APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1982, p. 1400–1405
0099-2240/82/061400-06$02.00/0

Vol. 43, No. 6

Perfusion Method for Assaying Microbial Activities in Sediments: Applicability to Studies of N_2 Fixation by C_2H_2 Reduction

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Received 21 September 1981/Accepted 3 February 1982

A perfusion method for assaying nitrogenase activity (acetylene reduction) in marine sediments was developed. The method was used to assay sediment cores from *Spartina alterniflora* (salt marsh), *Zostera marina* (sea grass), and *Thalassia testudinum* (sea grass) communities, and the results were compared with those of conventional sealed-flask assays. Rates of ethylene production increased progressively with time in the perfusion assays, reaching plateau values of 2 to 3 nmol · g of dry sediment^{-1} · h^{-1} by 10 to 20 h. Depletion of interstitial NH_4^+ was implicated in this stimulation of nitrogenase activity. Initial acetylene reduction rates determined by the perfusion assay of cores from the *Spartina* community ranged from 0.15 to 0.60 nmol of C_2H_4 · g of dry sediment^{-1} · h^{-1}. These rates were similar to those for sediments assayed in sealed flasks without seawater when determined over linear periods of C_2H_4 production. Initial values obtained by using the perfusion method were 0.66 nmol of C_2H_4 · g of dry sediment^{-1} · h^{-1} for sediments from *Zostera* communities and 0.70 nmol of C_2H_4 · g of dry sediment^{-1} · h^{-1} for sediments from *Thalassia* communities. In all cases, rates determined by simultaneous slurry assays were lower than those determined by the perfusion method.

The C_2H_2 reduction test has been adapted as a routine means of assessing nitrogenase activity in marine sediments (16). In situ (e.g., bell jar) applications of this method have been found to have serious shortcomings (14). Many studies have therefore employed sealed-flask assays, which entail considerable sample manipulation and, often, lengthy assay periods (3, 15, 18, 20). Acetylene is itself an inhibitor of N_2 fixation (9), as well as a variety of other important microbial processes in sediments (5, 10, 13). For sediments, it is therefore prudent to curtail the exposure of samples to C_2H_4 (4). As a further caution, sample disturbance and exposure to oxygen should be minimized since sediment nitrogenase activity occurs in a highly reduced environment and may be oxygen sensitive (3, 14, 19).

Perfusion methods have been used as a means of assessing microbial activities in soil systems (12). In this report, we describe a method of assessing microbial activity in marine sediments by a perfusion technique which rapidly assays a relatively unmanipulated sediment core. Essentially, the system consists of the passage of water through the sediment, a process that occurs in nature (2, 11), and the collection and analysis of gases (or nutrients or both) in the perfusion water. This report gives the details of preliminary observations on the utility of the system in assaying anaerobic bacterial N_2 fixation by the C_2H_2 reduction technique.

MATERIALS AND METHODS

Sample collection. Most samples were taken in the low salt marsh among the stands of tall *Spartina alterniflora* at Flax Pond, N.Y. The semidiurnal tidal amplitude is about 1 to 2 m and is enough to alternately drain and inundate the sample site. The average organic content of the material passing through a 1-mm mesh was about 2.5% (19 determinations on three dates). Sediment densities averaged about 1.5 ± 0.1 g · cm^{-3} (nine sampling dates). The sediment has been characterized as a peat consisting of a fibrous mixture of clay, silt, sand, rhizomes, and roots (6).

Rhizome sediment samples from *Zostera marina* communities were collected from a site near Bird Island in Eastern Great South Bay, N.Y. The site is located in about 1.0 to 1.5 m of water. Sediment densities averaged 1.4 g · cm^{-3}, and organic content was about 0.5%. The surficial sediment at this site has been characterized as a sandy mud containing about 87% sand, 9% silt, and 4% clay (8). High rates of submarine groundwater discharge have been found at various shallow sites throughout Great South Bay (2).

One experiment was also run with rhizosphere samples from the *Thalassia testudinum* community in Bimini Harbor, Bahamas. Sediment density at this site was about 0.9 g · cm^{-3}, and sediments contained about 81% sand and 19% silt-clay (P. Penhale, unpublished data).

In all cases, samples were manually collected in aluminum core tubes (internal diameter, 3.5 cm). For perfusion assays, 6- or 12-cm-long cores were used (to allow space for stoppering), and for flask assays, 15- to 20-cm-long cores were used. Each core was sealed upon collection with a black rubber stopper and returned immediately to the laboratory for assay.

Perfusion assays. For perfusion assays, cores were placed directly on a manifold, and the stoppers used to seal the cores in the field were replaced by those already plumbed for the perfusion system (Fig. 1). A small wad of glass wool was included in the lower end of each column above the lower stopper to prevent extrusion of sediment through the exit port. A peristaltic pump was used to draw and regulate the perfusion medium through the column. We purged columns of bubbles introduced while the stoppers were changed by tapping the columns after placing them on the manifold and before turning the pump on. Up to four columns could be run simultaneously from the same or separate reservoirs. All assays were run at room temperature (26°C). In preliminary experiments, unsupplemented deoxygenated seawater from the corresponding sample site was used as a percolation fluid, and flow rates were adjusted to enable a relatively rapid turnover of interstitial waters (ca. every 0.5 to 2 h). All preliminary experiments used 0- to 10-cm cores. It was later found that with shorter cores (4 cm), assays could be set up more rapidly and with more precise flow control. Thus, 0- to 4-cm core sections were used in all subsequent experiments.

Flask assays. In most experiments, comparable samples were also extruded into flasks and incubated in parallel by previously described methods (3). Briefly, sediment sections were directly dispensed into flasks with or without previously deoxygenated seawater (slurry or static, respectively). The operation was performed while the flasks were continously flushed with O_2-free N_2. The flasks were immediately sealed and placed in a shaking (100 rpm [rotary]) water bath at ambient (26°C) temperature.

Acetylene reduction assay. Nitrogen fixation was assayed by the C_2H_2 reduction method (9). In perfusion assays, C_2H_2 blended with pure N_2 to yield a C_2H_2 concentration of approximately 10 to 15% was vigorously bubbled in the reservoir. Nitrogen was used as the makeup gas to deoxygenate the reservoir. Samples collected in Vacutainers were vigorously shaken, and 100 μl of the gas phase was immediately assayed for C_2H_2 and C_2H_4 by gas chromatography (183- by 0.32-cm stainless steel column; 80- to 100-mesh Porapak-R; 60°C; N_2 flow, 40 ml · min^{-1}).

For flask assays, purified C_2H_2 (Linde Specialty Gases) was added to yield a final concentration in the gas phase of about 12%. Subsamples (100 μl) of the gas phase were obtained with a syringe through the rubber stoppers and directly injected into the chromatographic system. The time course of C_2H_2 production was monitored over 1- to 2-day periods in perfusion and flask assays. Comparisons of slurry and perfusion assays were made for all three sediment types. Flask assays of *Spartina* sediments without added seawater (static) were also compared with perfusion assays.

Although the majority of experiments utilized unsupplemented (deoxygenated) seawater as the assay medium, the effect of NH_4^+ on nitrogenase activity was studied in several experiments. For perfused cores, specific levels of NH_4^+ were achieved in the reservoirs by the addition of NH_4Cl. Direct determination of NH_4^+ concentrations in the effluent was made by standard methods (17). For flask assays, appropriate additions of NH_4Cl solutions were made with a syringe through the stopper.

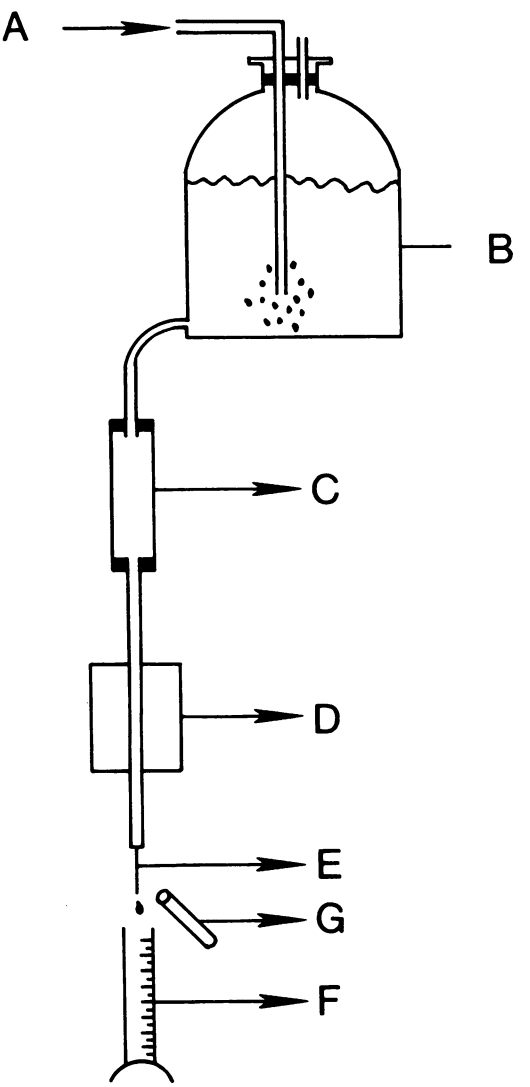

FIG. 1. Schematic representation of the components of the perfusion assay system. (A) Influent stream of gas, in this case the source of inhibitor (C_2H_2 in N_2). (B) Fluid reservoir containing seawater and appropriate supplements. (C) Sediment sample contained within the collection core. (D) Peristaltic pump. All tubing below the sample was made of polyvinyl chloride (Tygon or Technicon). A syringe needle was attached at the terminus (E). Samples (3 to 4 ml) of the effluent stream were collected in 7-ml Vacutainers (G). Between sample collections, we determined the flow rate by collecting the effluent in a graduated cylinder (F).

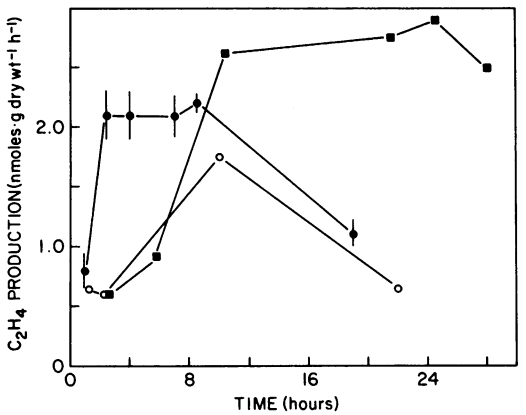

FIG. 2. Time course of C_2H_4 production rate for rhizosphere sediments from *S. alterniflora* (■), *Z. marina* (○), and *T. testudinum* (●) communities, as observed in the perfusion assay. Sediments of *S. alterniflora* were obtained on 29 November 1979.

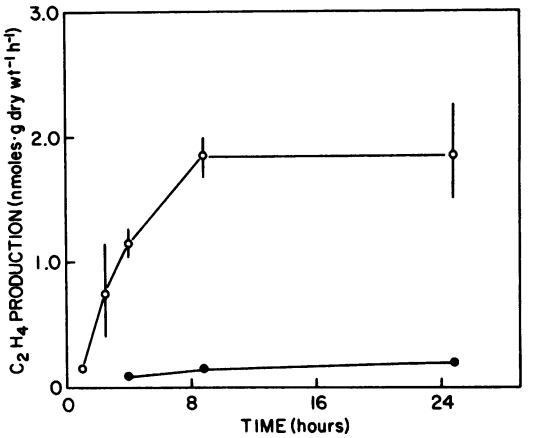

FIG. 3. Effect of flow rate on nitrogenase activity (C_2H_2 reduction) in *S. alterniflora* community rhizosphere sediments collected on 5 December 1979. Symbols: ○, average of duplicate cores maintained with a flow rate of 4 ml · h^{-1} · cm^{-2}; ●, average of two cores maintained with a flow rate of 0.9 ml · h^{-1} · cm^{-2}. Bars indicate standard error ranges.

Upon termination of all experiments, sediments were dried at 105°C for at least 24 h and then weighed. Determinations of sediment organic content were made by determining the loss on ignition at 450°C.

Calculations of results. For flask assays, rates of C_2H_4 production were determined by linear regression analysis over periods of linear C_2H_4 production. This usually occurred between 2 and 12 h. For perfusion assays, rates were calculated from the point at which the C_2H_2 concentration in the effluent became similar to that in the reservoir, indicating a steady state with respect to C_2H_2.

We computed the concentration of C_2H_4 for the effluent and influent samples by determining the gas-to-liquid ratios in the Vacutainers, the solubility of C_2H_4, and the volume of seawater collected. The rate of C_2H_4 production (nanomoles per milliliter) for the flow-through experiments was then calculated as follows: [(C_2H_4 in effluent) − (C_2H_4 in influent)] × flow rate (milliliters per hour) and expressed per dry weight gram of sediment or surface of core cross section.

Effect of increased fluid pressure on sediment flow characteristics. To determine whether channelization of perfusion water occurred in the assayed cores, we measured the flow rate for several marsh cores (0.4-cm depth) as a function of increasing headspace pressure by sealing the reservoir and attaching a line from the reservoir to a pressure source (high-pressure N_2) with a calibrated gauge. Flow rates were determined at pressures of 1.0 to 1.28 atm (101 to 130 kPa) above the liquid phase. For each increment in pressure, the flow was allowed to equilibrate for 5 min and was then measured over the next 10 to 20 min.

RESULTS

Ethylene production from C_2H_2 was readily detectable by the perfusion method in all three sediment types. Typical time courses for C_2H_2 reduction for all three sediment types are presented in Fig. 2. In all cases, rates of C_2H_4 production are presented for periods after C_2H_2 penetration of the column, i.e., when the C_2H_2 concentration in the effluent was equal to that in the reservoir. In the three examples presented, as in the majority of the experiments in which deoxygenated seawater and flow rates of 40 to 100 ml · h^{-1} (4 to 10 ml · h^{-1} · cm^{-2}) were used, rates of C_2H_2 reduction increased drastically over the first several hours. The high sustained rates of C_2H_2 reduction in the sediments from the salt marsh (Fig. 2), compared with those in the sediments from sea grass communities, might be attributable to the higher organic content of the former sediments. No C_2H_4 production was noted in cores perfused with C_2H_2-free water.

The effect of flow rate on the time course of C_2H_2 reduction was studied, and the initial rates in both flow regimes were similar: about 0.16 nmol of C_2H_4 · g of dry sediment^{-1} · h^{-1} at about 1 h at the higher flow rate (4 ml · h^{-1} · cm^{-2}) and at about 4 h at the lower flow rate (0.9 ml · h^{-1} · cm^{-2}) (Fig. 3). Over the 24-h period presented, the interstitial waters were replaced about 50 times at the higher flow rate and only 11 times at the lower flow rate (assuming a 50% porosity). Beyond 24 h, rates at the lower flow rate also accelerated, approaching that at the higher flow rate.

The rapid increase in the C_2H_2 reduction rate over the initial period of the experiments suggested that the inhibition of nitrogenase was relieved by the replacement of interstitial waters with ambient-temperature seawater. In several experiments, various concentrations of NH_4^+ were perfused through the cores, and the

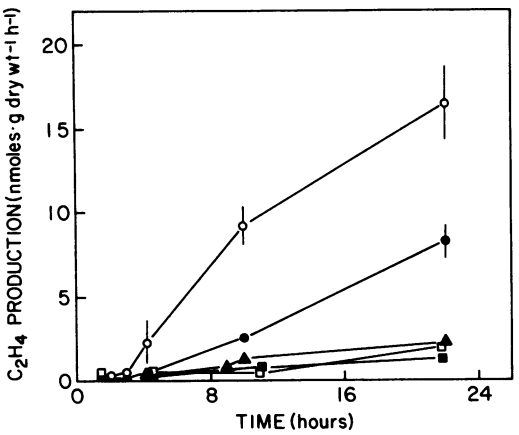

FIG. 4. Effect of NH_4^+ concentration on nitrogenase activity (C_2H_2 reduction) in rhizosphere sediments of a *S. alterniflora* community. Symbols: ○, samples collected and assayed on 28 May 1980 and perfused with ambient-temperature seawater; ●, samples collected and assayed on 28 May 1980 and perfused with seawater containing 200 μM NH_4^+; □, samples collected and assayed on 17 June 1980 and perfused with seawater containing 200 μM NH_4^+; ■, samples collected and assayed on 17 June 1980 and perfused with seawater containing 400 μM NH_4^+; ▲, samples collected and assayed on 23 September 1980 and perfused with seawater containing 500 μM NH_4^+. Means of duplicate determinations made on 28 May and 17 June and quadruplicate determinations made on 23 September are shown. Bars indicate standard error ranges.

changes in C_2H_2 reduction rate were noted. Increases in NH_4^+ concentration inhibited the stimulation of nitrogenase activity, although some stimulation occurred even at the highest NH_4^+ concentration (0.5 mM) (Fig. 4).

Samples of water first expelled from the column (before C_2H_2 penetration) were also analyzed for NH_4^+, and the average concentration on 17 June 1980 was 125 μM, but the concentration in the samples collected on 28 May was 300 to 400 μM. Ammonium and C_2H_2 in the effluent reached steady state at about the same time.

Table 1 presents a summary of the initial rates found in several of the perfusion experiments along with the concurrent static and slurry assay results for the three sediment types. We believe that the initial rates most closely approximate in situ nitrogenase activity. Fairly similar results were obtained by the perfusion method in a number of determinations performed on rhizosphere cores from a *Spartina* environment. Parallel static assays generally showed immediately linear rates of C_2H_4 production, which compared favorably with perfusion assay results. Linear rates of C_2H_4 production in slurry assays occurred after lags of 1 to 2 h, and these rates were consistently lower than that in either of the other two assays.

In the two sea grass sediment samples obtained during the fall of 1979, high C_2H_4 production rates were also noted and were greater than

TABLE 1. Initial C_2H_2 reduction rates in rhizosphere sediments of *S. alterniflora*, *Z. marina*, and *T. testudinum* communities[a]

Sediment from indicated community	Date (mo/day/yr)	Core depth (cm)	nmol of C_2H_2 · g of dry sediment^{-1} · h^{-1}		
			Perfusion[b]	Slurry[c]	Static[c]
S. alterniflora	8/23/1979	0–10	0.28 (2)[d]	0.10 ± 0.01 (3)	0.22 ± 0.05 (3)
	11/6/1979	0–10	0.29 ± 0.08 (4)		
	11/29/1979	0–10	0.60 (2)	0.08 (2)	0.44 (2)
	12/5/1979	0–4	0.17 (2)		
	12/5/1979	0–4	0.15 (2)[e]		
	5/28/1980	0–4	0.23 ± 0.06 (4)		
	6/17/1980	0–4	0.16 ± 0.03 (4)		
	9/23/1980	0–4	0.31 ± 0.04 (4)	0.12 ± 0.03 (3)[f]	0.48 ± 0.26 (3)
Z. marina	9/5/1979	0–10	0.66 (2)	0.16 ± 0.01 (4)	
T. testudinum	10/18 and 19/1979	0–10	0.70 ± 0.20 (5)	0.41 ± 0.04 (5)	

[a] All samples were incubated at room temperature (≈26°C). Mean results are presented, along with some standard errors. Numbers of replicates are in parentheses.

[b] Rates were calculated from the initial point at which C_2H_2 penetrated the core, usually after 1 to 2 h. Unless otherwise indicated, flow rates were 4 to 10 ml · h^{-1} · cm^{-2}.

[c] Rates were calculated by linear regression analysis of linear periods of C_2H_2 production, usually between 2 and 10 h after the injection of C_2H_2.

[d] The reservoir had subsaturating levels of C_2H_2 (partial pressure of C_2H_2 ≈ 0.05 atm) during the initial pulse. This value may therefore be somewhat low.

[e] Flow rates were adjusted to about 0.9 ml · h^{-1} · cm^{-2}.

[f] Cores were extruded into deoxygenated seawater containing 0.5 μM NH_4Cl.

the rates in concurrently assayed slurry samples. Although direct, simultaneous comparisons of perfusion and static assays were not made in this study, we have found that, in contrast to the results for cores from *Spartina* communities, similar results are obtained in slurry and static assays from sediments of sea grass communities (D. G. Capone, manuscript in preparation; P. Salvas and B. F. Taylor, personal communication).

For two cores collected from salt marshes, the flow rate increased as a linear function of increased pressure over the range 1 to 1.14 atm (101 to 116 kPa). Average flow rates of 29, 87, and 123 ml $\cdot$ h^{-1} $\cdot$ cm^{-2} were noted at 1.0, 1.07 and 1.14 atm (101, 108, and 116 kPa), respectively. These results suggest that the channelization of water flow did not occur at the flow rates typically used in the assays. Flow rates at 1.21 to 1.35 atm (123 to 137 kPa) in both cores leveled off (ca. 135 to 145 ml $\cdot$ h^{-1} $\cdot$ cm^{-2}), indicating that the sediment had been compacted (H. Bokuniewicz, personal communication).

DISCUSSION

The perfusion method is a relatively rapid, sensitive, and nondisruptive assay of nitrogenase activity in marine sediments. For salt marsh sediments, the perfusion method provided estimates of in situ nitrogenase activity comparable to those obtained in parallel static assays but considerably quicker (1 to 2 h versus >3 h). In several comparisons in which salt marsh sediments were used, slurry assays consistently yielded lower C_2H_2 reduction rates. Slurry assays of sediments from sea grass communities, compared with the perfusion assay (on a more limited basis), also yielded lower values for nitrogenase activity.

For *Spartina* cores taken from a depth of up to 4 cm, the rates obtained from perfusion and static assays ranged from 0.16 to 0.60 nmol of C_2H_2 $\cdot$ g (dry weight)$^{-1}$ $\cdot$ h^{-1}, or about 1.0 to 3.6 nmol of C_2H_2 $\cdot$ cm^{-2} $\cdot$ h^{-1} to a 4-cm depth. Whitney et al. (20) also assayed *Spartina* (tall form) rhizosphere sediments taken from a depth of up to 4 cm in this marsh by a short-term flask assay and reported substantially lower rates for summer samplings. Their estimate for average nitrogenase activity was 0.5 nmol of C_2H_4 $\cdot$ cm^{-2} $\cdot$ h^{-1}. Our own simultaneous slurry assays of *Spartina* sediments (Table 1) yielded values in good agreement with the results of Whitney et al. (20). We hesitate to compare our results with those of other studies of N_2 fixation in *Spartina* sediments because of the wide range of locations of these studies as well as the generally long-term (1 to 2 days) nature of the assays employed (14, 15, 18, 20).

Considering the uncertainty of the acceleration in nitrogenase activity in the perfusion assays, we suggest that the initial rate, i.e., the calculated effluent C_2H_2 rate first noted to be similar in concentration to that in the reservoir, most closely approximates an in situ rate for the sample since a minimum period would be allowed for enzymological response to the perfusate. This assumes that C_2H_2 becomes saturating during its initial pulse through the column and that C_2H_2 and C_2H_4 migrate through the column at similar rates. Effluent C_2H_4 concentration changes which are independent of flow rate or C_2H_2 concentration changes are therefore biological responses to the differing compositions of the interstitial water and the perfusate. As a corollary to this, the observed increases in the C_2H_4 production rate in the perfusion columns connote a physiologically responsive and sensitive N_2-fixing flora. This underscores the necessity of cautiously and carefully considering assay options and subsequent interpretations of data.

The perfusion rates used in our systems generally exceeded those observed in nature (2, 7, 11), thus allowing the relatively rapid assay of the cores and, in the use of the initial pulse of C_2H_2 to permeate the cores, thereby minimizing artifacts induced by prolonged exposure to C_2H_2 (1, 4). In assays which more closely simulated natural flow patterns (Fig. 3 and Table 1), initial rates were essentially similar to those at the higher flow rates. Also, channeling and rearrangement of the substrate at the elevated flow rates is less likely for the coarser-grained sands (Bokuniewicz, personal communication). Furthermore, for two cores tested, the flow rate increased up to 123 ml $\cdot$ h^{-1} $\cdot$ cm^{-2} as a direct function of applied pressure in the salt marsh peat, obeying Darcey's Law (7) and thereby indicating no induced channeling in these cores. The perfusion method may not be applicable to fine-grained mud samples, in which relatively small flow rate increases may cause compactation and particle rearrangement, thereby creating artificial flow regimes.

The discrepancies noted in this study among the various methods of assaying C_2H_2 reduction require further investigation and explanation. The quantitative interpretation assigned to the data collected in many earlier reports will remain questionable until the appropriateness of the assay methods are substantiated. Smith (16) has recently commented on the problem of comparison of N_2 fixation data collected and analyzed by different methods.

Additional work is also required to fully elaborate the factors responsible for the observed rate variations over time in our assays and, hence, the factors which may modulate nitrogenase

activity in these assays. The perfusion method is obviously applicable to the study of a number of other microbial parameters in marine sediments.

ACKNOWLEDGMENTS

We thank Steve Collins for the benefit of his experience in using perfusion methods and for valuable assistance during the initial stages of development. We also thank Alison Mitchell, Betsy Bass, Richard Murtagh, and John Budin for help.

Financial assistance was provided by National Science Foundation grant OCE 78-25444 from the Division of Ocean Sciences (Biological Oceanography), National Oceanographic and Atmospheric Administration/New York State Sea Grant 04715844009, and National Oceanographic and Atmospheric Administration/Office of Marine Pollution Assessment grants NA80RAD00057 and NA80RAD00062.

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