1 or 1 or 5 mM mannitol plus 2 g (wet weight) of ground, rinsed, heat-sterilized *Spartina* stem and leaf particles for type 2. Samples were incubated under dark and light, (200 microeinsteins $\cdot M^{-2} \cdot s^{-1}$ photosynthetically active radiation as cool-white and Grolux fluorescent light). Incubation temperatures corresponded to Beaufort Inlet water temperatures at the time of sampling. All samples were agitated by hand once a day and left stationary for the remainder of the time. Flocculent aggregates consisting of a variety of bacteria, diatom frustules, and unidentified particles embedded in an amorphous mucilaginous matrix appeared within 2 to 3 days of incubation.

Samples were intermittently tested for NA along with untreated seawater controls and deionized water blanks during the incubations. NA was determined by the acetylene reduction method (25, 37), modified for our samples as follows. Subsamples (20 ml) for each of three replicates were withdrawn and placed in 25-ml Erlenmeyer flasks which were then sealed with serum bottle rubber stoppers. Samples were then injected with 4 ml of high-purity acetylene (Matheson Scientific, Inc., Elk Grove Village, Ill.), shaken for 3 s, and left to incubate under static conditions for 3 to 4 h under dark and light conditions. At the end of the incubation 0.3-ml headspace samples were assayed with a Carle AGC-311 gas chromatograph, equipped with a flame Ionization detector and a 2-m-long Poropak T column. Column temperature was 80°C, and ultra-high-purity nitrogen (Matheson) was used as the carrier gas.

Additional subsamples from both freshly sampled cyanobacterium-bacterium and experimental maltose- and mannitol-*Spartina* aggregates were examined for localized reduction of the tetrazolium salt 2,3,5-triphenyl-3-tetrazolium chloride (TTC). This salt, which is colorless and water soluble under oxidized conditions, forms insoluble, microscopically observable formazan crystals when reduced (1). The conversion of TTC to formazan crystals requires reducing conditions ($E_{1/2}$ = +0.44 V), and will not occur if free oxygen is present (1). These properties make TTC useful for identifying both intra- and extracellular reduced microzone regions in aggregates (21, 24). Independent investigations have shown that localized TTC reduction (formazan deposition) is closely linked to sites of N_2 fixation in cyanobacteria (14, 24, 38), eubacteria (26), and both freshwater and marine detrital aggregates (21). Fay and Kulasooriya (14), Bryceson and Fay (4), and Paerl and Bland (24) have shown that TTC reduction strongly inhibits N_2 fixation (acetylene reduction) among a variety of cyanobacteria. The exact mechanism of this inhibition is unknown; however, competition for reductant between nitrogenase and TTC or morphological disruption near the site of N_2 fixation owing to the intracellular precipitation of crystals may be operating. We added TTC at a final concentration of 0.01% (wt/vol) 1 h before acetylene reduction assays. The impacts of TTC reduction on acetylene reduction, relative to TTC-free conditions, as well as observable patterns of formazan deposition were examined. Formazan deposition was examined microscopically after 0.5 to 3 h of incubation with TTC under both illuminated and dark conditions. After incubations and acetylene reduction gas withdrawal, 3% borate-buffered (pH 8.0) glutaraldehyde was added as a fixative. Samples were then stored in a refrigerator for subsequent microscopic observations. We used a Zeiss research-grade phase-contrast (oil immersion) microscope at 160× to 1,000× for observations and photomicrography.

Dissolved oxygen gradients associated with aggregates were measured with a 10-μm-wide (at tip) glass, polarographic, O_2-sensitive microelectrode (7, 30). Microelectrode response time ranged from 10 to 20 s. The microelectrode was polarized at −0.75 V through the use of a Diamond Electro-Tech Inc. model 1201 chemical microsensor. Air-bubbled (21% O_2) and argon-bubbled (0% O_2) seawater samples were used to calibrate the microelectrodes.

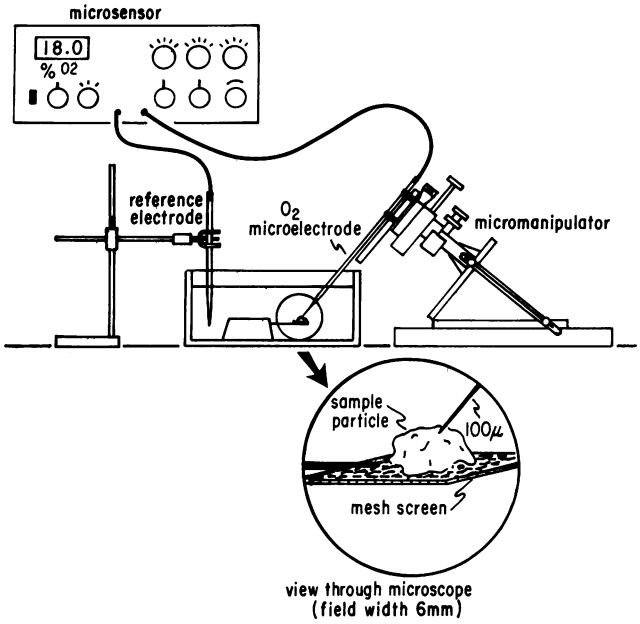

FIG. 1. Schematic diagram of microelectrode apparatus used to measure oxygen gradients associated with marine aggregates.

Output was read from the microsensor display or O_2 partial pressure and was later converted to O_2 concentration. The gradient measurement setup consisted of a square Plexiglas box in which a small (1-cm^2) Nitex screen was suspended by attachment to a plastic bar and secured to a rubber stopper (Fig. 1). After the box was filled with seawater, sample particles were gently placed by forceps onto the screen. Samples were allowed to equilibrate for approximately 30 min before measurements. A microscope was mounted on the side of the box so that the screen and particles could be observed during O_2 measurements. The O_2 microelectrode and reference electrode (Diamond Electro-Tech Inc. model 401) were both placed in the seawater, the O_2 electrode being attached to a micromanipulator. Using the microscope to locate the electrode tip in relation to the aggregate, the tip was first touched to the surface of the particle and then backed away 2 to 4 mm. Oxygen readings were taken at 0.1-mm increments as the electrode approached and pierced and the particle surface. Borate-buffered (pH 8.0) Formalin was later added to the seawater (0.1 to 4.0%, vol/vol) in the box, and O_2 readings were taken in the same manner as above to determine gradients in biologically inactivated particles.

RESULTS

Additions of sterile *Spartina* particles alone and in combination with 1 and 5 mM mannitol led to the formation of detrital aggregates within a 2- to 3-day period. Aggregates appeared similar in size (40 to 200 μm in diameter) and composition to particles previously observed in coastal waters bordering tidally flushed marshes, lagoons, and mudflats (21). In addition to containing partially decomposed *Spartina* particles, aggregates revealed silt, empty diatom frustules, and unidentified particles, all of which were populated by a variety of coccoid, rod-shaped, and filamentous bacteria (Fig. 2). Bacterial colonization was patchy; surface colonization densities typically varied from <2 to >30 bacterial cells per 100 μm^2. Attached populations were predominantly embedded in mucilaginous slimes which formed an amorphous glue promoting aggregation (Fig. 2). These observations are similar to earlier descriptions of both marine and freshwater detrital aggregates (21). In current experiments mucilaginous materials coating or embedded in aggregates are estimated to account for 20 to 50% of aggregate volumes.

Maltose additions increased both the incidence of bacterial colonization and mucilage production in *Spartina*-based aggregates. By itself, maltose enrichment promoted the formation of suspended bacterial flocs and aggregates incorporating empty diatom frustules, silt, and other small particles in the mucilaginous matrix associated with bacterial populations (Fig. 3). Bacterial densities for 500- to 1000-μm-diameter roughly spherical flocs varied from 2×10^3 to 5×10^4 cells per floc. Control conditions, receiving neither *Spartina* nor DOC additions, occasionally supported the production of small bacterium-particle aggregates, although they were much fewer in number and smaller in size than those seen under *Spartina*-DOC-amended conditions. Magnitudes of bacterial colonization and resultant mucilage production were distinctly lower under control as opposed to either *Spartina*- or maltose-amended conditions.

While freshly collected or stored Beaufort Inlet water consistently failed to exhibit NA, the addition of sterile *S. alterniflora* alone or in combination with maltose elicited NA within a 2-to 5-day incubation period (Fig. 4). Added alone, maltose also led to the development of NA (Fig. 4). Generally, NA appeared from 2 to 5 days after initial maltose additions. Among all treatments NA responses were at least as high under dark as under illuminated conditions. In a few cases NA was slightly higher under dark than under illuminated conditions, indicating potential inhibition of NA perhaps owing to O_2 evolved by photosynthetic microorganisms associated with aggregates. Microscopic observations revealed the occasional presence of what appeared to be viable coccoid and filamentous cyanobacteria as well as diatoms in aggregates.

Freshly sampled cyanobacterium-bacterium aggregates characteristically showed NA. Overall, NA rates per aggregate and per milligram (fresh weight) of filter-concentrated aggregates were highly variable among replicated samples (Fig. 5). Similarly, a great deal of variability existed between samples taken on different dates from the same location (Fig. 5). No significant differences between dark and illuminated incubations were observed at any time (Fig. 5). It therefore appeared that NA may have been confined to heterotrophic bacteria which appeared patchily distributed among aggregates. In a separate study (H. W. Paerl and B. Bebout, manuscript in preparation) several strains of N_2-fixing heterotrophic bacteria have been isolated from these aggregates.

Both patterns and magnitudes of TTC reduction showed distinct differences between control and *Spartina*- or maltose-amended samples. Among 50 control (unenriched seawater incubated for 2 days) aggregates examined only 3 (6%) revealed formazan deposition within a 2-h TTC incubation period. Formazan deposition was confined to small internal regions of these aggregates. In contrast, *Spartina*- or maltose-mannitol-based aggregates revealed much higher incidences of TTC reduction, with formazan deposition observed in localized regions of aggregates (Fig. 6A). Among 2-day-old *Spartina*-based aggregates, 36% exhibited internal formazan deposition after a 2-h TTC incubation. Maltose-based aggregates showed 65% TTC reduction, while com-

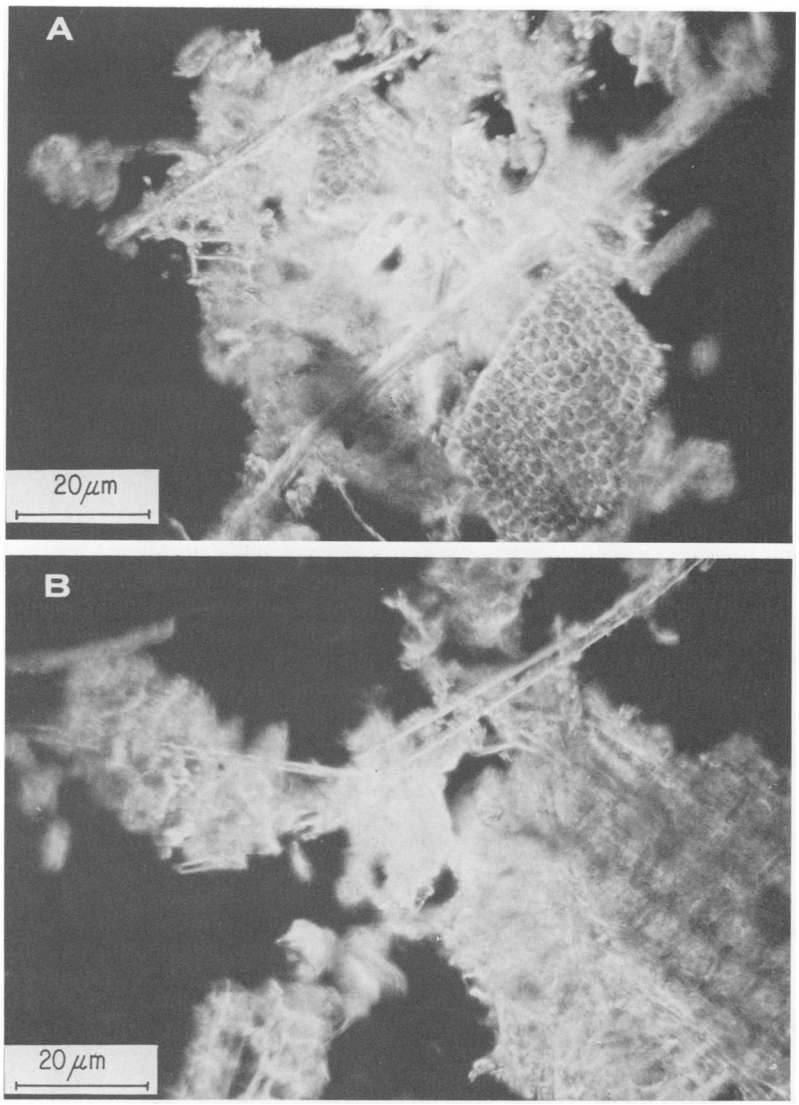

FIG. 2. (A) Darkfield photomicrograph of a suspended aggregate composed of *Spartina* fragments and associated organic matter. (B) *Spartina*-based aggregate, showing associated empty diatom frustules and silt particles glued to *Spartina* detritus with amorphous mucilaginous slimes. Bacteria commonly colonized such particles, being particularly numerous in organic slimes.

bined *Spartina*-mannitol-based aggregates exhibited 79% TTC reduction. Patterns of TTC reduction closely coincided with regions of extensive bacterial colonization among all aggregates observed. Resultant formazan deposits were observed both inside bacterial cells and within extracellular mucilaginous matrices in which bacteria were embedded (Fig. 6B).

Among freshly sampled cyanobacterium-bacterium aggregates, TTC reduction was observed (in a 2-h incubation) inside both *Oscillatoria* filaments and associated bacterial clumps (Fig. 6C). Bacterial colonies embedded in these aggregates similarly exhibited both intra- and extracellular (in mucilaginous slimes) TTC formazan deposition. Among 30 aggregates observed, 25 exhibited formazan deposition.

Patterns and intensities of formazan deposition appeared identical under illuminated and dark conditions. This observation suggests that heterotrophic processes were responsible for TTC reduction and that direct photoreduction (by fluorescent lights) of TTC played no significant part in observed patterns and magnitudes of formazan deposition.

Microscopic confirmation of TTC reduction was strongly correlated with detectable NA among all aggregates examined. Generally, whenever internal patches of formazan deposition were evident within a 2-h TTC incubation period, significant (at least three times the blank value) rates of NA were evident in parallel subsamples incubated with acetylene. Shaking (400 rpm) and O_2 enrichment (150 to 200% O_2 saturation) treatments each had negative effects on both TTC reduction and NA among aggregates examined, suggesting that internal reduced conditions were strongly linked to N_2-fixing potentials among such aggregates (Fig. 7).

When TTC reduction preceded (by at least 1 h) acetylene reduction assays in the same vessels, profound inhibition of NA was consistently observed (Fig. 8). Formazan deposition had to occur before the addition of acetylene to achieve inhibition. Parallel TTC and acetylene reduction assays

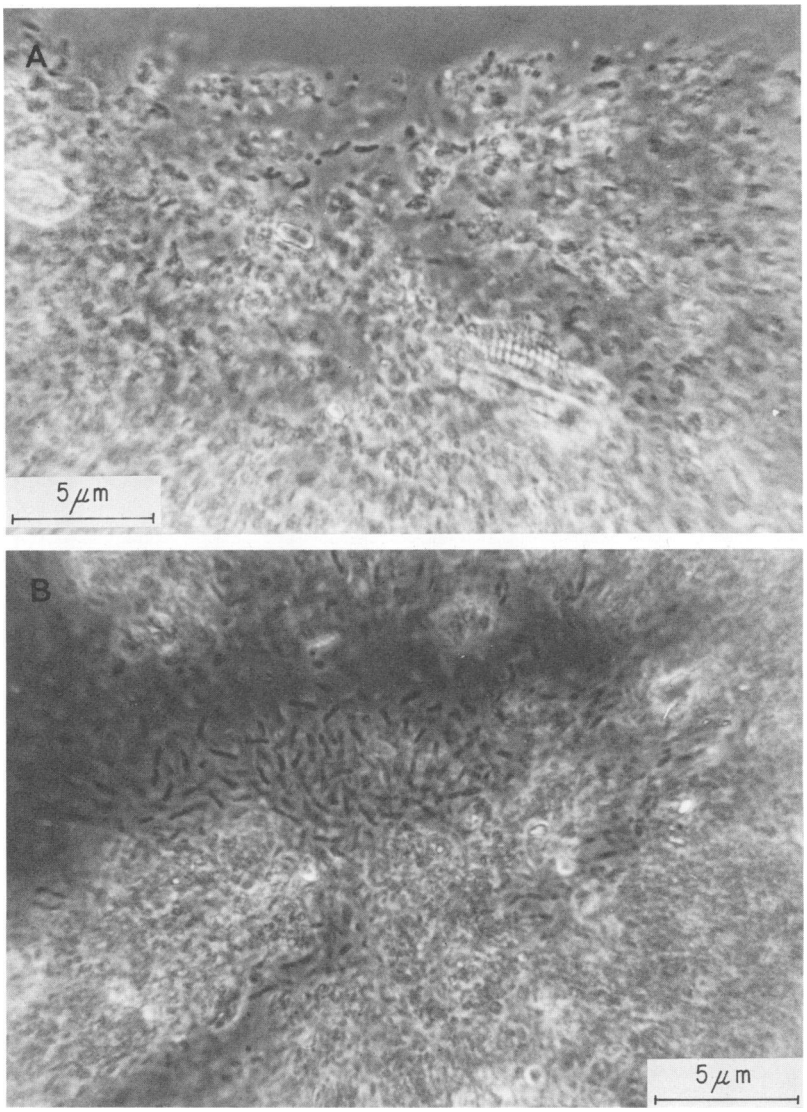

FIG. 3. (A) Edge view of a maltose-based marine aggregate photographed by phase-contrast microscopy. Both associated bacteria and remnants of an empty diatom frustule embedded in a mucilaginous slime matrix can be seen. (B) Another view of a maltose-based aggregate, revealing invaginated outer edge, hosting extensive bacterial colonization. Note the amorphous matrix forming the inner portion of aggregates. This matrix was largely composed of particulate matter, bacteria, and mucilaginous slime.

revealed little inhibitory impact of TTC addition on NA within the first 30 min of incubation (Fig. 8). During this time interval no formazan deposition was observed. It appears, therefore, that the physical formation of formazan crystals is a key step responsible for NA inhibition. At present it is uncertain whether the observed inhibition is due to the ability of TTC to outcompete acetylene for reducing equivalents (electrons) or whether formazan deposition alters the structural configuration of the nitrogenase enzyme complex, thereby inactivating N.A. This form of NA inhibition occurred with equal intensity and speed under both illuminated and dark conditions.

Microelectrode analyses revealed O_2 gradients associated with the three types of aggregates. Among bacterium-particle aggregates promoted by maltose additions, in situ O_2 gradients were quite pronounced as the electrode approached individual aggregates (Fig. 9). Within 2 mm of what could microscopically be determined as the structural body of the aggregate, O_2 levels dropped from 407 to 91 μM. The O_2 gradient going into the particle appeared somewhat less pronounced, ranging from 90 to 70 μM over 0.4 mm. Seven profiles taken within the same aggregate all appeared similar in magnitude and shape. Immediately after 4% (vol/vol) Formalin addition, O_2 gradients associated with these aggregates greatly diminished. Ten minutes after Formalin addition we observed further, although less dramatic, reduction of gradients.

Spartina-mannitol-based aggregates also revealed O_2-depleted conditions. However, these aggregates exhibited less pronounced O_2 gradients than aggregates resulting from maltose enrichment (Fig. 10). On average, O_2 gradients in *Spartina*-mannitol aggregates revealed -21 μM O_2 per mm change. Formalin additions (0.1%, vol/vol) led to a delayed response in terms of decreased O_2 gradients. Little impact of Formalin was observed until 35 min after addition. However, after 45 min of formalin exposure O_2 gradients were

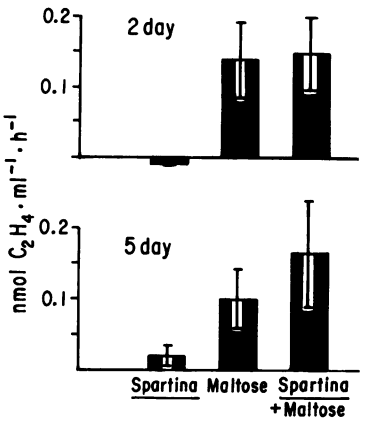

FIG. 4. NA (as nanomoles of C_2H_4 per milliliter per hour) elicited by maltose, *S. alterniflora*, and *Spartina*-plus-maltose additions made to seawater. revealed no detectable NA. Samples were assayed after 2- and 5-day incubations. Standard errors among triplicates are shown.

essentially gone. The apparent delay in O_2 gradient changes in response to Formalin, as compared with the maltose aggregate response, might have been due to both the relatively low Formalin concentration used (0.1 versus 4.0%) and the presence of extensive mucilaginous deposits in and around aggregates which can act as barriers to inward diffusion of Formalin as well as to other molecules, including O_2.

Microscopic observation of both maltose- and *Spartina*-based aggregates revealed that the flocculant, mucilaginous material in which the bacterial cells were embedded was transparent to semi-opaque, extruding from the readily identifiable aggregate surface into surrounding seawater. Possibly, such surface-associated extrusions helped to create the observed O_2 gradients external to the main body of such aggregates.

Similarly, freshly sampled cyanobacterium-bacterium aggregates revealed extensive mucilage production, resulting in a virtually transparent slime coating extending well away from the main aggregate structure. Gradients showed average changes of -40 μM O_2 per mm (Fig. 11). Formalin additions (2.0%) led to a breakdown of gradients. However, resultant gradient extinction was far from immediate. It took up to 12 h of Formalin exposure to eliminate gradients. The O_2 gradients of both *Spartina* and cyanobacterium-bacterium aggregates were not as smooth as those measured in maltose-based aggregates. Structural differences among respective aggregates may have been responsible for contrasting gradient curve characteristics. Maltose-based aggregates appeared much more homogeneous than either *Spartina* or cyanobacterium-bacterium aggregates. The former largely consisted of a variety of bacterial colonies aggregated within a mucilaginous matrix having relatively few additional inorganic (clay, silt) or organic (decomposing macrophyte and phytoplankton) particles. In contrast, both *Spartina*- and cyanobacterium-based aggregates appeared much more heterogeneous, including various particle shapes and sizes and patchy distributions of bacterial colonies and associated mucilaginous deposits wedged between *Spartina* particles and cyanobacterial filaments. It is likely that the irregular shapes of O_2 gradients are related to patchy microbial distributions in the latter types of aggregates. Since TTC reduction was most intense within microbial colonies distributed in aggregates, these colonies appeared to be the sites of the most highly reduced conditions. As microelectrode transects are taken through heterogeneous particles, such highly reduced patches could be responsible for observed small fluctuations in O_2 gradients.

The diameters of bacterial colonies found in aggregates varied from less than 10 μm to well over 80 μm. Colonies residing in the small size categories cannot be accurately measured with a 10-μm-wide microelectrode. Hence, our ability to resolve adequately microzones on this order of size is currently limited.

DISCUSSION

Previous studies have repeatedly shown that, except for sporadic late-summer blooms of the filamentous N_2 cyanobacterium *Trichodesmium* sp. and the occasional presence of suspended cyanobacterial *Oscillatoria* spp.-bacterium aggregates, NA is seldom detected in near-shore nitrogen-depleted Atlantic Ocean waters bordering North Carolina (21; Paerl et al., in press). Furthermore, rates of NA detected during N_2-fixing periods fall far short of phytoplankton community demands (21; Paerl et al., in press). This situation appears paradoxical, since persistent nitrogen depletion would be expected to favor the establishment and proliferation of an N_2-fixing community. It appears, therefore that environmental factors other than mere nitrogen depletion must regulate N_2 fixation in these waters.

Results presented here and elsewhere (Paerl et al., in press) indicate that the scarcity of NA among freshly sampled coastal waters cannot be attributed to the absence of N_2-fixing microbial taxa. The fact that NA can consistently be elicited in response to sterile particulate and dissolved organic matter additions indicates that heterotrophic bacteria (and possibly cyanobacteria) potentially capable of N_2 fixation are present. These results are in agreement with recent studies (16) demonstrating that a variety of bacterial N_2 fixers can be isolated from oligotrophic nitrogen-deficient marine waters, despite the fact that such bacteria may be inactive in situ.

Previous examinations of potential physicochemical factors controlling NA potentials of resident microorganisms have come to the following conclusions for North Carolina coastal waters. (i) The apparent absence of detectable rates of NA is not due to salinity characteristics of these waters (21, 22); (ii) iron and molybdenum, components of nitrogenase, are available in sufficient quantities in ambient waters;

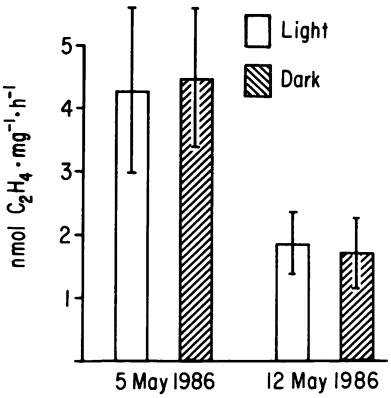

FIG. 5. NA among unamended, naturally occurring cyanobacterium-bacterium aggregates incubated under light and dark conditions on two sampling dates. Standard errors among triplicates show a high degree of variability.

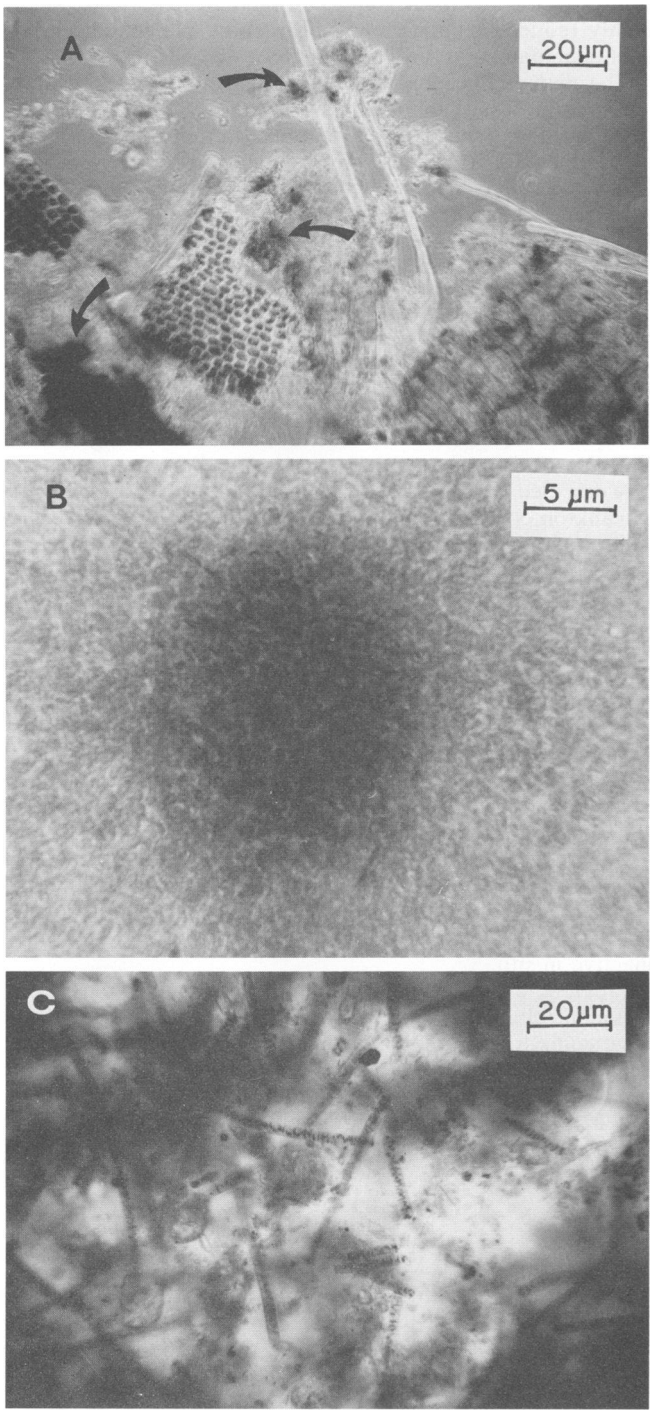

FIG. 6. (A) Localized TTC reduction in a *Spartina*-maltose-based aggregate. Note darkened regions (arrows) appearing both in mucilaginous slimes and in association with *Spartina* leaf fragments. Darkening in these regions is due to deposition of reduced formazan crystals, indicating oxygen-depleted microzones. During the course of TTC reduction surrounding waters remained fully oxygenated. (B) TTC reduction, shown as a dark patch, within internal regions of a maltose-based aggregate. Formazan deposition occurred both intracellularly (in bacteria) as well as in extracellular interstitial regions. (C) Localized TTC reduction in naturally occurring cyanobacterium-bacterium aggregates. Concentrated formazan deposits (darkened regions) were observed in bacterial colonies, associated mucilage, and inside cyanobacterial (*Oscillatoria* spp.) filaments.

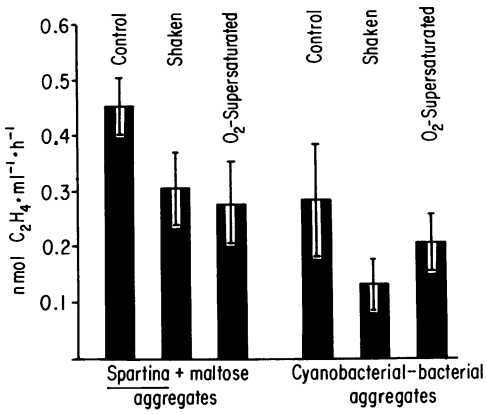

FIG. 7. Negative impacts of shaking (400 rpm) and oxygen supersaturation (150% O_2 saturation) on NA among both *Spartina*-maltose as well as cyanobacterium-bacterium aggregates. Standard errors among triplicates are shown. Controls indicate nonshaken, O_2-saturated (~100% O_2 saturation) conditions.

enrichment with these metals failed to elicit or enhance N_2 fixation among a large number of bioassays thus far completed (22; Paerl et al., in press); (iii) phosphate additions occasionally stimulated N_2 fixation, although results are highly variable in bioassays run thus far (L. E. Prufert and H. W. Paerl, manuscript in preparation); (iv) the same bioassay studies have shown that the addition of particulate matter (both organic and inorganic) and DOC compounds (simple five- or six-carbon sugars and alcohols) consistently led to the development and proliferation of N_2 fixation in these waters.

It is well known that submersed surfaces play key roles in eliciting and enhancing the growth of diverse microorganisms (3, 23), including photosynthetic procaryotes and eucaryotes, heterotrophic bacteria, and fungi. This surface effect is responsible for accelerated multiplication of microorganisms either stored in containers (47) or left associated with suspended and settled sediments as well as organic or inorganic detritus (22, 23).

Nitrogen fixation in response to particle (*S. alterniflora*) additions is linked to the fact that such particles provide both colonizable surfaces and a relatively concentrated source of organic matter enrichment, supplying readily metabolizable carbon sources needed for supporting this energy-demanding process. Further enrichment with soluble organic carbon compounds increases available energy supplies. In addition to providing necessary energy sources, the metabolic oxidation of organic matter results in O_2 consumption. Among surface and aggregate-associated microbial populations this leads to the formation of O_2-poor microzones. Owing to physical heterogeneity of particles and aggregates and resultant bacterial colonization in available niches, a patchy distribution of such microzones results. Such patchiness in turn leads to the formation of O_2 gradients in and around particles and aggregates.

Both TTC reduction and O_2 microelectrode analyses confirmed the existence and patchy distributions of reduced microzones and O_2 gradients associated with freshly sampled and *Spartina*-carbohydrate-based particles and aggre-

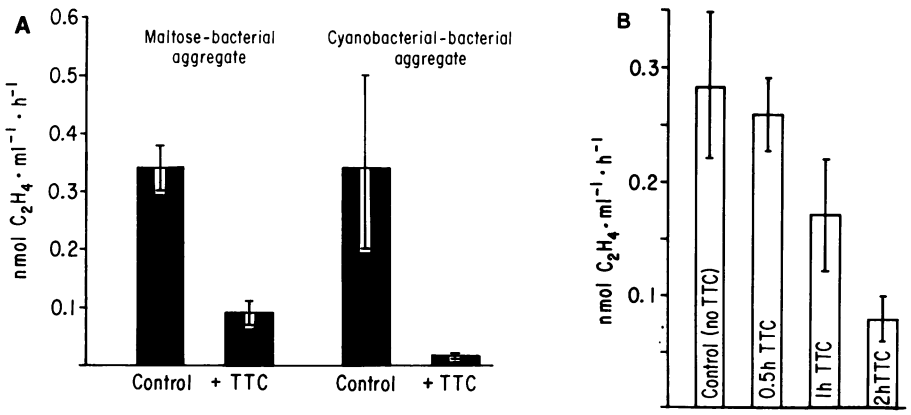

FIG. 8. (A) NA of control (untreated) versus TTC-treated maltose-bacterium and cyanobacterium-bacterium aggregates. TTC was added 1 h before initiation of acetylene reduction assays. Length of ensuing acetylene reduction assays was 2 h. Standard errors among triplicates are shown. (B) Effect of length of TTC incubation on relative (to controls) TTC inhibition of NA in *Spartina*-maltose aggregates. TTC was added from 0.5 to 2 h before conducting 1-h acetylene reduction assays.

gates. Furthermore, the development of reduced microzones appears to be crucial for both the development and proliferation of NA which in these experiments was attributable to selective stimulation of naturally occurring microorganisms; as a rule, conditions promoting extensive reduced microzone development favored maximal NA rates, while no NA was observed in samples having few or no aggregates. Last, the ability of localized TTC reduction to inhibit NA serves as additional evidence that sites of formazan deposition are likely loci of particle-associated N_2 fixation.

Our results suggest reduced microzone formation as being a key mechanism leading to the development and proliferation of marine N_2 fixation. Both organic matter and colonizable surface availability play joint roles in reduced microzone formation. The proper combination of these variables satisfies two requirements which eubacterial and nonheterocystous cyanobacterial genera must satisfy before conducting N_2 fixation: (i) an available energy source required by all heterotrophic N_2 fixers and (ii) localized O_2-poor conditions in which nitrogenase can actively function. Such requirements are not uniquely confined to marine planktonic habitats. Free-living heterotrophic sediment and soil microorganisms must encounter similar environmental conditions before initiating N_2 fixation (28), whereas among endosymbiotic N_2 fixers, these conditions are met by the host plant or animal (28, 39). However, among aquatic sediments, soils, and endosymbioses, organic matter availability, which ensures adequate energy supplies and localized deoxygenation, is generally less constrained than it is in the highly dilute water column. Accordingly, it is not surprising to find both reduced microzone development and associated N_2 fixation to be much more ubiquitous and quantitatively significant in the former habitats.

Because of its strong reliance on anaerobic conditions and its widespread presence in a variety of relatively primitive procaryotic microorganisms (including anaerobic heterotrophic decomposers, H_2S-utilizing photosynthetic bacteria, methanogens, and sulfate reducers), it is believed that N_2 fixation appeared relatively early during the biochemical evolution of procaryotes. In all likelihood early-free-living N_2 fixers relied heavily on geochemically formed organic matter as an energy and reductant source to support this process (10, 18). Presumably, such microorganisms had ready access to carbon sources in O_2-poor surface waters

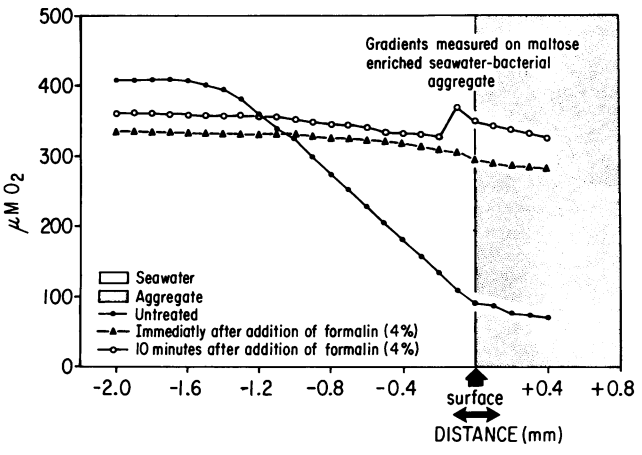

FIG. 9. Oxygen (micromolar O_2) gradients measured on a maltose-bacterium aggregate; control and Formalin-treated conditions are compared.

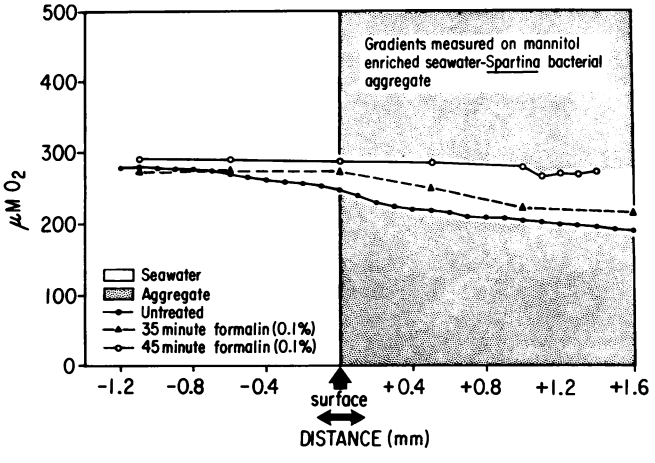

FIG. 10. Oxygen (micromolar O_2) gradients measured on a *Spartina*-mannitol aggregate; control and Formalin-treated conditions are compared.

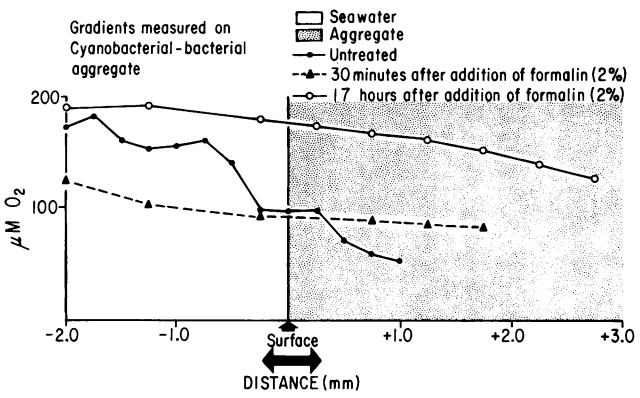

FIG. 11. Oxygen (micromolar O_2) gradients measured on a cyanobacterium-bacterium aggregate; control and Formalin-treated conditions are compared.

and soils before the development of O_2-evolving photosynthesis.

The advent of O_2-evolving (oxygenic) photosynthesis, attributable to cyanobacteria during the pre-Cambrian eras (34), has led to present-day confinement of many free-living N_2-fixing taxa to O_2-depleted microzones. Within a geological time frame, development and proliferation of heterotrophy among both procaryotes and eucaryotes has led to steady depletion of geochemically formed dissolved and particulate organic matter in the world's oceans, once (during the pre-Cambrian era) considered to be organic matter-enriched primordial soups (10, 17). Trends in organic matter depletion have paralleled increases in biospheric O_2 content, resulting from the evolution and diversification of oxygenic photosynthetic plants. Both trends have, from energetic and ecological perspectives, led to severe constraints on N_2-fixing microorganisms in the contemporary biosphere. The vast oligo- and mesotrophic planktonic marine environments of the world are perhaps the most extreme examples of forced confinement of N_2 fixation to microzones.

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