Pacific Science (1976), Vol. 30, No. 4, p. 385–393
Printed in Great Britain

Bacterial Nitrogen Fixation in a Polluted Coral Reef Flat Ecosystem, Kaneohe Bay, Oahu, Hawaiian Islands[1]

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ABSTRACT: Benthic nitrogen fixation was investigated in Kaneohe Bay, Oahu, Hawaiian Islands, which receives secondary sewage from two treatment plants. The range of nitrogen fixation rates (2 to 10 ng N_2 g^{-1} hr^{-1}) was similar to those reported by other workers for a variety of benthic systems. Enrichment cultures prepared from sediment samples from five stations revealed the existence of several distinct physiological types of nitrogen-fixing bacteria. It was found that 50 percent of the bacterial fixation in the southern sector was light-dependent. There was a significant relationship between the numbers of nitrogen-fixing bacteria detected and rates of nitrogen fixation measured in the sediments.

The presence and activities of nonsymbiotic nitrogen-fixing microorganisms have been demonstrated in a variety of environments. The contribution of such organisms to the overall nitrogen budget of the biosphere is considered by many to be of little significance. In marine environments, nitrogen fixation has been shown to occur both in nearshore and open ocean waters as well as in pelagic and estuarine sediments (Waksman, Hotchkiss, and Carey 1933; Pshenin 1963; Stewart 1965; Kawai and Sugahara 1971*a*, *b*; Brooks et al. 1971; Mague, Weare, and Holm-Hansen 1974; Gundersen et al. 1976). Stewart (1969) has reviewed the literature and tabulated the genera of potential nitrogen-fixing bacteria, blue-green algae, fungi, and yeasts.

Recently, an anaerobic spore-former ("clostridium-like") was found in an estuarine sediment where nitrogen fixation was being measured (Brooks et al. 1971). Keirn and Brezonik (1971) extended this survey of potential nitrogen fixers in some lacustrine sediments and reported the presence of a *Clostridium* sp., a *Thiospirillum* sp., a *Chromatium* sp., and nitrogen fixation. Howard et al. (1970) measured nitrogen fixation in lake sediments, and Kawai and Sugahara (1971*a*, *b*) counted the number of nitrogen-fixing organisms in seawater and marine sediments. But, unfortunately, in none of these studies were the types of organisms described.

In Kaneohe Bay, sewage effluents, nutrients in rainwater, and irrigation runoff and siltation from agricultural and domestic lands have been detrimental to most of the bay's natural reef communities (Smith, Chave, and Kam 1973). In this paper, the rates of nitrogen fixation in the sediments at several stations in the subtropical estuary of Kaneohe Bay have been determined and related to the numbers of various physiological types of nitrogen-fixing bacteria in the sediment.

Materials and Methods

Sampling and Determination of in situ *Nitrogen Fixation*

In situ nitrogen fixation was monitored by the acetylene reduction method of Stewart, Fitzgerald, and Burris (1967) and Hardy et al. (1968). Sediment samples were collected from the uppermost 0–10 cm of patch and fringing reefs

[1] This work was supported in part by the University of Hawaii Department of Microbiology and by National Science Foundation grant no. GA-27288. Manuscript accepted 22 September 1976.

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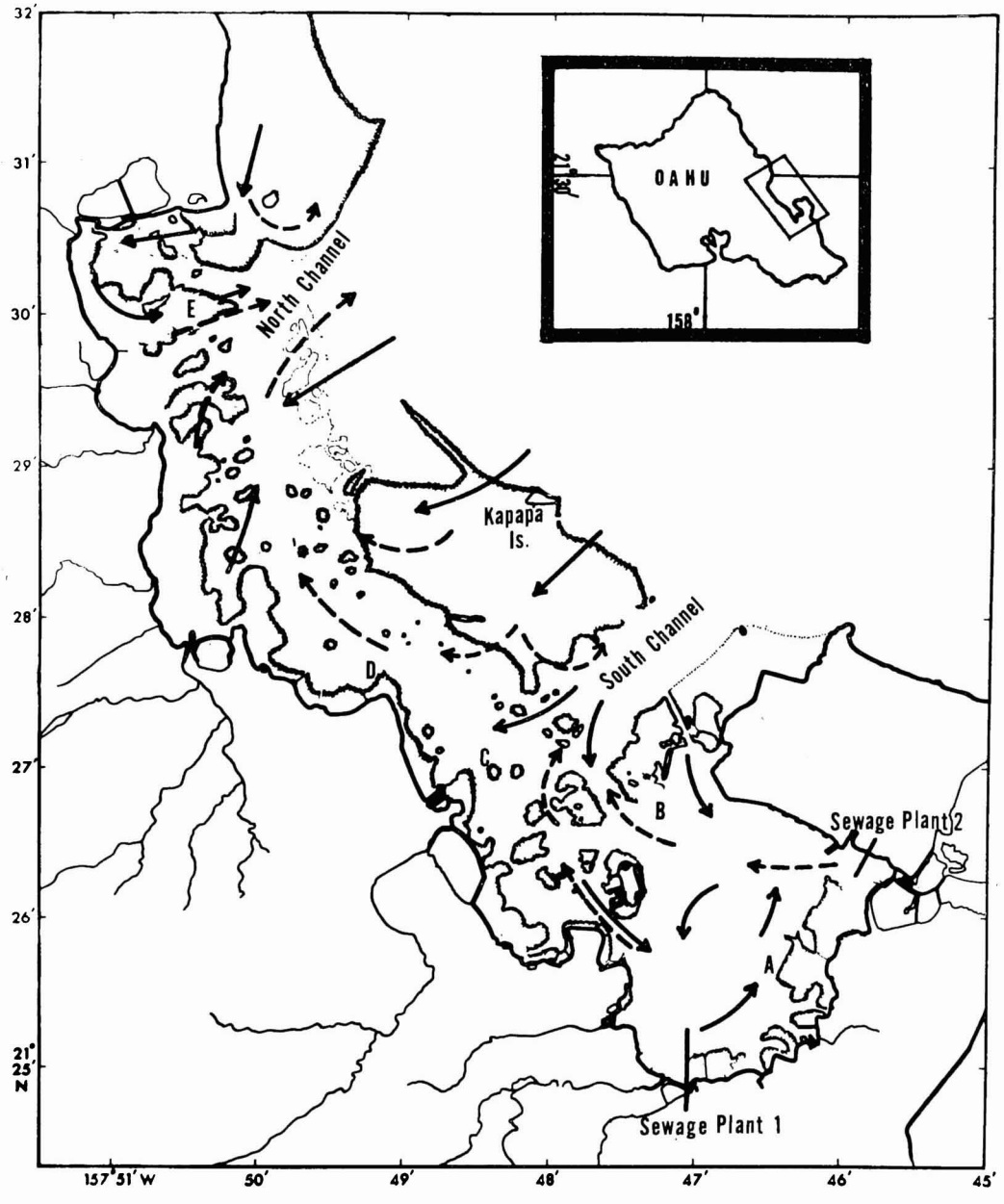

FIGURE 1. Kaneohe Bay and its location on the north-eastern coast of Oahu (insert). Sewage Plant 1 (Kaneohe Municipal) and Sewage Plant 2 (Kaneohe Marine Corps Air Station) with lines to outfall area. Basic current patterns: incoming tides, solid arrow; outgoing tides, broken arrow. The major navigational channels, North and South channels, are located at the respective ends of the extensive outer barrier reef which includes Kapapa Island. The five coral and sediment stations (lettered A to E) are located along a northwest to southeast transect of Kaneohe Bay.

in Kaneohe Bay at a total of five stations, designated *A–E*, along a southeast to northwest transect of the bay (Figure 1). The wet sediment samples were homogenized and 10–20 ml of the slurry were placed in three 38-ml serum bottles. Controls contained sediment slurry to which 5 ml of a saturated NH_4Cl solution was added to inhibit biological nitrogen fixation completely. The sample bottles were then returned to the reef sediment for incubation. After about 3 hours, the bottles were recovered and the amount of ethylene produced was collected in 4-ml Vacutainers. Fixation rates are given as weight of nitrogen fixed per gram dry weight sediment per hour. The reduction rate was determined during this period and converted to nitrogen fixed by the theoretical ratio 3:1 (Hardy et al. 1968). The rate of fixation was significantly correlated ($P < 0.05$; $r = 0.81$) to sediment dry weight.

Media and Enrichment Cultures

Samples of sediment of the same material used for the determination of *in situ* nitrogen fixation were returned to the laboratory for preparation of enrichment cultures. All media contained the basal salts mixture of AM medium (Antia and Kalmakoff 1965) but did not contain the added nitrogen.

BLUE-GREEN ALGAE: The ingredients of the basal medium were dissolved in 75 percent open-ocean surface water of low combined nitrogen content. The selective isolation procedure that was followed for blue-green algae was that of Allen and Stanier (1968).

HETEROTROPHIC NITROGEN-FIXING BACTERIA: Synthetic seawater, as described by Lyman and Fleming (1940), at 75 percent strength was used in the basal medium for bacterial cultures and was supplemented further with various carbon sources (Stanier, Doudoroff, and Adelberg 1970). I selected for spore-forming bacteria by pasteurizing the sediment samples for 10 minutes at 80° C and then growing the bacteria in basal medium enriched with either 1-percent glucose and 0.1-percent $CaCO_3$ or 0.5-percent Na-lactate and 0.1-percent $FeSO_4 \cdot 7H_2O$. Sulfate-reducing bacteria were also grown in the 0.5-percent Na-lactate, 0.1-percent $FeSO_4 \cdot 7H_2O$ medium, but the anaerobic cultures were prepared in glass-stoppered bottles that had been sparged with pure nitrogen gas for 3 minutes before inoculation. Aerobic heterotrophic bacteria were enriched in a medium containing 0.05-percent sucrose and 0.05-percent mannitol. The enrichment and isolation cultures of all the heterotrophic bacteria were incubated at 25° C in the dark, aerobic, liquid cultures on a rotary shaker.

PHOTOSYNTHETIC BACTERIA: The enrichment and isolation procedures followed for photosynthetic bacteria were those of van Niel (1944) and Pfennig (1967). The medium for the purple nonsulfur bacteria contained 0.5-percent Na-malate, 0.5-percent $NaHCO_3$, and 0.05-percent yeast extract (Difco); for the purple sulfur bacteria, the medium contained 0.1-percent Na_2S and 0.5-percent $NaHCO_3$; and for the green sulfur bacteria, the enrichment consisted of 0.05-percent Na_2S and 0.5-percent $NaHCO_3$ with the *p*H of the latter medium having been adjusted to 7.5. The incubation of enrichments for purple sulfur and nonsulfur bacteria was done at 25° C under continuous illumination by two incandescent 40-watt light bulbs; that for the green sulfur bacteria was at about one-half this light intensity. The shake culture tube method of van Niel (1944) was used for the isolation of photosynthetic bacteria.

All isolated microorganisms were tentatively identified by cell and colony morphology; gram stain reaction; type of motility, if present; and various physiological and biochemical tests that are described more completely in the results.

Enumeration of Potential Nitrogen-Fixing Microorganism in Sediments

The numbers of each potential nitrogen-fixing type of bacterium in the sediment were determined by the most probable number (MPN) method (American Public Health Association 1955). Sediment samples (1 g wet weight) were suspended in aliquots of the various enrichment media and serially diluted. Tubes containing the anaerobic media also contained 0.05-percent Na-thioglycollate and these were sparged for 3 minutes with nitrogen gas and then plugged with rubber stoppers. For each medium, dilutions were made in three replicate sets of tubes.

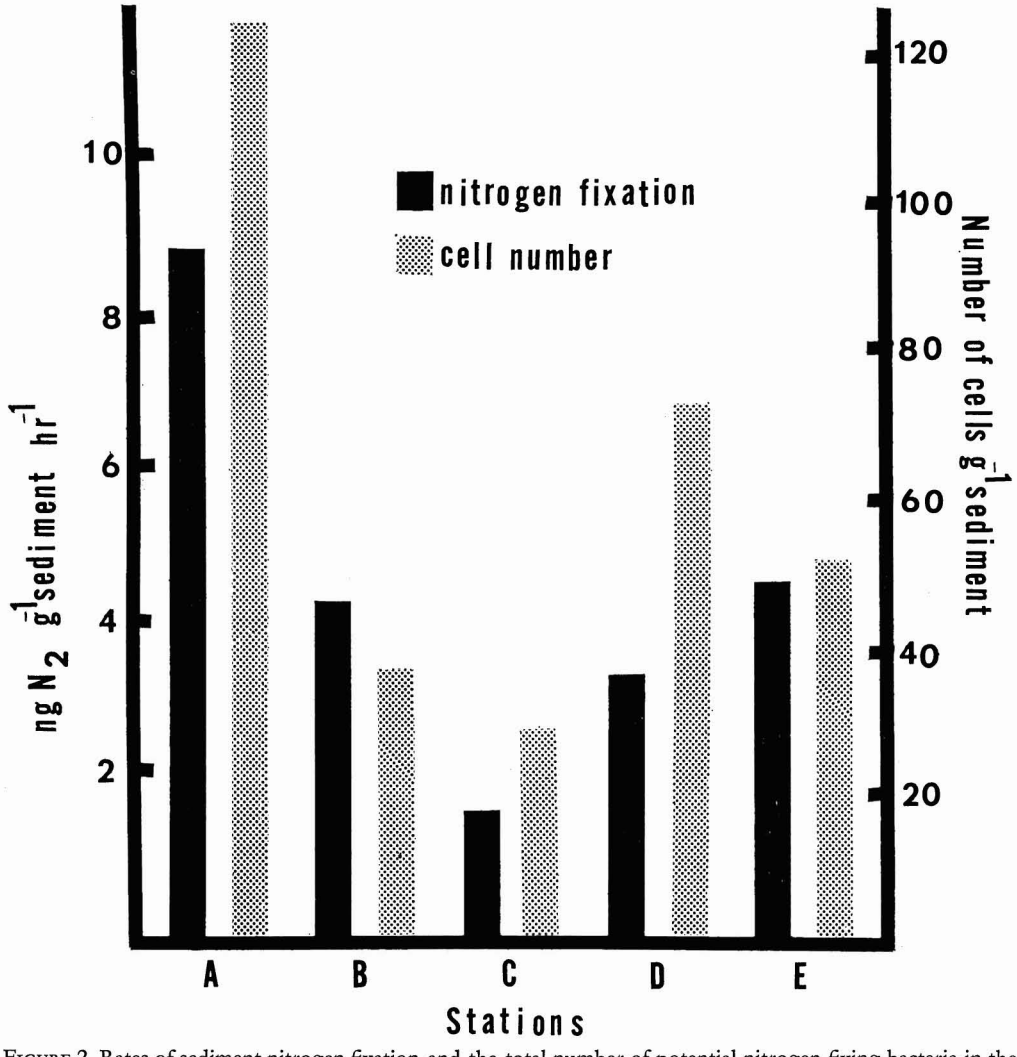

FIGURE 2. Rates of sediment nitrogen fixation and the total number of potential nitrogen-fixing bacteria in the sediment at each station in Kaneohe Bay, summer 1972.

Incubations were done at 25° C for 30 days. Tubes were scored positive when visible growth associated with pigment formation or black FeS precipitate in tubes of photosynthetic and sulfate-reducing bacteria, respectively, had developed and acetylene reduction could be demonstrated.

RESULTS

Nitrogen Fixation in the Sediment

The low rate of nitrogen fixation in the sediment made necessary the incubation of the reaction mixture for as long as 3 hours before gas sampling. In a time course experiment, acetylene reduction was found to be linear within 6 hours, after which time the rate decreased. The precision of the assay was calculated from the mean of four sets of sediment, in triplicate, which showed a coefficient of variation of 12 percent.

The nitrogen fixation rates in the reef flat sediments determined at all five stations are shown in Figures 2 and 3. The highest mean rate, 8.7 ng N_2 (g dry wt)$^{-1}$ hr^{-1}, occurred at station *A* of Kaneohe Bay. A lower mean rate, 1.3 ng

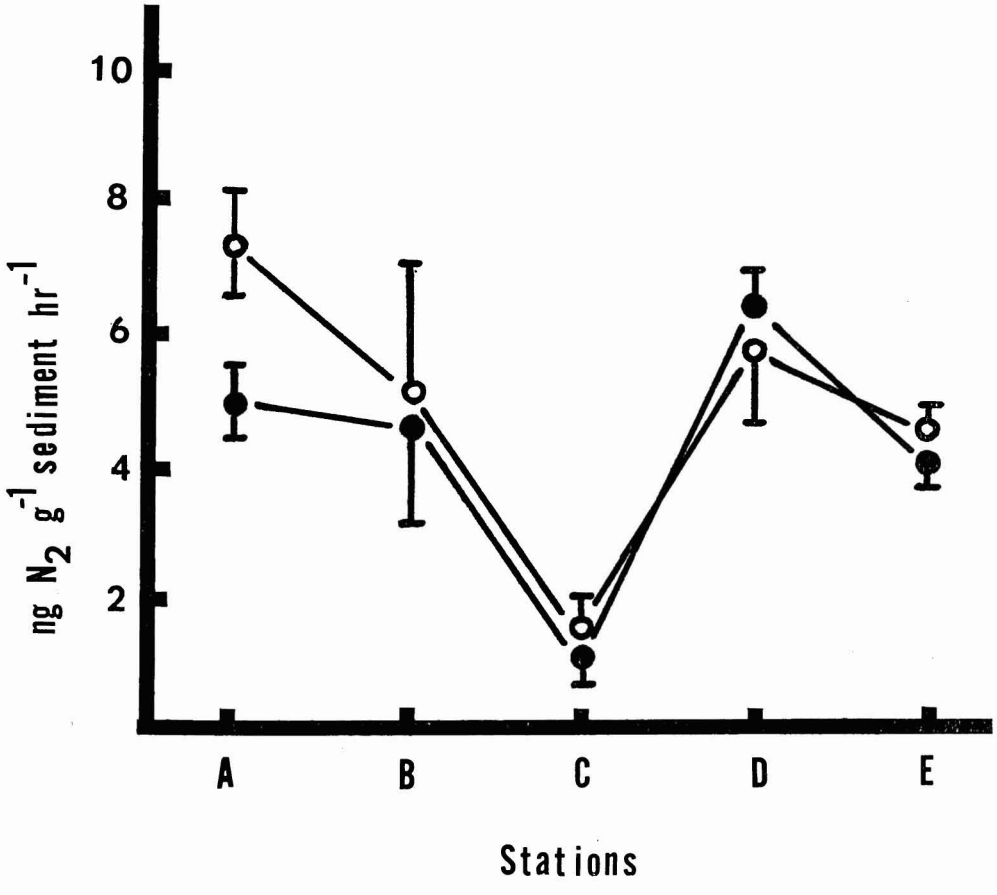

FIGURE 3. The rate of nitrogen fixation in the reef sediments at five stations in Kaneohe Bay, summer 1973: rates were measured in the 0–6 cm sediment stratum. White circles, incubation in light bottles; black circles, incubation in dark bottles. The vertical bars represent the range of fixation in four samples.

N_2 g^{-1} hr^{-1}, was found at station *C*; and a higher mean rate, 4.5 ng N_2 g^{-1} hr^{-1}, was measured at station *E*. Similar rates were measured through the months of June, July, and August, 1972 and 1973. No measurements were made September through May.

Nitrogen-Fixing Microorganisms

A variety of bacteria capable of fixing nitrogen was isolated from the various enrichment cultures. Nitrogen-fixing blue-green algae, however, were not found in the sediment, although such organisms were isolated from dead coral heads and from buoys (Hanson and Gundersen 1976, in press). It was observed that some of the nitrogen-fixing bacteria were restricted to certain localities in the bay. Two photosynthetic bacteria, a purple sulfur bacterium and a purple nonsulfur bacterium, were seen only in the enrichments from stations *A* through *D*, whereas an aerobic heterotroph and a green sulfur bacterium were found only at station *D*. Obligate anaerobic spore-formers and sulfate-reducing organisms occurred throughout the bay.

The photosynthetic bacteria were identified as belonging to the Athiorhodaceae (*Rhodopseudomonas*), Thiorhodaceae (*Thiocystis*), and Chlorobacteriaceae (*Pelodictyon*). Other nitrogen-fixing organisms belonged to the Bacillaceae (*Clostridium*), Spirillaceae (*Desulfovibrio*), and

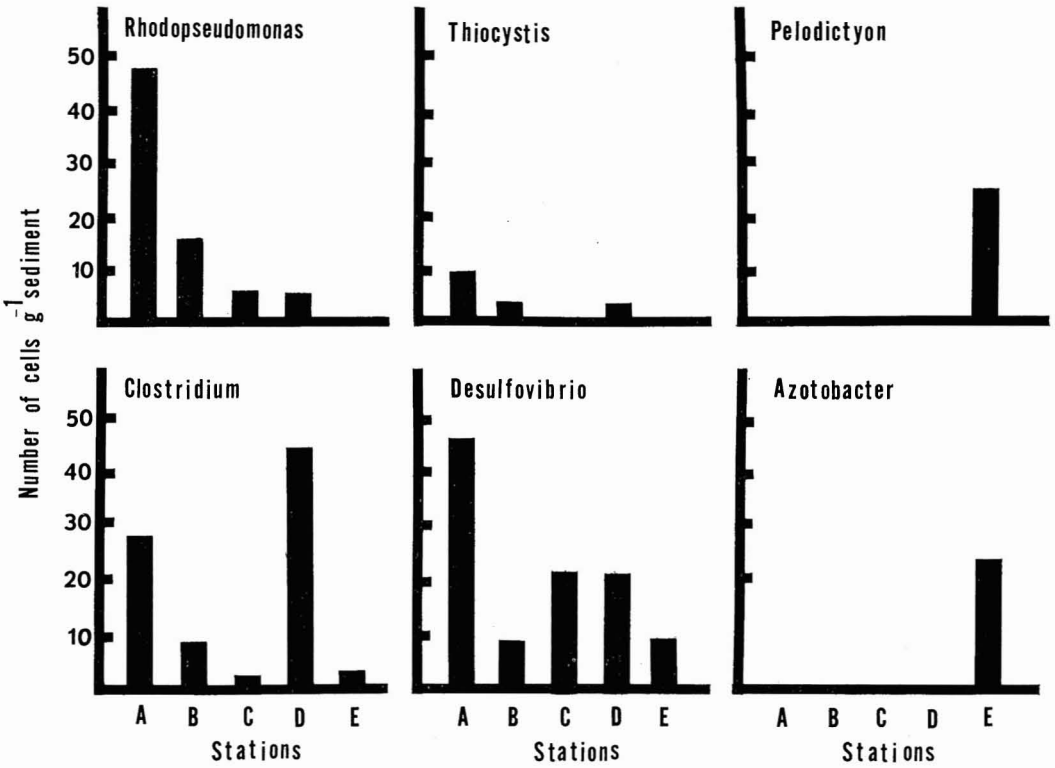

FIGURE 4. Distribution and number of potential nitrogen-fixing bacteria in Kaneohe Bay sediments. The numbers of organisms were determined by the most probable number method.

Azotobacteriaceae (*Azotobacter*). The isolates, grown under the enrichment conditions, showed rapid acetylene reduction.

The *Rhodopseudomonas* sp. grew in a selective medium consisting of Na-benzoate supplemented with *p*-aminobenzoic acid, suggesting that the photosynthetic bacterium was *R. palustris* (Pfennig 1967). The purple sulfur bacterium was identified as a *Thiocystis* sp. The green sulfur bacterium was morphologically similar to species of *Pelodictyon*, which show characteristic growth in the form of loose, irregular nets. The anaerobic spore-former was a gram-positive rod with a terminal spore. Since it did not grow aerobically, it was assumed to be a *Clostridium*. The odor of butyric acid in the enrichment culture suggested that it belonged to the butyric acid group. The only reported nitrogen-fixer among the nonspore-forming, sulfate-reducing bacteria was *Desulfovibrio desulfuricans* var. *azotovorans* (Postgate and Cambell 1966). The sulfate-reducer found in Kaneohe Bay sediments showed morphological and physiological similarities to this organism. The aerobic nitrogen-fixing organism was a motile, plump rod, which formed mucoid colonies but which did not produce pigments and did not grow at the expense of rhamnose or benzoate. These characteristics suggest that the organisms might have been *Azotobacter agilis*. Since this organism did not grow in a freshwater medium but only in seawater, it may be an obligate marine strain.

Numbers of Nitrogen-Fixing Organisms in the Sediment

Besides determining the physiological types of nitrogen-fixing bacteria in Kaneohe Bay sediments, we estimated the quantity of each type by the most probable number (MPN) method (Figure 4). From the station *A* to *D* sector, the

number of *Rhodopseudomonas* sp. decreased from 46 to 4.3 cells/g wet sediment while the number of *Thiocystis* sp. decreased from 4.3 to 0.9 cells/g wet sediment. Isolates of the *Pelodictyon* sp. and the *Azotobacter* sp., 24 and 13 cells/g wet sediment, respectively, again were isolated only at station *D*. The *Clostridium* sp. and the *Desulfovibrio* sp. were found throughout the estuary, ranging from 0.9 to 46 and 9.3 to 46 cells/g wet sediment, respectively.

The numbers of potential nitrogen-fixing bacteria within each physiological group were totalled and plotted with the rates of fixation from the same area (Figure 2). There was a definite trend between numbers of detectable nitrogen-fixers and the rates of nitrogen fixation.

Since the photosynthetic nitrogen-fixers represented a significant fraction of the total detectable number of bacterial nitrogen-fixers and since there was no culturable nitrogen-fixing blue-green algae in benthic samples from the southern end of the bay, the significance of the photosynthetic nitrogen fixation to the total nitrogen fixation was investigated. The sediment was incubated in light and dark bottles; its range of fixation is shown in Figure 3. At stations *B* to *E*, the rates found in the sediment incubated in light and dark did not differ significantly, whereas at station *A* the rates in the light were approximately 50 percent greater than those in the dark.

DISCUSSION

When we consider the high levels of organic matter and inorganic nutrients in Kaneohe Bay, which result from extensive urban development along its shores and from the existence of two sewage outfalls in the southern sector, we are not surprised to learn that nitrogen fixation occurred here and that a variety of nitrogen-fixing bacteria seemed to be involved in the process. The rates of nitrogen fixation have been found to be very low (0 to 10 ng N_2 g^{-1} hr^{-1}) in the sediments of most marine and freshwater ecosystems studied so far (Howard et al. 1970, Brooks et al. 1971, Keirn and Brezonik 1971, Jones 1974). The rates of nitrogen fixation (2 to 8 ng N_2 g^{-1} hr^{-1}) measured in the sediments of Kaneohe Bay were comparable to those reported by others. In Kaneohe Bay, the higher rates of fixation and the larger amount of detectable nitrogen-fixing bacteria always occurred in typically muddy sediments, whereas low rates and fewer bacteria were found in coarse sand and coral rubble sediments. The rates of fixation did not agree with the number of nitrogen-fixers found in the sediment. The MPN method has certain limitations and, if the rates of fixation are correct, is probably in error by at least two orders of magnitude. An increase in the number of replicates to 10 and a decrease in the dilution series to 1:2 would greatly increase the sensitivity of the method, but such changes were impractical.

The *in situ* benthic fixation rates at station *A* were found to be 50 percent greater in the light than in the dark, indicating the significance of the photosynthetic nitrogen-fixing bacteria at station *A* reef flat sediments. But it is not known if there is enough light for activity in the deeper regions of the bay. The restriction of the various physiological types to specific ecological localities in the bay sediments may be due, in part, to the nutrient regime in those areas. Heterotrophic bacterial fixation is dependent mainly on a suitable carbon source. It was estimated that 10^4 kg organic carbon (plankton and allochthonous material from sewage and streams) enter the sediment at the southern end of the bay daily (Steinhilper 1970). Although some of the carbon may be relatively refractory, sufficient amounts of oxidizable organic carbon should be available to maintain vigorous bacterial fixation.

Heterotrophic nitrogen fixation is considered minor in aquatic environments as compared to autotrophic nitrogen fixation by blue-green algae. Stewart et al. (1971) estimated that the total nitrogen fixed during a bloom of blue-green algae in the top 5 m of Lake Mendota, Wisconsin, was 2.4 kg N_2/ha/yr. A similar value, 2.9 kg N_2/ha/yr, was reported by Horne and Fogg (1970) for Lake Windermere, England. The Stewart et al. (1971) estimates were based on a 14-hour fixation period/day and a sampling period of 51 days, which covered almost all of the active period for the blue-green algal bloom. Since the temperature variation in Kaneohe Bay ranges from 23° to 27° C throughout the year (Bathen 1968), the biological activity in the sediment is probably relatively constant. The total amount of nitrogen fixed,

mainly by the heterotrophic population, in Kaneohe Bay sediment was estimated to be 6 kg/ha annually (Hanson and Gundersen, in press) or approximately two times greater than the seasonal fixation by blue-green algal bloom in the two temperate regions referred to above. Therefore, on an annual basis, the heterotrophic fixation in the tropics may be as significant to the nitrogen budget as the autotrophic process is in the temperate climates.

The contribution of bacterial nitrogen fixation to the nitrogen budget cannot be assessed without the measurement of denitrification. However, the process can be compared to the amount of nitrogen released by the sewage effluent in the estuary. The total nitrogen input from the sewage treatment plants, approximately 8×10^9 liters/yr, with an average total nitrogen content of 25 mg/liter (information obtained from the City and County of Honolulu, Division of Sewers, 1972), is 200×10^3 kg combined nitrogen annually. The annual nitrogen fixed by the heterotrophic bacteria in the sediment is approximately 6 percent of the nitrogen released in the sewage effluent. Therefore, biological nitrogen fixation in the sediment may contribute considerably to the total nitrogen entering the estuary from the sewage treatment plant and from other sources, provided that denitrification does not play a major role.

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