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Enumeration and Relative Importance of Acetylene-Reducing (Nitrogen-Fixing) Bacteria in a Delaware Salt Marsh

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Three groups of N_2-fixing bacteria were enumerated from the top 1 cm of the surface in four vegetational areas in a Delaware salt marsh. The results over the 9-month sampling period showed that there were no discernible seasonal patterns for any of the groups enumerated (*Azotobacter* sp., *Clostridium* sp., and *Desulfovibrio* sp.). *Azotobacter* sp. was present in numbers of 10^7 per g of dry mud, whereas the two anaerobic fixers were present in much lower numbers (10^3 to 10^4 per g of dry mud). There were no differences in the numbers of each group among the different vegetational areas, indicating that there was a heterogeneous population of N_2 fixers present. Additional studies indicate that the activity of sulfate reducers (*Desulfovibrio* sp.) may account for as much as 50% of the total observed acetylene reduction activity. Oxygen was found to exert little effect on the observed acetylene reduction activity, indicating that stable aerobic and anaerobic microenvironments exist in the surface layer of marsh sediments.

The importance of symbiotic nitrogen fixation in agricultural systems has been well known for many years. Only in recent years have "free-living" nitrogen-fixing bacteria been recognized to be important components of many ecosystems. One area receiving considerable attention in recent years has been the salt marsh system. We have previously presented data (10) which show that acetylene reduction activity (ARA) is seasonal, with peaks of activity occurring in the late summer and early fall. In addition, the majority of activity is heterotrophic in nature, and physiological control over ARA is exerted by the in situ levels of ammonia or nitrate or both. Previous workers have also studied nitrogen fixation in salt marsh systems, and our results agree closely with theirs in terms of the seasonality of ARA (6, 16, 23, 32, 38). These workers have also noted differences in ARA between different vegetational areas of the marsh. However, little work has been done concerning the organisms responsible for the observed ARA. Several workers have reported that various blue-green bacteria are the major fixers present (7, 23), whereas other workers have indicated that heterotrophic bacteria are of major importance (19, 38, 40). Patriquin and McClung (32) enumerated several groups of N_2-fixing organisms and found that anaerobes are present in numbers of 10^6 per g of wet rhizosphere soil of *Spartina alterniflora* whereas N_2-fixing microaerophiles are less abundant (10^5/g of wet rhizosphere soil). Nearly equal numbers of bacteria were reported to be present on washed *Spartina* roots. These workers also concluded that mud surface ARA may be associated with photosynthetic bacteria.

Little work has been done concerning the possible seasonal occurrence of heterotrophic N_2-fixing bacteria in marsh soils. In this study three groups of heterotrophic N_2 fixers were enumerated over a 9-month period, and their relative contributions to the observed ARA were estimated. In addition, the effects of O_2 on ARA were examined.

MATERIALS AND METHODS

Study site. Four sites were established in the Canary Creek marsh adjacent to The University of Delaware Marine Station facilities in Lewes, Del. The stations were located in four different vegetational areas of the marsh. Station 2 was located in a zone of mixed vegetation: *S. alterniflora* (Loisel), short form; *Distichlis spicata* (L.) Greene, and *Salicornia virginica*. Station 3 was located in an area of *S. alterniflora* (tall form), and station 4 was in an area composed entirely of short *S. alterniflora*. Station 5 was located at the boundary between the high marsh and a "tree island." The major vegetation at this site was composed of *Iva frutescens* L. var. *oraria* (Bart.) Fern. and Griscom, *Baccharis halimifolia* L., *Rhus radicans* L. More detailed station characteristics have been presented elsewhere (10).

Acetylene reduction assay. Samples were collected from the top 1 cm of the marsh surface from the four stations and assayed for ARA as described previously (10). For experiments dealing with the effect of O_2 on ARA, the oxygen concentration was varied to give gas mixtures containing 0, 5, 10, or 15% O_2. The acetylene level remained constant at 10%, and

the balance was argon. All reported rates of ARA are average 5-day rates (10). This method of analysis is likely to reveal the maximum ARA potential of a sample (10). Population densities of *Azotobacter, Clostridium,* and *Desulfovibrio* did not change during the 5-day ARA incubations (data not shown).

Bacterial enumeration and isolation. The numbers of presumed nitrogen-fixing *Azotobacter, Clostridium* sp., and *Desulfovibrio* sp. were determined on a bi-weekly basis from November 1977 to August 1978. *Azotobacter* was enumerated by the plate dilution frequency technique of Harris and Somers (20), whereas *Clostridium* sp. and *Desulfovibrio* sp. were determined by the most-probable-number method (18). All bacteria were enumerated on modifications of the medium of Smith and Rittenberg (37). The basic medium consisted of three parts: (i) mineral salts (grams per liter)—K_2HPO_4, 0.6; KH_2PO_4, 0.4; $CaCl_2 \cdot 2H_2O$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 20.0; (ii) trace metals as described by Allen (2); and (iii) Fe-ethylenediaminetetraacetic acid solution as described by Schmidt et al. (36). The pH for all media was 6.8 to 6.9. Agar was added for solid medium (15 g/liter). Mannitol (10 g/liter) served as the carbon source in the *Azotobacter* medium. Sucrose (20.0 g/liter), biotin (1 μg/liter), and *p*-aminobenzoic acid (1 μg/liter) were added to the medium for enumeration of *Clostridium* sp. Trisodium citrate (0.3 g/liter), sodium lactate (10.0 g/liter of a 60% syrup), and additional $MgSO_4 \cdot 7H_2O$ (2.8 g/liter) were added to complete the *Desulfovibrio* medium.

For *Azotobacter* enumeration, approximately 1 g of mud from the top 1 cm at each station was placed into a sterile, preweighed tube and brought up to a 10-ml total volume with sterile *Azotobacter* medium. Serial 10-fold dilutions were made, and 0.01 ml of the appropriate dilutions was spotted onto the appropriate agar plates as described by Harris and Somers (20). The plates were incubated at room temperature for 9 days before colonies were counted. The numbers of *Azotobacter* were calculated by referral to the table of Harris and Somers (20).

The most-probable-number technique involved placing approximately 1 g of mud from the top 1 cm at each station into a sterile, preweighed tube. The tube was flushed with N_2 gas and sealed with a black butyl rubber stopper. The volume in the tube was brought up to 10 ml with sterile *Desulfovibrio* medium (not previously flushed with N_2), and serial 10-fold dilutions were made. All tubes were flushed with N_2 gas after each manipulation and sealed with a black butyl rubber stopper. A set of five tubes of each medium (*Clostridium* and *Desulfovibrio*) was inoculated with 0.5 ml of each dilution (10^{-1} to 10^{-4}). The inoculated tubes were flushed with N_2 gas, and a cotton plug was placed in the top of each tube; 0.4 ml each of saturated solutions of pyrogallol and Na_2CO_3 was added to the cotton plugs, and the tubes were sealed with black butyl rubber stoppers (3, 15). The tubes were incubated at room temperature for 1 (*Clostridium* tubes) or 2 (*Desulfovibrio* tubes) months before being counted. A positive *Clostridium* tube was indicated by the presence of 10% or more gas in the inverted Durham tube. The production of gas in this medium was one of the criteria (35) used to establish the presence of *Clostridium*. A positive *Desulfovibrio* tube was indicated by the presence of a black precipitate (FeS). The numbers of *Clostridium* and *Desulfovibrio* were calculated after referral to appropriate most-probable-number tables (9, 18).

Isolation of acetylene-reducing bacteria was done by streaking from positive plates or tubes onto solid medium of the appropriate composition. *Azotobacter* was incubated aerobically, whereas *Clostridium* and *Desulfovibrio* were incubated in anaerobic GasPak jars (BBL Microbiology Systems, Cockeysville, Md.). Plates for *Clostridium* and *Desulfovibrio* isolations were preincubated anaerobically for a period of 1 to 2 weeks before use. This preincubation removed traces of O_2 from the solidified media and greatly enhanced growth of these anaerobes. Isolated colonies were first transferred to liquid medium devoid of added nitrogen, and after growth batch cultures (200 or 500 ml) were grown in yeast extract (0.1%)-supplemented medium. After growth cells were harvested by centrifugation at 5,000 rpm for 30 min, suspended in 10 ml of sterile medium, and stored at 6.0°C. To screen the isolates for ARA, 1.0 ml of the washed cell suspension was placed into 20 ml of the appropriate medium in a 50-ml serum bottle. The gas phase was evacuated and replaced with the following gas mixture: C_2H_2, 10%; O_2, 5%; argon, 85% (for *Azotobacter* isolates); or C_2H_2, 10%; argon, 90% (for *Clostridium* and *Desulfovibrio* isolates). To maintain anaerobic conditions in the latter set of bottles, a small vial was placed into the serum bottle and 1.0 ml each of saturated solutions of pyrogallol and Na_2CO_3 was added. The liquid medium for *Clostridium* and *Desulfovibrio* was preincubated anaerobically for 1 to 2 weeks before use. After 3 to 4 days of incubation the cultures were tested for ethylene production, as previously described (10). Tentative identifications were based on morphological and physiological features.

Bacterial activity. To determine the relative in vitro activity of the different groups of acetylene-reducing bacteria, a set of treatments was made. One set of bottles was pasteurized by heating at 85°C for 15 min, thus eliminating all but sporeforming acetylene-reducing bacteria (mainly *Clostridium* sp.). The activity of sulfate reducers was determined by adding a solution of $BaCl_2$ to a set of samples. The Ba^{2+} ions react with the SO_4^{2-} ions to form an insoluble $BaSO_4$ precipitate (3). Such a treatment will remove the terminal electron acceptor (SO_4^{2-}) used in respiration by this group of bacteria. The $BaCl_2$ was added by injecting 1 ml of either a 1.3 M (full strength) or a 0.13 M (1/10 strength) solution per 25 g of wet mud. The activity of sulfur-oxidizing, acetylene-reducing bacteria was screened by adding 1 ml of a thiosulfate solution (12.6 M, 2 g/ml) to 25 g of wet mud. The thiosulfate would presumably serve as an energy source for these bacteria if present. All of the amendment experiments included parallel duplicate unamended controls.

RESULTS

The number of three different groups of nitrogen-fixing organisms in marsh mud was investigated over a period of 9 months. One aerobic

fixer, *Azotobacter,* and two anaerobic fixers, *Clostridium* and *Desulfovibrio,* were enumerated. The results can be seen in Fig. 1. There were no discernible seasonal patterns in the number of bacteria at any of the four stations. Table 1 lists the average number of each bacterial group present at each station; the numbers were similar at all stations. The number of *Azo-*

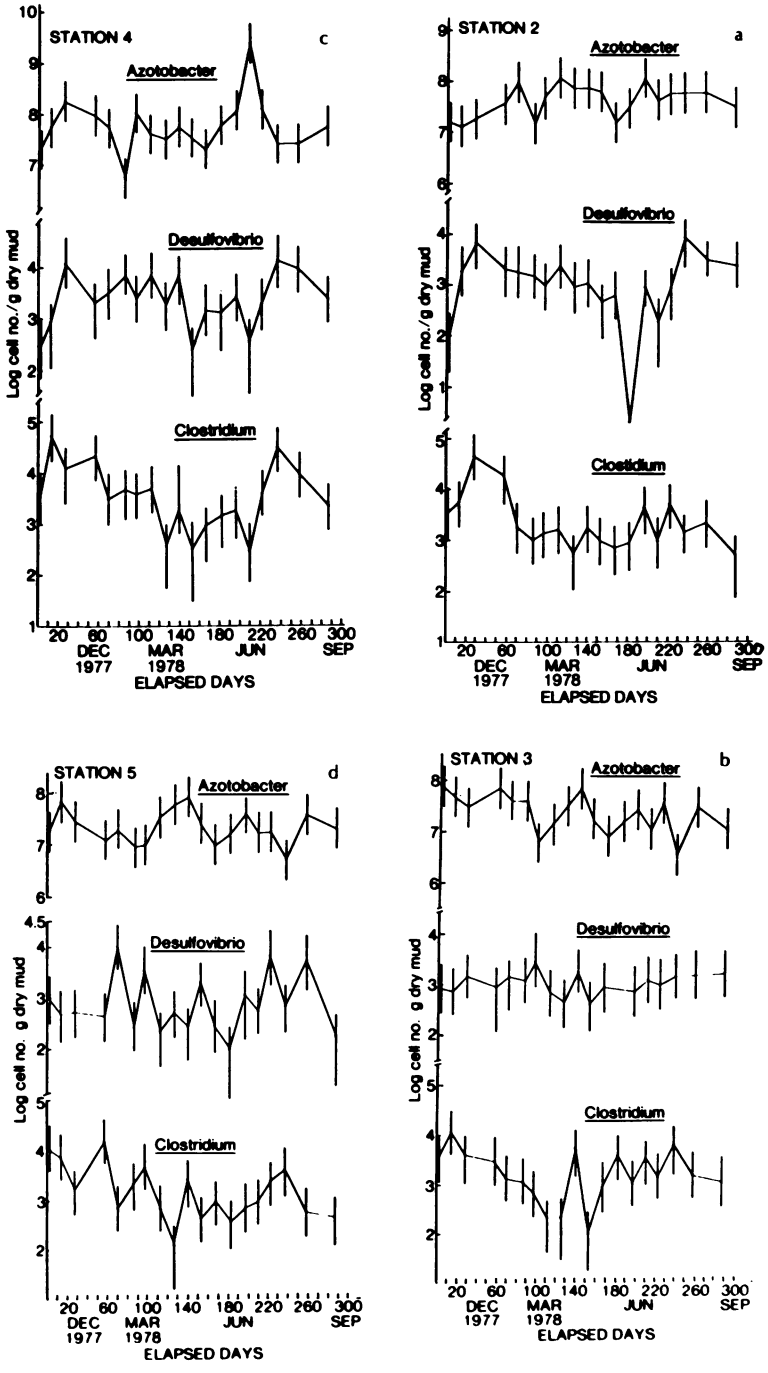

FIG. 1. *Numbers of Azotobacter, Clostridium, and Desulfovibrio in top 1 cm of marsh surface from November 1977 through August 1978. Bars represent the 95% confidence limits. (a) Station 2; (b) station 3; (c) station 4; (d) station 5.*

TABLE 1. *Average numbers of nitrogen-fixing bacteria in the top 1 cm of marsh surface from November 1977 to August 1978*

Station	Log_{10} no. of cells/g of dry mud ± standard deviation		
	Azotobacter	*Clostridium*	*Desulfovibrio*
2	7.6 ± 0.3	3.3 ± 0.5	2.9 ± 0.9
3	7.4 ± 0.4	3.2 ± 0.5	2.9 ± 0.7
4	7.4 ± 0.5	3.5 ± 0.7	3.3 ± 0.5
5	7.4 ± 0.3	3.2 ± 0.6	2.9 ± 0.6

tobacter was in excess of 10^7 per g of dry mud, whereas the numbers of clostridia and sulfate reducers were much lower (10^3 to 10^4 per g of dry mud).

The above results indicated that the observed seasonal patterns of ARA (10) were due not to a change in the numbers of acetylene-reducing organisms, but rather to a change in their activity. The above results also suggest that the relative importance of each group to the observed ARA was not the same. To ascertain the relative importance of the various groups enumerated at station 4, a series of treatments was used. To determine the importance of sporeforming, acetylene-reducing bacteria (mainly clostridia), a set of samples was pasteurized to eliminate all nonsporeformers from the sample before the acetylene reduction assay. A second set of samples was treated with $BaCl_2$ to eliminate the activity of sulfate-reducing, acetylene-reducing bacteria. Sulfur-oxidizing bacteria have been reported to be nitrogen fixers (26) and are also quite common in the Canary Creek marsh (unpublished data). To stimulate their activity, thiosulfate was added to a separate set of samples to serve as an additional energy source. The results of these treatments are listed in Table 2.

The pasteurized samples had an acetylene reduction rate that was only 2.3% of the untreated sample, indicating that there were few acetylene-reducing, sporeforming bacteria present in the sample. This does not necessarily indicate that sporeformers are not important, but rather that few spores were present at the time of sampling. The addition of $BaCl_2$ (full strength) caused a 50% decrease in ARA, suggesting that sulfate-reducing, acetylene-reducing bacteria may have been important in the observed ARA for that sample. The addition of thiosulfate resulted in a 90% decrease in activity, indicating that there were few sulfur-oxidizing, acetylene-reducing bacteria present. The high level of thiosulfate (12.6 M) may also have exerted inhibiting effects on all acetylene-reducing bacteria present.

In addition to the above treatments, the effect of varying the O_2 concentration on ARA was measured. Since nitrogenase is oxygen sensitive, it was believed that observed ARA could be affected by the amount of O_2 present in the gas phase. Samples were exposed to 0, 5, 10, or 15% O_2 and ARA was measured. The results (Fig. 2) show that the O_2 level had little effect on ARA at any station except station 4, where there was a large decrease in activity at 15% O_2. These results suggest that both aerobic and anaerobic microenvironments existed in the collected samples, and these sites were relatively stable with respect to changes in external O_2 levels.

Isolates from each of the three experimental groups were examined for growth in media devoid of fixed nitrogen and for their ability to reduce acetylene to ethylene. Bacteria tentatively identified as *Azotobacter* sp. were isolated from stations 2, 4, and 5. These isolates were capable of growth in liquid mannitol medium devoid of added combined nitrogen and were able to reduce acetylene to ethylene in pure

TABLE 2. *Effect of pasteurization, $BaCl_2$, and $Na_2S_2O_3$ on rates of acetylene reduction in samples collected from station 4*[a]

Experimental condition	nmol of C_2H_4/g of dry mud per day	
	Control rate	Experimental rate (% of control)
Pasteurization	340.6	7.8 (2.3)
$BaCl_2$ (full strength)	25.7	12.3 (47.7)
$BaCl_2$ (1/10 strength)	25.7	17.3 (67.4)
$Na_2S_2O_3$	25.7	2.9 (11.1)

[a] Rates are the average 5-day rates. All values are the averages of at least duplicate samples which agreed within 20%.

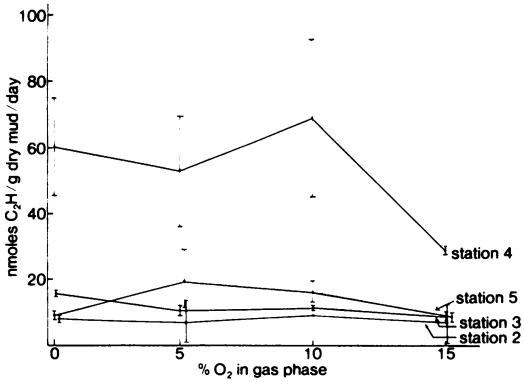

FIG. 2. *Effect of oxygen concentration on the rate of acetylene reduction at stations 2, 3, 4, and 5. Bars represent the range of values obtained for a particular treatment.*

culture. The isolates were also capable of forming cysts or cystlike structures upon the depletion of the carbon source, indicating that they were *Azotobacter* sp. Higher cell densities were achieved when the cultures were not shaken. Isolation of a gas-producing, sporeforming bacterium (*Clostridium* sp.) was achieved from station 4. This isolate is capable of growth in liquid, nitrogen-free medium but was not observed to reduce acetylene to ethylene, and hence its status as a nitrogen fixer remains in doubt. Spiral-shaped, sulfate-reducing bacteria were isolated from stations 2, 4, and 5, and these isolates were capable of reducing acetylene in pure culture under anaerobic conditions. Examination of morphological features indicated that the isolates belong to the genus *Desulfovibrio* and not to the genus *Desulfotomaculum*. Isolation of nitrogen-fixing *Desulfovibrio* sp. has been previously reported (33).

DISCUSSION

The numbers of three groups of nitrogen fixers were determined from samples collected from four different areas of a salt marsh. It was initially expected that the low marsh station (station 3, tall *Spartina*) would contain large numbers of the anaerobic fixers (clostridia and sulfate reducers) since the sediment there appeared to be more reduced than that at the other stations (based on the presence of black metal sulfide precipitates). Conversely, station 5 was expected to contain large numbers of *Azotobacter* since the sediment there was noticeably coarser than that at the other stations and should have had a greater degree of aeration. In addition, this station was flooded by the tide only infrequently, which would also maintain aerobic conditions. That each of the three groups of bacteria was present in essentially equal numbers at all stations suggests that there is a heterogeneous population of fixers present throughout the marsh and that aerobic and anaerobic microenvironments are commonly in close proximity.

The numbers of acetylene-reducing (nitrogen-fixing) bacteria reported here are similar to those given in several previous reports. Abd-El-Malek (1) has reported *Azotobacter* numbers in excess of 10^7 per g of dry soil in many Egyptian soil samples. Similarly, R. D. Ralph (Ph.D. thesis, University of Delaware, Newark, 1978) reported up to 10^7 *Azotobacter* per cm^3 of rhizosphere sand associated with the roots of *Ammophila breviligulata*. However, many other workers have reported lower numbers of *Azotobacter* in soil or soil-root systems (5, 11, 21, 24). These workers found only 10^3 to 10^4 *Azotobacter* per g of dry soil. Chang and Knowles (7) reported that the presence of *Azotobacter* was quite sporadic in several Quebec agricultural soils. Numbers of nitrogen-fixing *Clostridium* and *Desulfovibrio* are also variable. Several workers (5, 7, 35) reported numbers of clostridia similar to those obtained here (approximately 10^3 per g of dry soil). Abd-El-Malek (1) reports much higher numbers (up to 10^8 per g of dry soil). Herbert (21) has reported numbers of *Desulfovibrio* to be 1×10^3 to 4×10^3 per g of dry sediment. Patriquin and McClung (32) have reported that nitrogen-fixing sulfate reducers are present in numbers of 10^6 per g of dry rhizosphere soil from a Nova Scotia salt marsh. In these studies, as in the present study, there was little correlation between ARA and bacterial numbers.

In addition to the acetylene-reducing bacteria, the numbers of ammonia-oxidizing and sulfur-oxidizing bacteria at station 4 were also observed to remain constant over the same period (R. B. Ketcham, unpublished data). In these studies there were no large deviations in bacterial numbers from one sampling period to the next. The most-probable-number method used in these studies would realistically only detect a 10-fold change in bacterial numbers. Thus, small-scale (i.e., 10-fold) deviation in bacterial numbers would not be observed. This suggests that the marsh may be a steady state (at least in terms of bacterial numbers) and that ARA and other metabolic activites are controlled not by the number of bacteria present, but rather by their physiological condition. The data obtained here indicate that *Desulfovibrio* sp. may be one of the major contributors to the observed ARA despite their relatively low numbers. Such a finding has been reported by Herbert (21) in his study with estuarine sediments.

It appears that we have accounted for the majority of marsh ARA with the three bacterial types we isolated. The $BaCl_2$ experiment (Table 2) indicates that sulfate-reducing bacteria perform about 50% of the ARA. Although the activity of *Azotobacter* could not be delineated as precisely, the large populations (10^7 per g) of these notable nitrogen fixers must be presumed to contribute significantly to our measurements of ARA in natural samples. We have concluded that ARA and population densities of nitrogen-fixing bacteria are not well correlated. This conclusion could be weakened to the extent that bacteria other than those we isolated fix nitrogen in marsh sediment. Since we have accounted for the bulk of the system ARA potential, this possibility is unlikely to be important in our conclusion.

One of the major parameters reported to control ARA is oxygen partial pressure. Much work has been done concerning the effect of O_2 on

Azotobacter in pure culture, and this work has shown that actively fixing cultures of *Azotobacter* grow best at reduced O_2 levels (8, 15, 28, 29, 39). In addition, numerous workers have reported that ARA in soil or soil-root systems is greater under anaerobic than under aerobic conditions or greater in waterlogged than in aerated soil (4, 5, 7, 12, 21, 22, 27, 34, 35, 42). In contrast, there are several workers who have found that the O_2 level has little effect on ARA. Jones (23), working in an English salt marsh, found that ARA was the same whether the samples were incubated aerobically or anaerobically. Patriquin (30, 31) found that O_2 affected ARA in excised roots of *S. alterniflora* only if the roots were shaken vigorously during incubation. If the roots were shaken during the acetylene reduction assay, ARA was greatest at 0.02 to 0.03 atm of O_2. However, activity was also dependent on the presence of O_2, a situation which Patriquin termed "oxygen dependent but oxygen sensitive" acetylene reduction. The results of the present study are in agreement with the findings of Jones (23) and Patriquin (30, 31) in that O_2 exerted little effect on ARA.

The insensitivity of ARA to O_2 levels may have resulted from poor diffusion of O_2 through the mud sample. It has been reported that pO_2 decreases sharply toward the center of a soil core and away from the soil surface (17). Marsh sediments similar to those found at station 3 have been found to be composed of over 30% each silt and clay (unpublished data). Such sediments tend to restrict gas diffusion, especially when near the water-holding capacity as the samples often were. The diffusion of acetylene through the interstitial water would not be affected nearly as much as O_2 since it is more than 30 times more soluble (in water) than O_2 (41). Thus, only the outer 2 to 3 mm of the samples was actually affected by the change in O_2 concentration while the remainder of the sample was under anaerobic or anoxic conditions. The measurement of ARA at reduced O_2 levels may therefore more closely approximate in situ O_2 levels than initially predicted.

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