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MICROBIAL HYDROGEN PRODUCTION POTENTIAL IN SHALLOW OCEANIC NEPHELOID LAYERS

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ABSTRACT - Seawater samples taken from shallow suspended particle maxima (20-57m) at or near the base of the surface mixed layer in various oceanic areas were tested for their potential to produce hydrogen (H₂) gas. Results suggest that hydrogen can be bacterially produced through a fermentative process in microanaerobic niches within particulate matter. The organisms responsible for this process are ubiquitous within the temperate and tropical waters sampled. Enumeration of potential H₂-producing bacteria using fluorescent antibody techniques yielded a maximum at the base of the surface mixed layer, the same depth where the greatest H₂ gas production potential was observed. Hydrogen-producing bacteria comprised up to 15 percent of the total bacterial direct count (AODC). These findings indicate that bacteria associated with shallow nepheloid layers are capable of producing hydrogen gas and thus contributing to the observed supersaturated gas concentration in the marine environment.

Key words: microbial, hydrogen, potential, nepheloid layer, fluorescent antibody.

RÉSUMÉ - Des échantillons d'eau de mer contenant des particules en suspension ont été prélevés en zone peu profonde (20 à 57 m) sur ou près de la couche brassée de surface en différents points de l'océan et ont été testés pour leur potentiel de production d'H₂. Les résultats suggèrent que l'hydrogène peut être produit, par les bactéries, lors d'un processus fermentatif dans des microniches anaérobies de la matière particulaire. Les organismes responsables de ce processus sont ubiquistes dans les échantillons provenant des eaux tempérées et tropicales. Les numérations maximales de bactéries potentiellement productrices d'hydrogène, faites par des techniques d'anticorps fluorescents, sont obtenues à la base de la couche brassée de surface ; à cette même profondeur est observé le potentiel de production d'hydrogène le plus important. Les bactéries productrices d'hydrogène constituent jusqu'à 15 % des numérations bactériennes totales, effectuées par comptage direct (AODC). Ces observations indiquent que les bactéries associées aux couches nephéloïdes peu profondes sont capables de produire de l'hydrogène et contribuent ainsi à la sursaturation en gaz observée dans le milieu marin.

Mots clés: microbien, hydrogène, potentiel, couche nephéloïde, anticorps fluorescent.

INTRODUCTION

Hydrogen is one of several reduced trace gases present in oceanic waters. Studies of its distribution show a consistent pattern: in tropical and subtropical regions surface waters are supersaturated with hydrogen with respect to the atmosphere but deep waters are near atmospheric equilibrium or undersaturated. Polar regions are generally undersaturated throughout the water column (Bullister, *et al.*, 1979; Herr and Barger, 1978; Herr *et al.*, 1981; Scranton *et al.*, 1982; Seiler and Schmidt, 1974).

The vertical distribution and small scale (10² meters) horizontal variability (Scranton *et al.*, 1982) of dissolved hydrogen indicates a biological source for the gas. Also, physical or chemical processes cannot account for the observed variations in hydrogen saturation (Herr *et al.*, 1981). Nitrogen-fixing microorganisms and fermentative bacteria are the two

most likely biological sources. Herr *et al.* (1981) and Scranton *et al.* (1982) suggest that nitrogen-fixing microorganisms are the dominant hydrogen producers in the ocean. While these microorganisms undoubtedly are capable of evolving hydrogen, there is little direct evidence to suggest that they are the main source of the dissolved hydrogen in the water column.

On the other hand, there is evidence that fermentative bacteria are active in marine hydrogen production. In the Gulf of Cadiz, Seiler and Schmidt (1974) noted a large hydrogen maximum at the interface between Mediterranean and North Atlantic water masses. From the same area, Junge *et al.* (1972) isolated several species of heterotrophic hydrogen-producing bacteria from near the interface, at depths of 700 and 900 meters. At several stations in the north Atlantic, Herr and Barger (1978) observed hydrogen maxima associated with the pycnocline. Suspended particulate maxima sometimes occur at sharp gradients in water densities (Pak *et al.*, 1980) and Bishop (1977) found that bacteria compose from 20 - 45 % of the organic carbon in a $<53 \mu\text{m}$ particulate fraction from the equatorial Atlantic. These facts suggest that bacteria associated with particulate maxima could be responsible for the observed hydrogen maxima. Particles could serve as anaerobic microenvironments (Knowles, 1978) where hydrogen production could take place.

To test the hypothesis that hydrogen-producing bacteria are associated with oceanic particulates, we have collected samples from particulate maxima, tested them for hydrogen production, and isolated hydrogen-producing bacteria from them. On previous cruises to the Mediterranean Sea and Caribbean Sea, we have detected hydrogen production in samples of concentrated particulates from surface waters but cultures from the samples lost viability within a few weeks (Schropp and Schwarz, unpublished data). On a recent cruise to the subtropical Atlantic, we extended our observations of the potential for hydrogen production and were able to successfully isolate pure cultures of hydrogen-producing bacteria from oceanic water against which antisera were produced. These antibodies were then utilized in an indirect fluorescent antibody assay procedure to enumerate the number of hydrogen producing bacteria present in nepheloid layer water samples.

MATERIALS AND METHODS

Station locations.

Samples for these experiments were collected on cruise 710-82 of the U.S.N.S. *Lynch*, 4-25 June, 1982 and cruise 84-G-5 of the R/V *Gyre*, 1 - 13 May, 1984. Microbiological sampling was done at five stations in the subtropical Atlantic, located within or near the Sargasso Sea (Fig. 1).

Sample collection.

Water samples were taken with 30-l Niskin bottles mounted on a rosette sampler (General Oceanics). The rosette was positioned in the particle maxima by means of real-time light scattering data from a rosette-mounted nephelometer or transmissometer.

The samples from the U.S.N.S. *Lynch* cruise were concentrated using reverse-flow filter units, usually containing a $3\text{-}\mu\text{m}$ pore diameter membrane filter (Schropp and Schwarz 1983). The filter units were constructed of clear plexiglass and had a volume of 2.2 l in both the upper and lower chambers. Before each use the filter units, Niskin bottles, and connecting tubing were washed with ethanol. A very gentle concentration of suspended matter was achieved at a flow rate of *ca.* 1 l min^{-1} . At each station, 58 l of water were concentrated by a factor of 26.5:1. At station 11, effluent from the reverse flow units

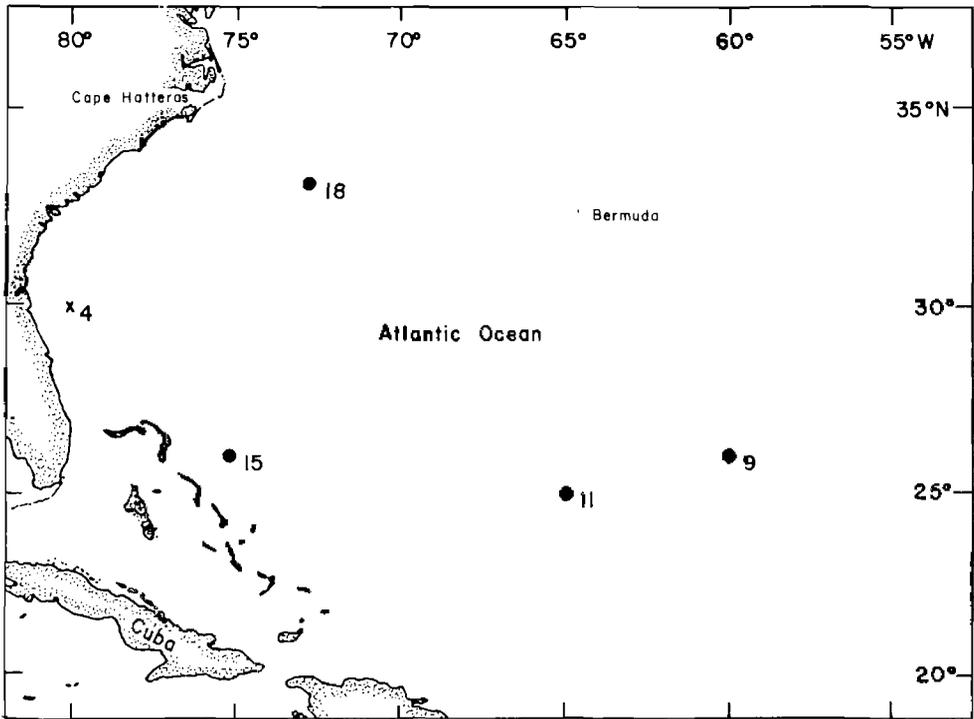


Figure 1 : Location of stations sampled during U.S.N.S. LYNCH cruise (●) and during R/V GYRE cruise (x).

($3\ \mu\text{m}$ size fraction) was also collected and processed. At station 18, two units were connected in series, the first with a $30\text{-}\mu\text{m}$ mesh filter and the second with a $3\text{-}\mu\text{m}$ filter, to obtain $> 30\ \mu\text{m}$ and $3 - 30\ \mu\text{m}$ particle size fractions. Samples from the R/V Gyre cruise were utilized without any concentration of the seawater.

Growth of hydrogen-producing bacteria.

Twenty-five ml of the concentrate was pipetted into each of nine sterile 50-ml serum vials. The vials were then closed with butyl stoppers and aluminum seals and flushed with argon for a minimum of 15 minutes to remove oxygen. Each vial was then injected with 0.25 ml each of various stock solutions to yield the following final concentrations (mg l^{-1}): cysteine hydrochloride, 0.5; yeast extract, 1.0; $\text{K}_2\text{H}_2\text{PO}_4$, 0.25 each; $(\text{NH}_4)_2\text{SO}_4$, 0.5; and resazurin, 0.05. Three of these vials each received an injection of glucose ($1.25\ \text{mg l}^{-1}$); three received lactate ($1.25\ \text{mg l}^{-1}$), and three received no additional carbohydrate. Nine control vials were prepared as above but with the concentrate filtered through $0.2\ \mu\text{m}$ pore diameter membrane filters to remove particulates and microorganisms. All vials were incubated in the dark at room temperature.

Hydrogen analyses.

Periodically, 1-ml portions of the headspace gas were withdrawn from the experimental and control vials for hydrogen analysis by gas chromatography. An equivalent volume of argon was injected into each vial to maintain a constant gas pressure.

The gas sample was injected into a Hewlett-Packard 5880 gas chromatograph equipped with dual thermal conductivity detectors. The analytical column was a 2.75 m x 3 mm stainless steel column packed with molecular sieve 5A. Argon carrier gas was used at a flow rate of 30 ml min⁻¹. Operating temperatures were (°C); injection port, 35; oven, 35; detector, 250.

The total hydrogen in a vial was calculated using the Bunsen solubility coefficients of Wiesenburg and Guinasso (1979) and corrected for any gas removed during previous analyses. The value for total hydrogen was converted to hydrogen per liter of original unconcentrated seawater, and unless otherwise noted, results expressed as nl H₂l⁻¹ are on the basis of a liter of unconcentrated seawater.

Isolation of hydrogen-producing bacteria.

Hydrogen-producing bacteria were isolated from the incubated gas-producing samples. At sea, 0.25 ml of each hydrogen-producing sample was transferred to a serum vial prepared as above with 25 ml sterile Instant Ocean (Aquarium Systems, Inc.) in place of seawater. When transfers of these crude cultures were returned to the lab, they were plated on Difco marine agar 2216 and grown aerobically. Thirty-eight representative colonies were picked and restreaked three times to ensure purity. The pure isolates were then tested for hydrogen production using the anaerobic serum vial system described above. Hydrogen-producing isolates were keyed to genus using the taxonomic scheme of Oliver (1982) for marine bacteria.

Bacterial enumeration. Portions of concentrated and unconcentrated water were preserved with glutaraldehyde (2% final concentration) for bacterial enumeration. Total cells were counted using a modification of the acridine orange staining procedure (AODC) described by Hobbie *et al.* (1977).

Fluorescent antibodies were prepared in rabbits against pure cultures of the three primary hydrogen-producing strains present in the seawater samples. The procedure used for antisera preparation and indirect immunofluorescent staining is a modification of that described by Ward and Perry (1980) and Ward (1982). Portions of the preserved samples were also reserved for observation by scanning electron microscopy (SEM) by Dr Paul LaRock (Florida State University).

RESULTS

Physical and chemical data.

Nephelometry indicated the water column to be quite low in suspended particulate matter (except for Station 4). Particle maxima were generally poorly developed and the microbiological samples were taken at depths of 22 to 57 m, where light-scattering data indicated particle maxima. The microbiological samples confirmed the paucity of suspended matter in the Sargasso Sea; very few particles or planktonic organisms were visible in the concentrate. Dissolved nutrient concentrations in the surface waters in the Sargasso Sea were quite low as had been previously reported for this region (Carpenter and McCarthy, 1975).

Bacterial counts (AODC).

Unconcentrated seawater contained from 7.3 x 10⁵ to 2.4 x 10⁸ cells ml⁻¹ (Tab. 1). The number of cells was lowest in the central portion of the Sargasso Sea, increasing towards its periphery and highest off the Florida coast as Station 4. At station 18 bacteria were counted in three different size fractions. The bacteria were distributed such that 12%, 7%, and 80% of the cells were in the >30 μm, 3 - 30 μm and <3 μm fractions, respectively.

	Station	Number of bacteria ml ⁻¹
U.S.N.S. Lynch	9	8.2 (1.9) x 10 ⁵
	11	7.3 (2.8) x 10 ⁵
	15	1.3 (0.3) x 10 ⁶
	18	1.8 (0.6) x 10 ⁶
R/V Gyre	4	2.4 (1.0) x 10 ⁸

Table 1 : Total direct bacterial count at each station enumerated as acridine orange direct counts (AODC).

Scanning electron micrographs showed the bacterial population to be composed mainly of small coccoid forms, <1 μm in diameter. The cells were observed to be free, in clumps, and attached to particles. When grown on a nutrient-rich artificial seawater medium, the majority of the cells assumed a short rod shape although a few cocci were still present.

Hydrogen production.

Hydrogen production was observed in samples from all stations. Production occurred in the experimental vials only; hydrogen in the 0.2 μm filtered controls was negligible, totalling <0.1% of the amount in the experimental vials. Gas production was detectable after 19 - 24 hours of incubation. Once hydrogen production began, the hydrogen concentration in the headspace increased rapidly until about 50 - 60 hours, when there was no further increase in concentration. The maximum hydrogen concentration at all stations was near 10⁶ nl H₂l⁻¹, regardless of nutrient supplements. There was no indication of hydrogen consumption up to 124 hours of incubation. A plot of hydrogen production at Station 9 is shown in Figure 2; plots for the other stations were quite similar and are not shown. The <3 μm fraction at Station 11 and both the 3 - 30 μm and >30 μm fractions at Station 18 produced hydrogen in quantities comparable to the other stations.

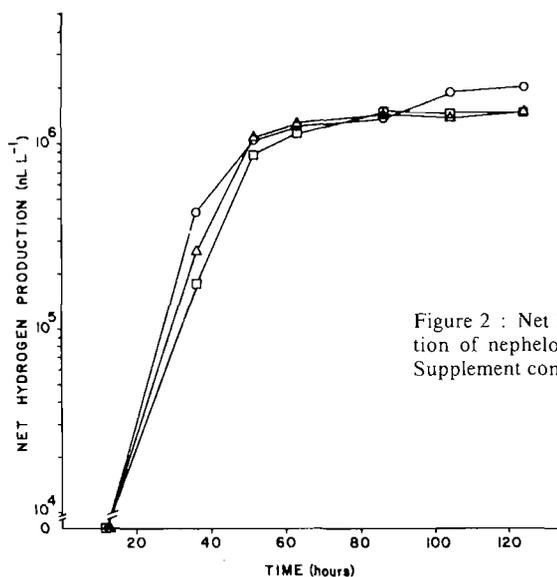


Figure 2 : Net hydrogen production measured during incubation of nepheloid layer water samples taken from station 9. Supplement concentrations were as indicated in text.

Station 9. U.S.N.S. Lynch. 12 June 1982.
Supplements: Glucose, □; Lactate, Δ; None, ○.

Hydrogen-producing isolates.

Thirty-eight isolates were obtained from the vials which produced hydrogen, 17 of which were capable of producing hydrogen after isolation. All were short, gram-negative rods. Fifteen of the isolates appeared identical and keyed out to be members of the Enterobacteriaceae. The remaining two isolates appeared to belong to the genus *Photobacterium*.

Fluorescent antibody enumeration.

Hydrogen-producing bacteria present in water samples from the R/V Gyre cruise were enumerated using the immunofluorescence techniques previously described. Antisera, prepared against pure cultures isolated from the U.S.N.S. Lynch two years prior, were reacted with freshly preserved water samples taken from various depths during the R/V Gyre cruise. The physical data depicted in Figure 3 indicates the base of the mixed layer to be at approximately 50-60m. A strong transmissometry minimum (88.2 percent) was also present at 55m. The peak in hydrogen-producing bacteria, enumerated using fluorescent antibodies, was right above this depth at 45-55m (Fig. 4). The initial hydrogen production rates in the incubated serum vials, as measured by gas chromatography, also displayed maximum production in the vials containing water from 45-55m depth (Fig. 4).

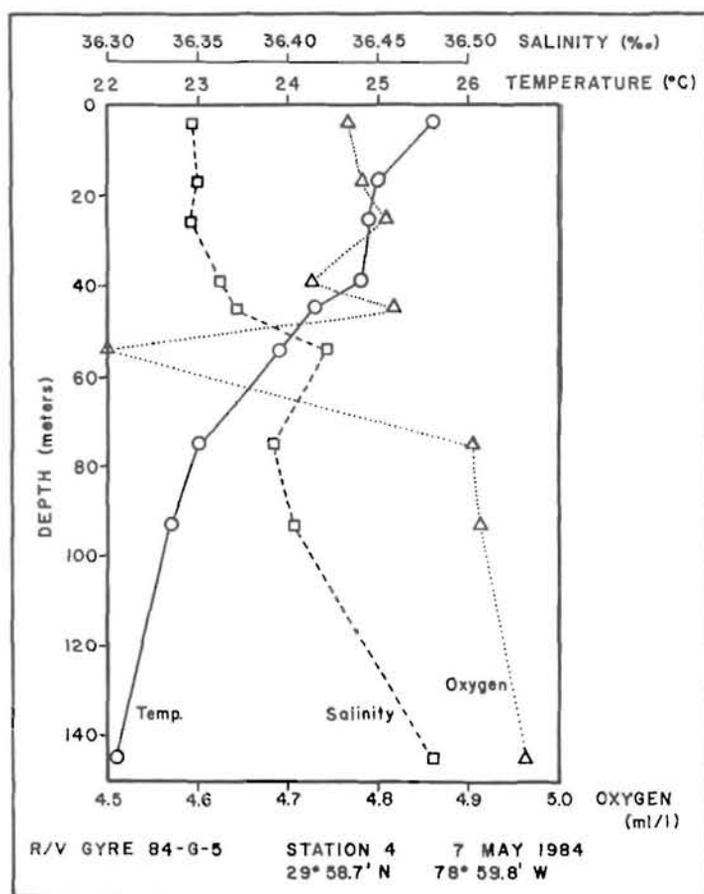


Figure 3 : Temperature, salinity and oxygen concentration versus depth at station 4.

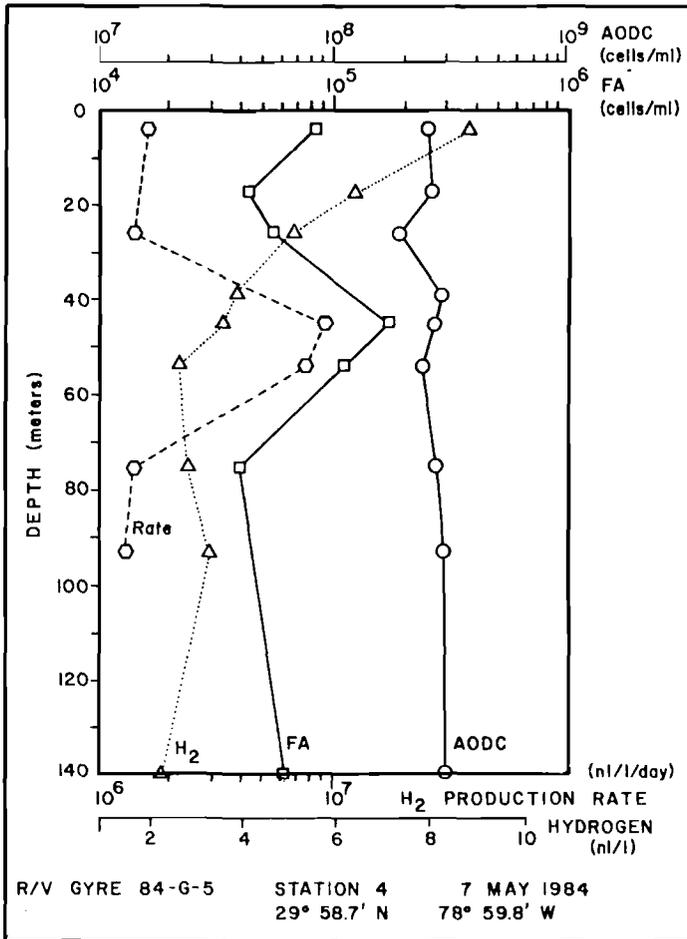


Figure 4 : Total bacterial direct count (AODC), fluorescent antibody enumerated bacterial count (FA), hydrogen-production rate *in vitro* (Rate) and *in situ* hydrogen gas concentration (H₂) versus depth at station 4.

DISCUSSION

The total number of bacteria detected in the central Sargasso Sea is low ($7.3 - 8.2 \times 10^5$ cells ml⁻¹) when compared to other oceanic areas. Direct counts (AODC) usually indicate at least 10^6 cells ml⁻¹ in ocean surface waters (Griffiths *et al.*, 1978; Hobbie *et al.* 1977, Kogure *et al.*, 1979; Zimmerman *et al.*, 1978). The low number of cells is indicative of the nutrient-poor conditions in the Sargasso Sea. ATP values were also quite low in the Sargasso Sea, *ca.* 4 - 17 ng l⁻¹ (Paul LaRock, personal communication). The greater bacterial numbers at Stations 15 and 18 reflect more normal oceanic conditions on the periphery of the Sargasso Sea. Within the Sargasso Sea, low nutrient concentrations result in poor productivity and as a result the water column contains little suspended matter and poorly developed particulate maxima.

Most of the cells seen in the SEM photographs were less than $1 \mu\text{m}$ in diameter. Small size is a characteristic of starved marine bacteria (Amy and Morita, 1983) so it is not surprising that most cells in the nutrient-depleted Sargasso Sea are quite small. These

small cells would pass through the 3 μm pore diameter membrane filter, the smallest size used in the reverse flow concentrators. Thus, despite the concentration of larger particles, 80 % of the cells were found in the $<3 \mu\text{m}$ size fraction.

Although the amount of suspended particulate matter in the Sargasso Sea is low, particulates are a likely, if not essential, habitat for active, fermentative hydrogen-producing bacteria. Since we are most likely dealing with fermentative bacteria, anaerobic microenvironments must be present within the aerobic water column for *in situ* hydrogen production to occur and particulate matter is a candidate for such microenvironments. At a station in the eastern equatorial Atlantic, bacteria accounted for up to 40% of the organic carbon in particulates (Bishop *et al.*, 1977). The bacteria that were concentrated in the $>30 \mu\text{m}$ size fraction at Station 18 clearly were associated with particulate matter, since the small free bacteria should easily pass through the 35 μm mesh. Station 4 samples, containing unconcentrated water, had relatively high amounts of particulate matter within which bacterial hydrogen production could occur.

It is difficult to evaluate the role of particulate matter with respect to microbial activities in the water column because most sampling techniques are not capable of collecting intact particulates. Trent *et al.* (1978) found that marine waters contain fragile aggregate particles called marine snow, up to 9 cm long, that are destroyed by conventional sampling methods. The aggregates are diverse microhabitats containing bacteria, ciliates, dinoflagellates, and diatoms. Nutrients are more concentrated in aggregates than in the surrounding water and it has been suggested that microbial activity in the nutrient rich aggregates could alter the microenvironment (Shanks and Trent, 1979). In terms of hydrogen production, this means that intensive microbial activity could deplete oxygen to a level at which facultative anaerobes could begin fermentative metabolism, releasing hydrogen. Production of hydrogen in the $<3 \mu\text{m}$ size fraction at Station 11 indicates that facultative anaerobes are free in the water column, as well as attached to particles, and could rapidly take advantage of any developing microscale anaerobic habitats.

Our incubation technique, using serum vials, does not permit a quantitative estimate of the number of hydrogen producers in the water; it is designed to detect the potential for hydrogen production and allow isolation of the gas-producing organisms. Thus, our hydrogen production figures must be considered maximum potential production value. The maximum amount of hydrogen produced from each station's samples was relatively constant at about 10 nl l⁻¹. The added nutrients did not affect either the rate of production or total amount of hydrogen (Fig. 2). The other organic supplements, yeast extract and cysteine, which all of the vials received, provided adequate nutrients for hydrogen production without the addition of glucose or lactate. The relatively constant amount of hydrogen produced indicates that the hydrogen itself may inhibit further production. In several species of bacteria, increasing partial pressures of hydrogen inhibit hydrogen evolution (Zajic *et al.*, 1978), so this is a reasonable mechanism to explain the constant upper limit to our measured hydrogen concentrations.

Our serum vial sample incubation technique has been further modified to permit the use of unconcentrated water and to allow estimation of the initial rates of hydrogen gas production. When water samples from various depths were incubated individually, differences in initial rates of gas production were observed as shown in Figure 4. Production rates were greatest and exhibited a marked maximum at the base of the mixed layer (45-55m). This is the same depth range where the greatest number of hydrogen-producing bacteria were enumerated through the use of fluorescent antibody techniques (Figure 4, FA). Thus, it appears that hydrogen gas-producing bacteria are associated with the particulates found at the base of the mixed layer, have the highest gas production potential there, and are present in the greatest numbers there.

Taxonomic characterization of the hydrogen-producing isolates showed them to consist of only two groups of bacteria. These organisms were the ones which responded best to the artificial conditions in the experimental system, so we cannot exclude the possibility that other hydrogen producers such as anaerobic microaerotolerant bacteria are present in the water but do not grow on agar plates. Nevertheless, these results suggest that if *in situ* fermentative hydrogen production does occur, it will be from the activity of relatively few species.

Other authors have suggested that nitrogen-fixing microorganisms, particularly the cyanobacterium, *Oscillatoria*, are primarily responsible for dissolved hydrogen maxima in marine waters (Herr *et al.*, 1981; Scranton, 1983). However, Scranton *et al.* (1982) found no *Oscillatoria* associated with dissolved hydrogen maxima in the Mediterranean Sea. Carpenter and McCarthy (1975) concluded that *Oscillatoria* in the Sargasso Sea had very slow growth rates, too slow to account for much nitrogen fixation and so, by inference, much hydrogen production. Doremus (1982) has argued that on a large scale, nitrogen fixation in the oceans is insignificant in the long term because of nutrient limitations. Thus, nitrogen-fixing cyanobacteria apparently cannot be responsible for all of the dissolved hydrogen maxima in the oceans. Localized blooms of cyanobacters do occur and could account for some hydrogen production. However, on a larger scale, it appears that some other biological mechanism must contribute to dissolved hydrogen production.

On two previous cruises we also detected fermentative hydrogen production from $>35 \mu\text{m}$ particle fractions (Schropp and Schwarz, unpublished). Junge *et al.* (1972) were able to isolate hydrogen-producing bacteria from the Gulf of Cadiz, just below the depth of a dissolved maximum. The results of all the experiments indicate that hydrogen-producing bacteria are ubiquitous in marine waters. Under the appropriate conditions these organisms readily produce hydrogen. We believe that particulate matter can be conditioned so that fermentative metabolism can proceed *in situ*. Our gas production rate and fluorescent antibody enumeration studies (Fig. 4) along with the physical data (Fig. 3) would support this hypothesis. Particulates tend to be concentrated at a pycnocline since their sinking rate decreases as the density of the water increases. Fermentative bacteria associated with the particles could thus contribute to the observed hydrogen maxima associated with density gradients.

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